THE OXIDATION OF WHEAT FLOUR

I. Measurement of Sulfhydryl Groups [1]

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ABSTRACT

Although *p*-chloromercuribenzoate (PCMB) reacts specifically with free sulfhydryl groups, these groups can still be measured in the presence of PCMB by amperometric titration with mercuric chloride. Mercuric chloride apparently displaces the PCMB from –SH groups, the mercuric chloride possessing a stronger affinity for the –SH groups than PCMB. This provides a useful technique for protecting –SH groups during measurement and results in higher values.

The –SH content of flour is about equally divided between the water-soluble and the gluten proteins. No –SH has been found in the lipid or starch fraction of flour. The lipids of germ do contain sulfhydryl groups.

The maturing of flour is one of the most challenging and intricate problems in the field of cereal chemistry. The present status of the subject has been reviewed recently (10,13), It is generally believed that sulfhydryl groups are involved in the improvement of the physical properties of flour by aging and by maturing agents, but, in spite of the large amount of work on the subject, the entire mechanism remains obscure.

It is well known that –SH-containing compounds, such as glutathione, cysteine, and thioglycollic acid, soften the gluten and decrease the viscosity, plasticity, and mixing time of flour doughs (10,13). Free gluthathione is found in significant amounts in wheat germ (0.2 to 0.5% as cysteine) and to a lesser degree in bran and low-grade flour, but none is found in shorter-extraction flours. The nature of the –SH-containing substances in flour is not known. Presumably they are

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cysteine residues of the protein. Flour contains very small amounts of sulfhydryl; the range reported according to most acceptable methods varies from 0.2 to 1.5 microequivalents sulfhydryl per g., depending on the method and the sample. Until recently, the major obstacle in studying the whole problem was the lack of an accurate method for sulfhydryl or there were limitations such as inadequate dispersions of gluten or flour to release all –SH groups, precipitation of the protein at the electrode, and other conditions of a particular method that gave results of doubtful value. This situation is not unique for flour but applies to many other complex biological materials. Bloksma (2) and Sokol, Mecham, and Pence (11,12) have discussed some of the problems in the measurement of thiol groups by amperometric titration.

Because of such problems in the quantitative measurement of sulfhydryl and the difficulty of obtaining adequate separations of constituent parts of flour, there is no unanimity concerning the location of –SH groups except that the water- and salt-soluble fractions of flour (albumins and globulins) contain sulfhydryl. There are conflicting results on the presence of –SH-containing compounds in the so-called gluten proteins and in the lipids.

This paper will discuss the measurement of sulfhydryl in the water extract of various milled fractions of a spring wheat, in the lipids separated from these fractions, and in some gliadin, albumin, and globulin preparations. A new technique, using PCMB to protect the –SH groups from oxidation during measurement, was employed to determine the total as well as the water-soluble –SH content of flour.

Materials and Methods

The method outlined by Kolthoff, Stricks, and Morren (4) using mercuric chloride as the titrant has given the most consistent results for flour. When gliadin or any lipid or other alcohol-soluble material is to be measured, the method of Benesch, Lardy, and Benesch (1) using silver nitrate is preferred.

Distribution of Sulfhydryl in Water Extract of Milled Fractions. Samples of a short patent flour, a first clear flour, a second clear flour, bran, and germ were taken at the same time from a spring wheat mix of 14.0% protein. Forty grams of the flours and 20 g. of the bran and the germ were shaken in a nitrogen atmosphere with 100 ml. of glass-distilled water and centrifuged. The supernatant liquid was decanted, recentrifuged, and a suitable aliquot (usually 10 ml.) titrated amperometrically with 0.001*M* mercuric chloride, according to the method of Kolthoff *et al.* (4). The titration was carried out in 60 ml. of a solution that was 0.05*M* to borax and 0.1*M* to potassium chloride.

Sulfhydryl of Lipids. The same mill fractions and, in addition, a soft wheat sample were shaken in an atmosphere of nitrogen with water-saturated n-butanol and centrifuged. The supernatant extract was decanted and recentrifuged after drying with anhydrous sodium sulfate. An aliquot of the extract was titrated amperometrically using a rotating platinum electrode and 0.001*N* silver nitrate, according to the method described by Benesch *et al.* (1). Nitrogen and phosphorus (8) in the lipid were also determined.

Sulfhydryl of Protein Fractions. Three albumin and three globulin preparations described by Pence and Elder (9) were measured by the argentometric amperometric titration.

