Direct Colorimetric Assay of Free Thiol Groups and Disulfide Bonds in Suspensions of Solubilized and Particulate Cereal Proteins[1]

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ABSTRACT

A direct colorimetric method that simultaneously combines measurement of solubilized and insoluble thiol groups and disulfide bonds in corn meal-based materials is described. Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid), which reacts specifically with thiol groups, or disodium 2-nitro-5-thiosulfobenzoate, which reacts with cysteine and thiol groups formed after reduction of disulfide bonds with sodium sulfite, were reacted directly with corn meal in the presence of surfactants (urea and/or sodium dodecyl sulfate), releasing the soluble chromophore 2-nitro-5-thiobenzoate. After a clarification step to remove suspended material, absorbance at 412 nm was read. This assay was highly reproducible, and measurements agreed with direct amino acid analysis. Twin-screw extrusion of corn meal at 150°C at moisture levels of 16 and 18% had no significant effect on cysteine or disulfide bond levels. Other possible changes such as disulfide bond rearrangements could not be determined by the mixed-phase assay. This method provides a rapid and convenient means for screening thiol and disulfide levels in insoluble proteinaceous materials.

Disulfide bonds are thought to play an important role in the texture of cereal-based products. However, because of the hydrophobic and insoluble nature of cereal proteins, quantification of thiol and disulfide bonds has proven difficult. Complete extraction of corn meal protein with sodium dodecyl sulfate (SDS), urea, and a reducing agent leads to cleavage of disulfide bonds. Furthermore, since extraction with SDS and urea results in only partial solubilization of the protein, assays based on solubilization followed by measurement of thiol and disulfide content often yield highly variable results and underestimates of true thiol and disulfide group content. A technique that avoids this initial protein solubilization step would largely eliminate these problems.

This article describes a solid-phase assay that simultaneously combines quantification of soluble and particulate thiol and disulfide groups in cereal-based proteins. The principle of this method is to suspend the entire sample in urea and to react it with a color reagent that will simultaneously react with both soluble and insoluble proteins, with release of a soluble chromophore. Ellman's reagent, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), which reacts specifically with thiol groups (Ellman 1958, Riddles et al 1983), and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$), which is used to quantify disulfide group content (Thannhauser et al 1987), are ideally suited for this purpose, since reaction with either results in the release of the 2-nitro-5-thiobenzoate anion (NTB^{2-}), which is soluble in aqueous solution. Following the removal of insoluble material by clarification steps, absorbance at 412 nm is then read. This method was used to assess the effects of twin-screw extrusion processing on thiol and disulfide levels in corn meal.

MATERIALS AND METHODS

Materials

Corn meal (12% moisture, 7% protein, 0.7% oil, 0.5% fiber, 0.4% ash, and 79.4% N-free extract) was obtained from Lauhoff Grain Co., Danville, IL. DTNB and $NTSB^{2-}$ were obtained from Aldrich Chemical Co., Milwaukee, WI. Extrusion was conducted in a Brabender type 2003 single-screw extruder with an axially ground barrel; length-to-diameter ratio, 20; screw diameter, 1.9 cm (0.75 in.); screw length, 38.1 cm (15 in.); and compression ratio, 1:3.

Solid-Phase Assay for Free Thiol Content

Colorimetric reactions were conducted under the conditions described by Ellman (1958, 1959). Unless otherwise indicated, samples (30 mg ground to 40 mesh and dried in vacuo) were suspended in 1.0 ml of reaction buffer consisting of 8*M* urea, 10 m*M* DTNB, 3 m*M* ethylene-diaminetetraacetic acid (EDTA), and 0.2*M* Tris-HCl, pH 8.0. SDS (1%) was present where indicated. Samples were incubated under N_2 for various intervals. To remove particulate matter, samples were centrifuged at 13,600 $\times$ *g* for 10 min in a microcentrifuge. A 0.1-ml aliquot of supernatant was removed and diluted with 0.9 ml of 8*M* urea, 1% SDS, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 8.0. This solution was centrifuged at 13,600 $\times$ *g*, and its absorbance was read at 412 nm.

