Sulfhydryl Analysis. I. Determination of Free Sulfhydryls in Wheat Flour Doughs

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ABSTRACT

Methodology was developed for the determination of free sulfhydryls in wheat flours and doughs using NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole). The solubilization of protein by sonication in sodium dodecyl sulfate (SDS) was compared to proteolysis with Proteinase K in SDS. Oxidation during proteolysis and disulfide cleavage during sonication are the likely reasons for observed differences between the two solubilization methods. The sulfhydryl determination and both extraction procedures work effectively on model doughs and full bread formula.

The free sulfhydryl content of wheat flours may be an important determinant of the rheological properties and the action of chemical improvers in wheat flour doughs. Sulfhydryl-disulfide interchange reactions have been studied extensively. However, questions about their function still exist. One of the major obstacles in the measurement of free sulfhydryls in flour has been that they occur in very small concentrations. Furthermore, the dispersion of the flour matrix to obtain a homogeneous solution requires harsh treatment. This has made simple, reproducible determination difficult.

A number of methods for measuring free sulfhydryls in flour have been developed. One of the first and most widely used was the amperometric titration of free thiols with silver nitrate (Sokol et al 1959). The results obtained by Sokol and his coworkers using this method ranged from 0.66 to 1.30 μmols/g of material. Variations of the method were proposed by Hird and Yates (1961), who used methyl mercuric iodide as a titrant, whereas Sullivan et al (1961) found the method worked best using mercuric chloride. The results for the number of sulfhydryls obtained by both studies were in the same range as those obtained by Sokol et al (1959). Hird and Yates (1961) showed values of ~1.00–1.30 μmols/g of material, while Sullivan et al (1961) found a sulfhydryl content of 0.40 μmols/g of material. Clearly the results depend on the flour used, but all are in the same range. Tsen (1968) found that the procedure worked better in the absence of urea. He found sulfhydryl contents in the range 0.70–1.05 μmols/g of material.

Other work by Lee and Samuels (1962) used the radioactive tracer, C^{14} labeled S-(N-ethylsuccinimido)-L-cysteine, to measure the sulfhydryl content. However, the method is very time-consuming. Generally, the amperometric titration method continued to be used for many years with minor variations. In 1988, for example, the same basic method was used by Ewart (1988a) with phenyl mercuric acetate as the titrant.

In 1988, Ewart (1988b) used Ellman's reagent 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) to spectrophotometrically determine thiols in glutenin. Ellman's reagent reacts specifically with free thiols quantitatively releasing the nitro-thiobenzoate anion that exhibits a characteristic absorption at 412 nm in solutions where pH > 8. The concentration of thiol may be found from the molar extinction coefficient of $13{,}600 M^{-1}$ cm^{-1} for the anion (Flashner et al 1972). This use of DTNB appears much easier and less prone to errors than amperometric titration for measuring sulfhydryls.

This study investigated the use of 7-chloro-4-nitrobenzo-2-oxa-4,3-diazole (NBD-Cl), an alternative reagent for the spectrophotometric determination of sulfhydryls in wheat flour and doughs. The compound NBD-Cl contains a reactive chloride that readily reacts with sulfhydryls producing a product with a characteristic absorption at 420 nm.

The solubilization of the flour proteins before the assay of sulfhydryls was performed by either sonication or proteolysis. The sonication method is widely used for the study of wheat proteins. Work by Singh et al (1990) showed that a 95% extraction of unreduced proteins was possible from strong and weak flours using sonication in a sodium dodecyl sulfate (SDS)/phosphate buffer. This work also showed that the extracted protein size distribution depended on the time of sonication. They found that sonication (at 10W power) beyond 30 sec extracted almost no more protein, and, in some cases, caused unwanted heating. The results tended to suggest that factors maintaining protein aggregation were being irreversibly modified.

Proteolysis of flour proteins has been used with less frequency, although it was used before sonication. In one of the first attempts to measure free sulfhydryls, Kong et al (1957) used trypsin to completely solubilize the flour proteins. During the development of the amperometric sulfhydryl determination procedure, Sokol et al (1959) also used trypsin to digest the flour proteins. They stated that this seemed to work well, but problems were later encountered and the process was abandoned with no reason given. More recently Guthrie and Gruen (1991) have used pepsin in a study of disulfides in gluten.