Gliadin. Gliadin was isolated from a spring wheat patent flour and titrated in alcohol solution amperometrically by the method of Benesch *et al.* (1) using "tris" buffer. A commercial sample of gliadin secured from the Huron Milling Company also was tested.

Sulfhydryl of Gluten. Gluten recovered by the conventional washing process contains significant amounts of albumins and globulins, as has been emphasized by Pence *et al.* (10). Therefore, –SH results on washed gluten would be high as a result of the adsorbed albumins and globulins. In order to eliminate this possibility and to find out if the more insoluble, higher-molecular-weight proteins contained sulfhydryl, several techniques were tried. Kong, Mecham, and Pence (5) used a tryptic digest to solubilize the flour protein. Their amperometric titration values of dilute buffer extracts of untreated and trypsin-treated flour showed –SH groups probably to be absent from gluten.

In this laboratory, several experiments using trypsin were tried, but the results were not satisfactory. They varied widely, depending on the temperature of the digest, and were generally unreliable. Sokol *et al.* (11) showed, in further work, that maximum sulfhydryl values obtained for trypsin digests of flour were only about one-third as large as those of flour dispersions.

Some further preliminary tests were conducted on wet gluten washed out in the conventional manner from 10 g. of spring wheat patent flour (0.39% ash and 12.30% protein at 14.0% moisture). The gluten was dispersed in 8*M* urea and acetic acid under nitrogen and titrated amperometrically with 0.001*M* mercuric chloride. Lower values were obtained than those calculated as the difference between total sulfhydryl and water-soluble sulfhydryl of the flour from which the gluten was washed. The low values were probably the result of incomplete dispersion and of precipitation at the electrode. When the wet gluten was washed with 0.5*M* sodium chloride, the gluten still

contained about half of the total sulfhydryl.

Effect of Temperature. Another factor that has caused discrepancies in results is temperature. Sokol *et al.* (11) found that a rapid loss of sulfhydryl occurs in flour-buffer-urea dispersions when the dispersions are made at room temperature. Significantly higher results were obtained at 2°C. than at 25°C. A similar trend has been observed in this laboratory. The following experiment was designed to measure the effect of temperature on –SH determinations of a untreated spring wheat, straight-grade flour.

Five grams of a straight-grade flour were dispersed under nitrogen in 100 ml. 8*M* urea in a Stein Mill operating 5 seconds per minute for 10 minutes. The resulting solution was made 0.5*M* to potassium chloride and 0.05*M* to borax and titrated under nitrogen with 0.001*M* mercuric chloride. The temperature of the dispersion and the temperature of the titration were both maintained at just below 10°C. in one experiment and at room temperature (25°C.) in another test. Then the temperature of the dispersion was kept under 10°C. but the titration conducted at 25°C., and, finally, the temperature of the dispersion was maintained at room temperature but the titration conducted at just below 10°C.

Protection of –SH Groups from Oxidation during Measurement. *p*-Chloromercuribenzoate (PCMB) reacts specifically with –SH groups. Some observations by Dahle and Sullivan (3) indicated that, although PCMB reacts with free sulfhydryl groups, these sulfhydryl groups can still be measured by amperometric titration with mercuric chloride. Presumably, mercuric chloride displaces the PCMB from a –SH group, the former having a stronger affinity for the –SH group than the latter. This offers a useful technique for protecting –SH groups during measurement. This was checked by measuring the sulfhydryl of two glutathione (GSH) solutions, identical except for the presence or absence of PCMB.

Sixty milliliters of buffer (0.05*M* borax, 0.1*M* potassium chloride) were purged with nitrogen until very little oxygen was present, as indicated by deflection of the galvanometer from zero position; 2 ml. of a GSH solution were added and titrated, measuring 1.91 ml. 0.001*M* mercuric chloride. An identical procedure was followed using 60 ml. of buffer containing 5 micromoles PCMB; this measured 1.88 ml. of 0.001*M* mercuric chloride. The difference of the two values lies within the limits of accuracy of measurement. Although the GSH solution had been made to be 0.001*M*, it is likely that the molarity was slightly less since the GSH was not subjected to a preliminary drying before weighing.