Solid-Phase Assay for Total Sulfhydryl Group Content

Colorimetric reactions were conducted under the conditions described by Thannhauser et al (1987). Unless otherwise indicated, samples (30 mg ground to 40 mesh and dried in vacuo) were suspended in 1.0 ml of reaction buffer consisting of 8*M* urea, 0.1*M* sodium sulfite, 3 m*M* EDTA, 0.2*M* Tris-HCl, pH 9.5, and 10 m*M* $NTSB^{2-}$, synthesized from DTNB in the presence of sodium sulfite and O_2 as described in Thannhauser et al (1987). SDS (1%) was added where indicated. To remove particulate matter, samples were centrifuged at 13,600 $\times$ *g* in a microcentrifuge for 10 min. A 0.1-ml aliquot of supernatant was removed and diluted with 0.9 ml of 8*M* urea, 1% SDS, 0.1*M* sodium sulfite, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 8.0. This solution was centrifuged at 13,600 $\times$ *g*, and its absorbance was read at 412 nm. Disulfide group content was calculated as the difference between thiol group content before and after reduction of disulfide bonds with sulfite. Total cysteine was calculated as [-SH] + 2[-S-S-].

Two-Step Method for Sulfhydryl-Disulfide Assay

Unless otherwise indicated, samples (60 mg ground to 40 mesh and dried in vacuo) were extracted under N_2 with 2.0 ml of buffer consisting of 8*M* urea, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 8.0. On the basis of a time course of protein solubilization (Fig. 1A), extractions were conducted for 3 hr under N_2. To remove particulate matter, samples were centrifuged at 16,000 $\times$ *g* for 30 min in a Sorvall RC5C preparative centrifuge (Sorvall Instruments, Du Pont, Wilmington, DE). Aliquots of 0.2 ml of supernatant were removed and brought to 1.0 ml with appropriate DTNB- and $NTSB^{2-}$-containing reaction mixtures (see above) for spectrophotometric determination of thiol and disulfide groups.

RESULTS

Development of the Method

Protein solubility. With insoluble proteins, the extent of protein solubilization is dependent on solvent composition, exposure to O_2, and solubilization time. Figure 1 shows time courses of corn

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meal protein solubilization that confirm that maximal solubilization is obtained only when reducing agents are added. Furthermore, solubilization is enhanced when O_2 is excluded (Fig. 1). On the basis of Kjeldahl assays of protein remaining in the pellets, even under optimal conditions (urea, SDS, and β-mercaptoethanol combined) we found that only 85% of the protein could be solubilized. The pH of the solubilization mixture was varied between 7 to 10, and similar levels of protein were solubilized over this range. Therefore, to directly assay for thiol and disulfide levels present in the entire sample and to avoid tedious fractionation procedures, the direct colorimetric assay was developed.

Chemical and physical aspects of the direct colorimetric assay. A schematic of the mixed-phase method is shown in Figure 2. The key feature is that regardless of whether DTNB reacts with soluble or insoluble protein, the product of this reaction, the chromophore NTB^{2-}, is soluble in aqueous solution. NTB^{2-} gives a yellow color with an extinction coefficient of $13{,}600 M^{-1}$ cm^{-1} at 412 nm. These reactions are conducted in the presence of urea or urea and SDS to maximize the reactivity of any thiol or disulfide groups that may be buried within the hydrophobic protein matrix. In samples analyzed in tandem, disulfide bonds are determined indirectly with $NTSB^{2-}$ (Thannhauser et al 1987), which is added to protein suspensions with sodium sulfite. At pH 9.5, sodium sulfite completely cleaves disulfide bonds. One of the sulfur atoms forms a sulfated derivative, whereas the other forms the thiolate anion, which, with thiol groups from cysteine, reacts with $NTSB^{2-}$. Thus, $NTSB^{2-}$ quantifies total thiol and disulfide content, and disulfide content is then calculated by difference. Corn meal did not contain high enough levels of compounds that absorbed at 412 nm to interfere with measurement of NTB^{2-}.