The aim of this investigation was to develop methodology for determining free sulfhydryl groups in flour doughs using NBD-Cl as a spectrophotometric reagent. The methodology was applied to flour proteins dispersed by either sonication or proteolysis.

MATERIALS AND METHODS

Wheat Samples

This study concentrated on a single, commercial Australian wheat cultivar, Janz (protein content = 10.9%, N × 5.7, 14% mb). Details for other cultivars will follow in subsequent reports. The sample was obtained from the 1991 Interstate Wheat Variety Trials, milled on a Buhler test mill fitted with an entoleter (Butcher and Stewart 1972), and stored at 15°C.

Chemicals and Equipment

NBD-Cl was purchased from Sigma. Proteinase K was purchased from Boehringer Mannheim. All other reagents were analytical grade.

The reaction of NBD-Cl was followed in a Cary 4E spectrophotometer with a six-position autocell accessory. Absorbance readings were recorded at 1-min intervals with a read time of 2 sec for each cuvette. Position 1 in the autocell accessory was left vacant so that the NBD-Cl blank solution was not exposed to light during the delay period of each cycle. Absorbance readings were fitted to the equation:

$$A(t) = [A_\infty - A_0] \times [1 - \exp(-kt)] + R \times t + A_0$$

where $A(t)$ is the absorbance at 420 nm at time t; A_0 is the

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absorbance at $t = 0$; A_∞ is the absorbance at $t = \infty$ (when $R = 0$); k is the first-order rate constant of the reaction between NBD-Cl and sulfhydryl; and R is the residual rate constant. Values for these parameters were obtained from the nonlinear regression program supplied by the manufacturer. The value of $(A_\infty - A_0)$ was used to compute the moles of sulfhydryl titrated. The residual rate constant covers other much slower reactions occurring during the primary reaction.

An estimation of A_∞ may be obtained by extrapolating absorbance measurements at 30, 45, and 60 min back to 0 time. Our results indicate that this value is 19 ± 6% higher than $(A_\infty - A_0)$.

The suitability of the methodology for sulfhydryl estimations was verified using L-cysteine, thioglycolic acid, and β-mercaptopropionic acid. Standard solutions were derivatized. The data in Figure 1 demonstrates good linearity up to 80 μM sulfhydryl. The NBD-Cl concentration in the reaction mixture is 500 μM to ensure the derivatisation follows first-order kinetics. The molar extinction coefficient was 12,800M^{-1} cm^{-1} and compares well with the value of 13,000M^{-1} cm^{-1} for the NBD-thio derivative of glutathione quoted by Birkett et al (1970).

Treatment of Flour

Flour doughs were prepared for sulfhydryl analysis by mixing 80 g of dough with 48 ml of water (distilled, deionized) until the dough cleared, The dough was mixed in a four-prong National pin mixer (Finney modified). The dough was immediately frozen then freeze-dried, powdered, and stored at −20°C. Analysis was performed within two weeks of preparation to minimize natural oxidative effects.

Free Sulfhydryl Determination Using Sonication Extraction

Flour or freeze-dried dough (40 mg) and degassed 1% (w/v) SDS (1.0 ml) was sonicated for 30 sec in an Eppendorf tube using a micro-tip sonicator at 10 W. The resulting solution was centrifuged (11,640 × g for 5 min) and the supernatant removed.

The supernatant (0.2 ml) was added to 30 mM MOPS/1 mM ethylenediaminetetraacetic acid (EDTA) buffer (1.0 ml) at pH 7.0 (adjusted with 1M NaOH) in a 1.5-ml cuvette. A blank of 1% SDS and buffer was similarly prepared.

After positioning the cuvettes in the spectrophotometer, the reaction was started by the addition of 20 µl of a freshly prepared solution of NBD-Cl in dimethyl sulfoxide (6 mg/ml). The absorbance at 420 nm (A_{420nm}) was recorded at 1-min intervals for 70 min.