Titrations of total sulfhydryl of flour were now attempted in the presence and absence of PCMB. The procedure involved sifting flour from a salt shaker into 8*M* urea previously purged with nitrogen, with constant swirling of the beaker to effect dispersion of the flour. The flour dispersion was made 0.05*M* to borax and 0.5*M* to potassium chloride and then titrated. The optimal amount of flour that could be titrated was found to be 5 g. dispersed into 200 ml. 8*M* urea.

Attention was now turned to a measurement of the water-soluble portion of flour sulfhydryl, where oxidation is more of a problem. The best procedure found was to drop 10 g. of flour in a single portion into 200 ml. of water (previously purged with nitrogen) in a 500-ml. cylinder, stopper the cylinder, and shake manually for 1 minute. The flour seemed completely dispersed after this treatment. The flour dispersion was centrifuged 10 minutes at 1,500 r.p.m. One hundred milliliters of the supernatant extract were pipetted and transferred to a beaker containing 48 g. of urea. To this were added 25 ml. of buffer (2.75*M* potassium chloride, 0.275*M* borax). The solution then was titrated.

Results

Distribution of Sulfhydryl in Water Extract of Milled Fractions. The results on the distribution of sulfhydryl in the water extract of milling separations from a spring wheat are shown below. The

Product	*–SH Content per g. Product* μeq.
Patent	0.12
First clear	0.17
Second clear	1.33
Bran	4.75
Germ	27.75

sulfhydryl in germ and bran is due to glutathione. No free glutathione can be found in flour except for low-grade or second clear flours containing significant amounts of germ and the scutellum layer.

Sulfhydryl of Lipids. Table I gives results on the sulfhydryl, nitrogen, and phosphorus of lipids extracted from a soft wheat and from milled products of a spring wheat. All lipid fractions gave negative results for sulfhydryl with the exception of the germ.

Starch free from gluten showed a negative test for sulfhydryl, as would be expected and has been shown previously by work in this laboratory and by other investigators (Sokol, Mecham, and Pence, 11).

All investigators are agreed that the soluble proteins contain –SH groups, but reports have varied concerning the presence of sulfhydryl

TABLE I

ANALYSES OF LIPIDS OF MILLED PRODUCTS[a] AND A SOFT WHEAT

	NITROGEN IN LIPID	PHOSPHORUS IN LIPID	MOLE-RATIO N:P	SULFHYDRYL IN LIPID
	%	%		%
Patent	0.63	0.97	1.2:1	0
First clear	0.50	0.76	1.4:1	0
Second clear	0.44	0.52	2:1	0
Bran	...	...	...	0
Germ	...	...	...	0.01
Soft wheat	0.70	0.70	1.9:1	0

[a] The milled fractions were obtained from a spring wheat mix of 14.0% protein.

in the more insoluble proteins.

Table II gives some figures for the –SH content of some albumin and globulin preparations. Both these fractions occur in the water extract of flour. Albumins and globulins are adsorbed to some degree on washed gluten.

TABLE II

SULFHYDRYL CONTENT OF ALBUMIN AND GLOBULIN FRACTIONS

	VARIOUS COMPONENTS	–SH PER GRAM PROTEIN
	%	μeq.
Albumins[a]		
Comanche		4.5
Pentad		6.1
Thatcher		6.6
Globulins[a]		
Germ	70 , 20 ,	6.1
Germ	20 , 68 , 11	6.1
Flour	44 , 46 , 7	6.6

[a] These samples were a few years old and there may have been some oxidation of –SH.

Sulfhydryl of Gluten Proteins. The gliadin preparation isolated in our laboratory from a spring wheat, patent flour contained 1.2 μeq. sulfhydryl per g. A commercial sample of gliadin showed 1.3 μeq. sulfhydryl per g. After this gliadin solution had stood for 62 hours, the –SH value dropped to 0.2 μeq. per g. As has been mentioned, wet gluten dispersed in 8*M* urea and acetic acid and measured amperometrically with mercuric chloride gave lower –SH values than those calculated as the differences between total flour sulfhydryl and water-soluble sulfhydryl. While the low results on gluten may be partly explained by precipitation at the electrode, other factors such as temperature affect the results of the –SH determination. Thus, a straight-grade flour gave the following figures with changes in the temperature of the dispersion and the temperature of titration:

Temperature of Dispersion	*Temperature of Titration*	*–SH per g. Flour*
		μeq.
<10°C.	<10°C.	0.42
room	room	0.23
<10°C.	room	0.23
room	<10°C.	0.26

The end point was not as sharp at low-temperature dispersion and low-temperature titration. The higher values obtained at lower temperature may be attributed to some labile compound that acts on sulfhydryl but at a lesser rate at lower temperature, or some configuration that is altered at relatively higher temperatures. It is further suggested by these data that the phenomenon is an irreversible one. Subjection of a dispersion to cold did not result in a higher value after the dispersion had once been at room temperature.