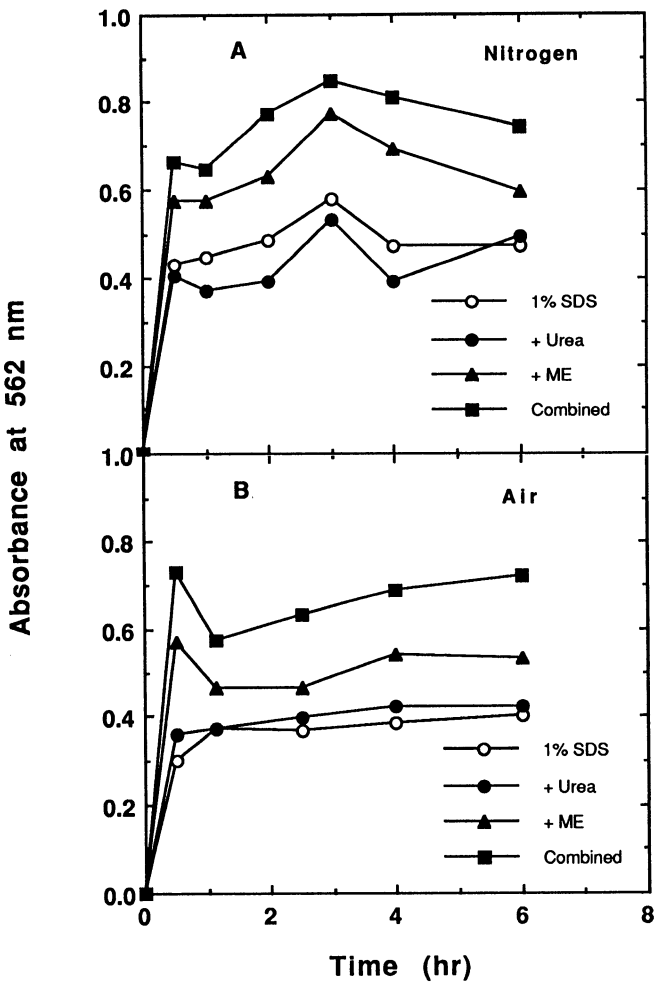

Fig. 1. Time course for protein solubilization from corn meal under nitrogen (**A**) and air (**B**). Native corn meal (60 mg) was solubilized at room temperature in 2 ml of 1% sodium dodecyl sulfate (SDS), 1% SDS and 8 *M* urea, 1% SDS and 2% β-mercaptoethanol (ME), or all combined in 12.5 m*M* sodium tetraborate buffer (pH 10.0) for various times as indicated. The samples were then centrifuged at 14,500 × *g* for 30 min, and aliquots ranging from 10 to 30 μl were transferred to a microcentrifuge tube and mixed with 1 ml of water. Soluble protein was assayed colorimetrically by the bicinchoninic acid assay with trichloroacetic acid precipitation (Brown et al 1989).

Clarification of protein suspensions. To complete this procedure, a clarification step must be conducted to remove suspended particles that would otherwise interfere with obtaining an accurate colorimetric reading. Various combinations of four procedures were evaluated (Table I): 1) centrifugation at 13,000 × *g* in a preparative centrifuge; 2) centrifugation at 13,000 × *g* in a microcentrifuge; 3) spin filtration through a 1-ml bed of glass wool in a microcentrifuge at 13,000 × *g;* and 4) dilution of the reaction mixture by 1:10, followed by centrifugation at 13,000 × *g* in a microcentrifuge.

The combination of all four steps resulted in complete clarification, but the process was time-consuming and labor-intensive. The combination of steps 2 and 4, microcentrifugation and dilution followed by a second microcentrifugation, proved most rapid and gave complete clarification (trial 5, Table I). This condensed procedure produced results equivalent to that of the four steps combined and was therefore incorporated as part of the standard procedure.

Time course and concentration dependence. Figure 3 shows time courses of both colorimetric reactions with corn meal. By 20 min or even less, each of the reactions reached completion. These time courses also demonstrated that NTB^{2-} is stable for at least 1 hr.

Figure 4 shows that absorbance was linear with the amount of sample. It should be noted that above 40 mg, assay mixtures would become viscous and difficult to dilute during the clarification step. Below 10 mg, increased weighing errors were apparent. On the basis of these considerations, a suitable sample weight range for corn meal is between 10 and 40 mg.