Free Sulfhydryl Determination Using Proteolytic Extraction

Flour or freeze-dried dough (40 mg) was suspended in 0.8 ml of Proteinase K (0.16 mg/ml) and 0.2 ml of 5% (w/v) SDS. The Proteinase K was prepared in 1mM $CaCl_2$. This could be stored at room temperature for up to one week. The flour suspension was mixed for 30 min using a small magnetic flea in an Eppendorf tube. The solution was then centrifuged as before and the supernatant treated as described above.

An example of the spectrophotometric trace obtained for an extracted flour sample is shown in Figure 2. Typical values of k and R for the reaction conditions employed were: $k = 0.14$ min^{-1} and $R = 1.5 \times 10^{-4}$ min^{-1}.

For comparison of extraction efficiencies between samples, the absorbance of a 1:6 dilution of the supernatant in buffer at 280 nm was measured. An absorbance of 1.0 at 280 nm was assigned the arbitary protein concentration of 1 mg/ml (Whitaker and Granum 1980, van Iersel et al 1985).

RESULTS AND DISCUSSION

A comparison of the two extraction methods is illustrated in Figures 3 and 4. From the regression lines in Figure 3, it appears

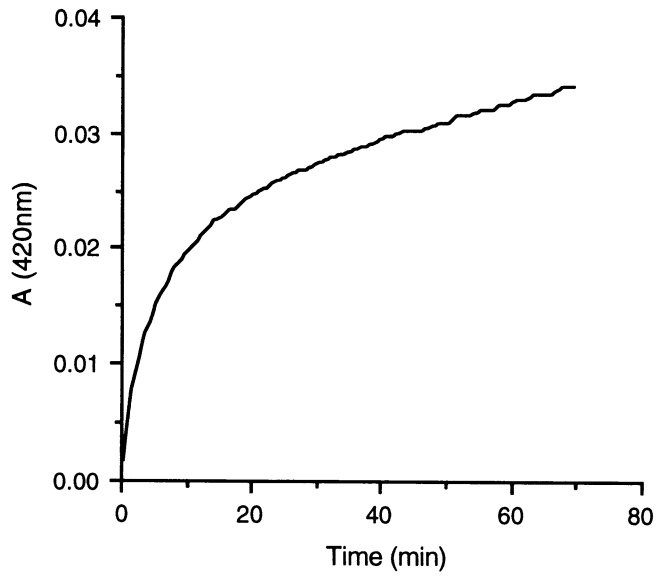

Fig. 2. Typical trace of absorbance vs. time for the reaction of NBD-Cl with an extract from Janz flour.

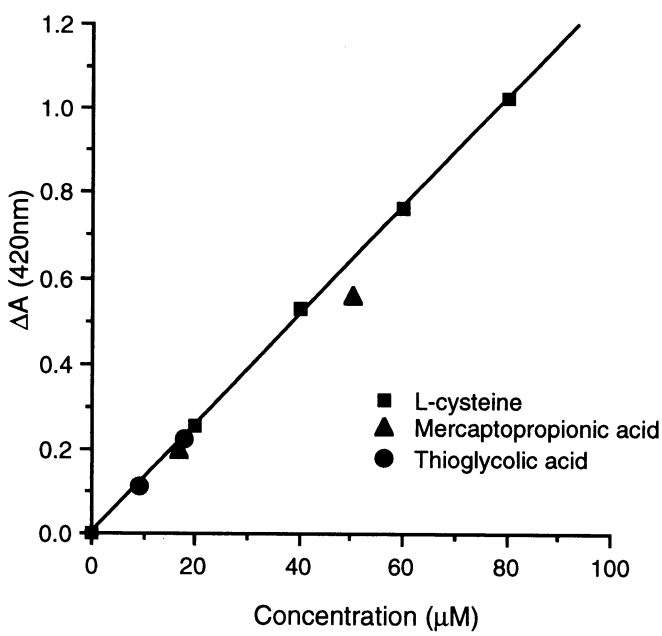

Fig. 1. Standard curve for sulfhydryl compounds derivatized with NBD-Cl.