It was apparent that, since starch and lipids of flour contain no –SH groups and since gluten cannot be separated without some adhering globulins and albumins, a measurement of total flour sulfhydryl minus water and/or salt-soluble sulfhydryl would have to suffice as an index of the –SH content of the gluten proteins. Some investigators have reported that gluten proteins contained no sulfhydryl, perhaps because of the great difficulty in adequately dispersing the sample and the lability of the –SH groups.

Protection of –SH Groups from Oxidation during Measurement. As has been noted, PCMB can be used to protect –SH groups during measurement. Matsumoto and Shimoda (7) had observed that PCMB did not lower the titration value obtained by the mercurimetric titration. Results differed from those obtained with silver nitrate in the presence of PCMB. In a more recent paper, Matsumoto and Hlynka (6) reported on the –SH content of various flour fractions and, further, that PCMB decreased the –SH content of water-soluble and acid-soluble fractions of dough by 76 and 86%, respectively, when measured amperometrically with silver nitrate.

Five grams of untreated straight-grade spring wheat flour dispersed in urea measured 2.4 ml. 0.001*M* mercuric chloride to give a value of 0.56 μeq. sulfhydryl per g. of flour (dry basis). An identical procedure using 200 ml. of 8*M* urea containing 5 micromoles PCMB measured 0.62 μeq. sulfhydryl per g. of flour (dry basis). The water-soluble extract, which is more vulnerable to oxidation, measured 0.35 ml. 0.001*M* mercuric chloride to give a value of 0.08 μeq. sulfhydryl per g. of flour (dry basis). An identical procedure using 200 ml. of water containing 10 micromoles PCMB measured 1.2 ml. 0.001*M* mercuric

chloride or 0.28 μeq. sulfhydryl per g. A tabulation of these values follows:

–SH	–SH per g. Flour (dry basis)	
	No PCMB	*With PCMB*
	μeq.	μeq.
Total	0.56	0.62
Water-soluble	0.08	0.28
Difference	0.48	0.34

Since it is not possible to maintain a completely air-free environment in the procedure for the determination of water-soluble sulfhydryl, it is likely that a loss is effected through air oxidation. The higher the values obtained, the shorter the time between dispersion of flour and measurement of the supernatant liquid. Since sulfhydryl in the presence of PCMB ought to be protected from loss by other agents such as air or oxygen, the PCMB values might be considered as the true figures. The difference of total and water-soluble sulfhydryl might be regarded as representing the nonwater-soluble sulfhydryl in the procedure using PCMB.

Using the PCMB values as a reference, it might be said that $((0.62 - 0.56)/0.62) \times 100$, or 9.7%, of total sulfhydryl is lost in the procedure deleting PCMB, and $((0.28 - 0.08)/0.28) \times 100$, or 71%, of water-soluble sulfhydryl is lost in the procedure involving its measurement. Assuming air oxidation to have a negligible effect in the measurement of *total* sulfhydryl (either in the presence or absence of PCMB), it must be said that the loss of water-soluble sulfhydryl is much greater when PCMB is not present. The difference in *water-soluble sulfhydryl* measured with and without PCMB is 0.20 $(0.28 - 0.08)$, whereas the difference in total sulfhydryl with and without PCMB is 0.06 $(0.62 - 0.56)$. Thus, there is about four times as much sulfhydryl lost in the procedure for water-soluble as for total sulfhydryl. We believe this loss is due to oxidation of sulfhydryl by thioctic acid monoxide (3). There is probably a more intimate mixture of sulfhydryl and –SH-reacting factors in a water extract of flour than in a total flour dispersion where adsorption could inhibit their free movement. The reason for this loss will be explained in a later paper.

Generally, with all precautions taken, such as minimizing or eliminating oxidation by blowing nitrogen through solutions, by keeping temperatures low, and by protecting available –SH groups by PCMB, the results shown in this paper average somewhat lower than many figures reported by other investigators.

With the use of the best techniques currently available, –SH groups of flour appear to be about equally distributed between the water-soluble proteins and the gluten proteins, with none present in the lipid and none, of course, present in the starch.

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