Comparison to literature values. As a positive control, the thiol and disulfide group content of lysozyme was determined. In SDS and urea the thiol content of lysozyme was measured to be 0.35 mol/mol and the disulfide content 3.87 mol/mol. These compared favorably with the literature values of 0 mol/mol and 4 mol/mol, respectively (Phillips 1974). SDS was critical. Without it,

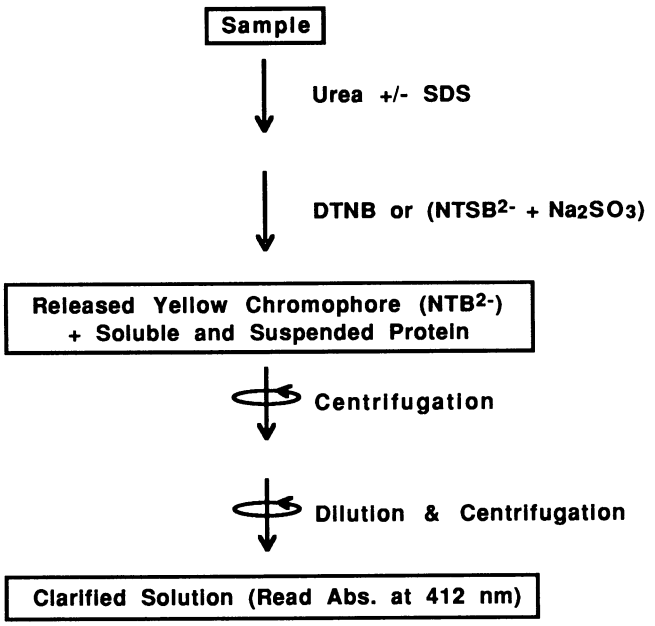

Fig. 2. Schematic of the direct colorimetric assay for thiol and disulfide groups. Samples were suspended in urea and/or sodium dodecyl sulfate (SDS) and reacted directly with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) or disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$). Clarification was conducted to remove suspended particles that would otherwise interfere with an accurate colorimetric reading.

TABLE I
Effects of Clarification Steps on Absorbances of 5,5′-Dithiobis (2-Nitrobenzoic Acid) (DTNB) and Disodium 2-Nitro-5-Thiosulfobenzoate ($NTSB^{2-}$)

Trial	Clarification Steps[a]				Absorbance, 412 nm	
	Preparative Centrifugation[b]	Microcentrifugation[c]	Spin Filtration[d]	Dilution and Microcentrifugation[e]	DTNB	$NTSB^{2-}$
1	−	−	−	−	1.212	1.685
2	+	+	+	+	0.093*[f]	0.343*
3	+	+	−	+	0.099*	0.365*
4	−	+	+	+	0.084*	0.340*
5	−	+	−	+	0.084*	0.354*
6	+	−	+	+	0.102*	0.358
7	+	−	−	+	0.122	0.358*
8	−	−	+	+	0.093*	0.312
9	+	+	+	−	0.284	0.414
10	+	+	−	−	0.248	0.458
11	−	+	+	−	0.170	0.444
12	−	+	−	−	0.160	0.496
13	+	−	+	−	0.262	0.497
14	+	−	−	−	0.480	0.667
15	−	−	+	−	0.203	0.408
16	−	−	−	+	0.143	0.364*

[a] +, Step used; −, step not used.
[b] Centrifugation at 16,000 × *g* for 30 min in a Sorvall centrifuge (model RC5C, Sorvall Instruments, Du Pont, Wilmington, DE).
[c] Microcentrifugation at 13,600 × *g* for 10 min using a Fisher microcentrifuge (model 235A, Fisher Scientific, Pittsburgh, PA).
[d] Conducted by adding 0.5 ml of sample to a 0.5-ml microcentrifuge tube filled with glass wool with a hole punched in its bottom. This was inserted into a 1.5-ml microcentrifuge tube and centrifuged for 3 min at 13,600 × *g*. The clarified solution was recovered from the lower tube.
[e] Tenfold dilution of the clarified solution from each step described above with either 8*M* urea, 1% sodium dodecyl sulfate (SDS), 3 m*M* ethylenediaminetetraacetic acid (EDTA), and 0.2*M* Tris-HCl, pH 8.0, for thiol group assay, or with 8*M* urea, 1% SDS, 0.1*M* Na_2SO_3, 3 m*M* EDTA, and 0.2*M* Tris-HCl, pH 9.5, for disulfide group assays. Dilution was followed by microcentrifugation (13,600 × *g*).
[f] Asterisks signify the absence of turbidity, defined by the lowest absorbance values.