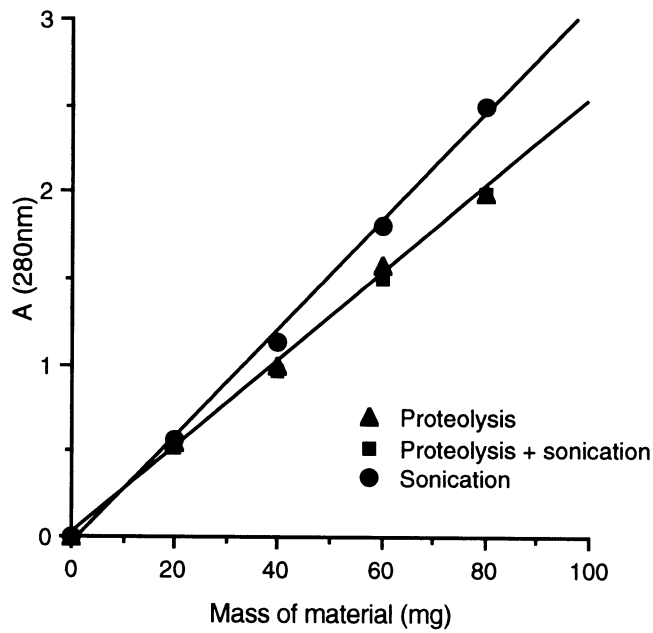

Fig. 3. Effect of different extraction procedures on protein content (measured by absorbance at 280 nm) of the same dough sample.

that ~20% less protein is extracted using proteolysis compared to sonication. The question of whether sonication was releasing more protein was addressed by sonication of samples after 30 min of proteolysis. The samples were sonicated in the proteolysis solution containing SDS, then centrifuged and the supernatants analyzed for protein as before. The data obtained is included in Figure 3 and suggests that some protein, otherwise extractable with sonication, is not extractable by sonication after treatment with Proteinase K. The data in Figure 4 suggests that the additional protein extracted by sonication contributes to a substantially higher (~37%) quantity of free sulfhydryls in the extracted solution. An analysis of the parent flour from which the freeze-dried doughs were derived showed an even larger variation. Parent flour extracted by sonication produced a value of 0.95 μmol -SH/g of flour, whereas proteolytic digestion produced a value of 0.45 μmol -SH/g of flour.

This discrepancy between the apparent number of free sulfhydryls obtained by each extraction method may be explained as either: 1) a decrease in free sulfhydryl groups during the time of the proteolysis due to oxidation; 2) the sonication method produces an elevated number of free sulfhydryls through a cleavage of disulfide linkages in the native gluten structure; or 3) a combination of 1 and 2.

The data in Figure 5 indicates that the free sulfhydryl content decreases by ~10% over a 30-min period of proteolysis with Proteinase K. However, sonicated samples stirred for up to 60 min show little or no decrease in free sulfhydryl content. This suggests that air oxidation is negligible under these conditions, and that the Proteinase K preparation may possess some thiol oxidase activity. The 10% decline, however, is still insufficient to account for the substantial difference (37%) between the two extraction methods. It is uncertain whether this thiol oxidase activity exists as a separate enzyme, or whether it is an inherent activity of the Proteinase K enzyme.

The work of Singh et al (1990) suggests that sonication can produce smaller molecules when it is extended beyond an optimum period. It is not inconceivable that a significant number of free sulfhydryls might be produced when the large cross-linked gluten molecule is subject to the shearing forces generated during the sonication process. This view is shared by Khan et al (1994) and Weegels et al (1994). The disulfide content of flour and bread has been reported elsewhere (Tsen and Anderson 1963, Hird et al 1968, Bloksma 1972), and typical values are in the range of 10–16 μmol -SS-/g of flour. A shearing of a small percentage