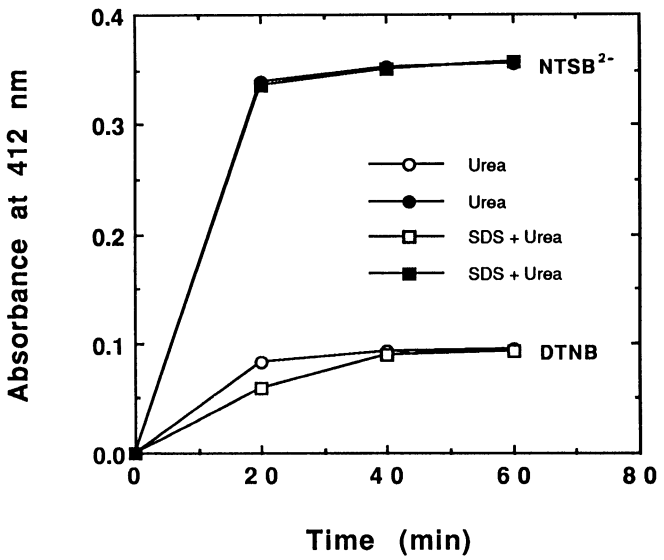

Fig. 3. Time courses of colorimetric reactions with corn meal. Samples were incubated at room temperature in 8*M* urea and 1% sodium dodecyl sulfate (SDS) and 8*M* urea with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) as described in Materials and Methods.

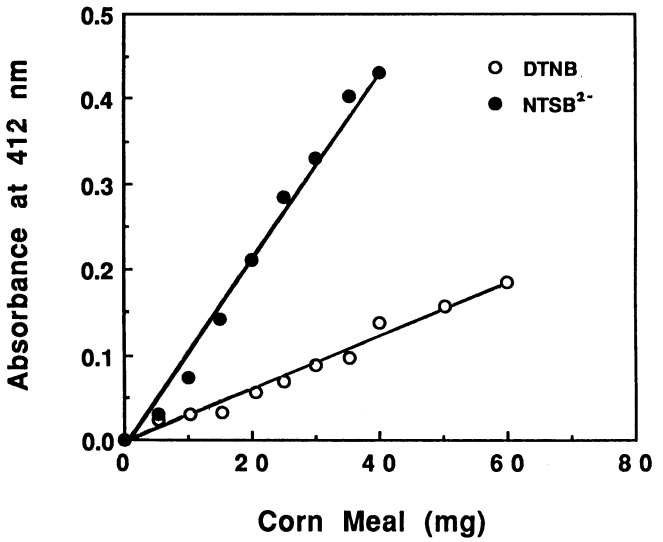

Fig. 4. Concentration dependence of the colorimetric reactions. Corn meal was incubated at room temperature in 8*M* urea with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$) and assayed as described in Materials and Methods.

the disulfide content was only 0.65 mol/mol, implying that thiol groups in lysozyme are buried and must be exposed before addition of color reagents. The total cysteine content of corn meal was 196.4 nmol/mg of protein, comparing favorably with the literature value of 199.7 nmol/mg (Lasztity 1984).

Comparison of the direct colorimetric assay with the two-step method. The direct colorimetric assay was compared with a two-step assay. In the latter, protein was first solubilized from samples with urea and SDS. After centrifugation, an aliquot of the supernatant was analyzed for free and total cysteine content with DTNB and $NTSB^{2-}$. Two major differences between each assay were observed (Table II). First, higher readings were obtained by the direct colorimetric assay. Most likely, this is due to the fact that some of the protein resists solubilization in SDS and urea, and it is therefore less available to react with DTNB and $NTSB^{2-}$ in the two-step assay. Second, the standard error was always significantly higher in the two-step assay. This increased variability also can be attributed to the incomplete release of protein during the solubilization step.

Application of the Method

Effect of extrusion on the thiol and disulfide group content of corn meal. The effect of twin-screw extrusion on the thiol and disulfide content of corn meal was determined. Table III shows that in the presence of 8*M* urea, extrusion caused an apparent 25–30% increase in free thiol content relative to that of the control. This was accompanied by a small decrease in disulfide groups. At most, only about 5% of the total cysteine content was affected, suggesting a small amount of extrusion-induced

rupturing of disulfide bonds. Total cysteine content did not change as the result of extrusion, indicating that significant oxidation or heat damage did not occur.

An observation of possible significance was decreased reactivity of thiol and disulfide groups after extrusion when SDS was added to reaction mixtures in the presence of 8*M* urea (Table III and Fig. 5). Figure 5A illustrates that addition of increasing levels of SDS to native corn meal had no effect on reactivity with either DTNB or $NTSB^{2-}$. However, in extrudates, addition of SDS at levels as low as 0.1% caused a significant (30%) decline in reactivity with both color reagents. (Fig. 5B and C). In light of the presumption that SDS, a powerful denaturant, would be expected to enhance the reactivity of thiol and disulfide groups found at the surface or buried within hydrophobic proteins, this finding was unexpected. Because SDS did not decrease the reactivity of native corn meal, it is unlikely that this decline is due to interference with the color reactions.

DISCUSSION

The direct colorimetric assay is a rapid and convenient method for quantifying the cysteine and cystine content of suspensions that contain a mixture of soluble and insoluble proteins. Here, assays were conducted directly on corn meal using the color reagents developed by Ellman (1958, 1959) and Thannhauser et al (1987). To maximize reaction of the protein with DTNB and $NTSB^{2-}$, surfactants (combinations of urea and SDS) were added to reaction mixtures. A clarification step was added to remove suspended material before reading of absorbance at 412 nm. The critical feature of this assay is that the NTB^{2-} chromophore is released into solution and does not covalently bind to protein.

With the exception of some radioisotopic (Schofield et al 1983) and amperometric (Redman and Ewart 1971) methods and direct amino acid analysis (Ewart 1988), most previous attempts to quantify thiol and disulfide content of insoluble samples required extraction of protein with denaturants and reducing agents and removal of the reducing agent before assay by colorimetric (Burgess and Stanley 1976, Hager 1984, Abe et al 1985) or other (Draper and Catsimpoolas 1978, Neumann et al 1984, Alary and Kobrehel 1987) means. The semidirect method described by Schofield et al (1983) for detection of thiol groups in gluten was based on the reaction of gluten with ^{14}C-iodoacetamide. Nonetheless, quantification of incorporated radiolabel required subsequent fractionation steps. The amperometric assay of Redman and Ewart (1970) was conducted directly on glutelin suspended in guanidine HCl. Incomplete extraction of protein and losses during fractionation steps could have led to significantly underestimated values for thiol and disulfide group content. Two major differences observed between the direct solid-phase and two-step

TABLE II
Comparison of the Direct Colorimetric Assay with a Two-Step Method[a]

Method	Sulfhydryls	Disulfides	Total Cysteine
Direct colorimetric	2.29 ± 0.08	6.28 ± 0.09	14.86 ± 0.20
Two-step[b]	2.09 ± 0.45	3.29 ± 0.73	8.68 ± 1.52

[a] Mean ± standard error of values for five replicates. Values are nanomoles per milligram of sample.
[b] The two-step assay, analogous to Hager (1984), consisted of solubilization in 1% sodium dodecyl sulfate and 8*M* urea, followed by centrifugation and assay of an aliquot of supernatant for thiol and disulfide group content (see Materials and Methods).

TABLE III
Effect of Twin-Screw Extrusion on the Free Thiol and Disulfide Group Content of Corn Meal[a]

	Extrusion Conditions				
	Temperature (°C)	H_2O (%)	Thiol	Disulfide	Total Cysteine
In 8*M* urea					
Native			31.0 ± 0.9	82.7 ± 1.1	196.4 ± 2.4
2/13-2	151	16.3	40.4 ± 1.2	80.3 ± 2.2	200.9 ± 4.6
3/13-1	152	18.2	38.2 ± 2.5	81.2 ± 3.7	200.6 ± 7.9
In 8*M* urea + 1% sodium dodecyl sulfate					
Native			30.4 ± 1.1	83.2 ± 1.3	196.9 ± 2.8
2/13-2	151	16.3	22.9 ± 2.2	77.6 ± 3.0	178.0 ± 6.3
3/13-1	152	18.2	25.4 ± 1.5	77.0 ± 2.7	179.4 ± 5.5

[a] Mean ± standard error of values for three replicates. Values unless otherwise noted are nanomoles per milligram of protein.