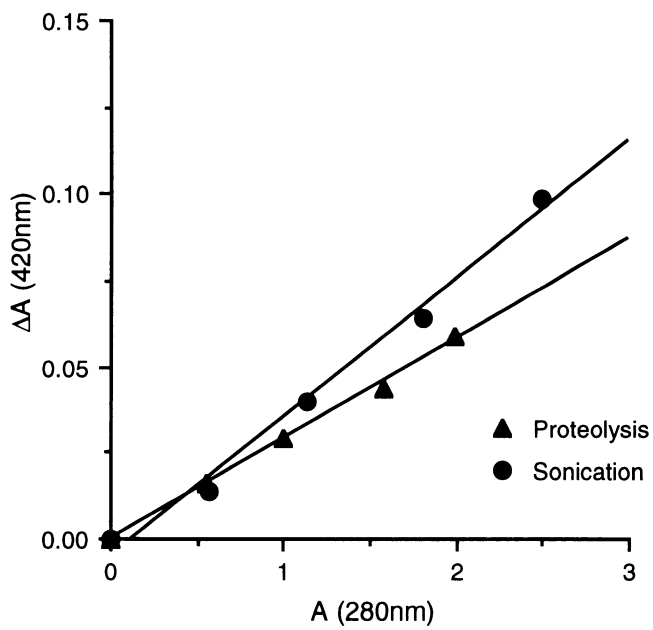

Fig. 4. Comparison between the protein content (A_{280nm}) and change in absorbance at 420 nm for proteolytic and sonication extraction procedures.

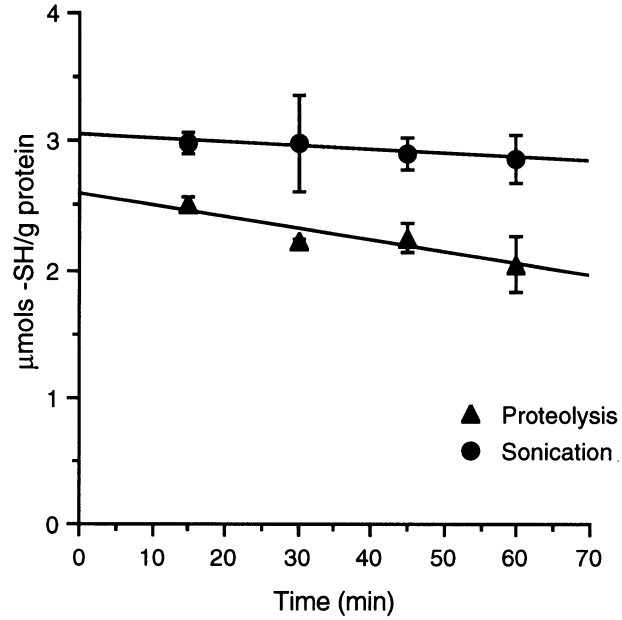

Fig. 5. Free sulfhydryls extracted from the same dough sample over a 1-hr time period using proteolytic and sonication extraction procedures.

of these disulfide linkages would produce a significant effect on the free sulfhydryl content of the extracted material. Proteolysis of dough before sonication would result in cross-linked peptides more resistant to bond shearing.

Sokol et al (1959) expressed concerns about proteolysis using trypsin. It is thought that the use of Proteinase K and SDS may be more effective. Proteinase K is an endopeptidase with no pronounced cleavage specificity. It is not inactivated by sulfhydryl reagents, but is stimulated when used in conjunction with SDS. This makes it a good proteolytic enzyme for flour, as the SDS will increase flour protein solubility. It also has a pH optimum around pH 7.0, and is unlikely to affect the pH of the MOPS/EDTA buffer used for sulfhydryl derivatization.

The levels of free sulfhydryls obtained in this study were approximately the same as those found by others (Sokol et al 1959, Sullivan et al 1961, Tsen 1968). Although the results depend on flour variety and other factors, such as heat and added improvers, the results were consistent with those found for medium protein flours.

The determination method was run (in triplicate), using both extraction procedures, on a full-bread dough (flour, water, fat, salt, and yeast). In addition to the basic bread sample, another sample contained the improvers malt flour and 100 ppm ascorbic acid. Both samples were freeze-dried and powdered after baking. The results displayed good reproducibility and showed that the sample with added improvers had less free sulfhydryls than the nonimproved sample. This is to be expected, as theory suggests that improvers convert free sulfhydryls to disulfides. It appears that the addition of salt, fat, yeast, and improvers has no effect on the free sulfhydryl determination procedure.

CONCLUSION

The method developed using NBD-Cl as a reagent for measuring free sulfhydryls is quick, relatively easy, and effective. Two extraction procedures for wheat flour and dough were studied and showed minor discrepancies in the quantitation of sulfhydryls. However, the authors favor enzyme digestion, as it is easier to control the reaction conditions and is not subject to the uncertainty of disulfide cleavage by the sonication process.

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