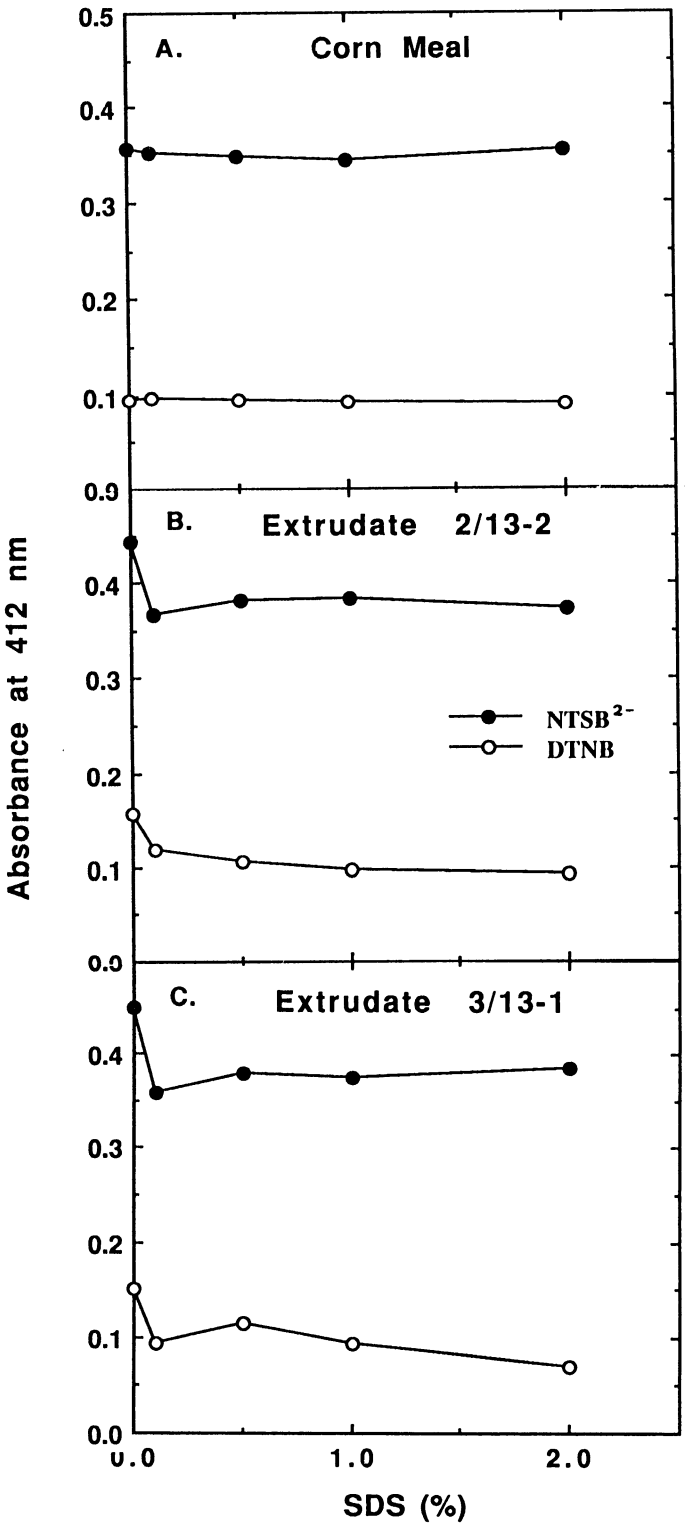

Fig. 5. Effect of sodium dodecyl sulfate (SDS) on reactivity with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5-thiosulfobenzoate ($NTSB^{2-}$). Native corn meal and extrudates 2/13-2 and 3/13-1 (**A–C**, respectively) were incubated at room temperature in 8*M* urea with 0–2% SDS with DTNB and $NTSB^{2-}$ and assayed as described in Materials and Methods.

methods (Table II) are that higher readings were obtained by the direct colorimetric assay because there is no opportunity for protein to be lost, and that standard errors were always significantly higher in the two-step assay.

The solubility of cereal proteins is strongly affected by reducing agents and O_2. The effect of O_2 in the solubilization time course was an observation of note (Fig. 1). In both the absence (Fig. 1A) and presence (Fig. 1B) of O_2, the first 30 min was characterized by a rapid release of protein. Under N_2, solubility increased in the presence of reducing agent for up to 3 hr (Fig. 1A); in air, protein solubility markedly declined between 30 min and 1 hr (Fig. 1B). Furthermore, total extractability is improved by exclusion of O_2, with about 12% more protein solubilized (Fig. 1A at 3 hr vs. Fig. 1B at 30 min). Since the rapid decline in solubility after 30 min in air (Fig. 1B) did not occur in the absence of reducing agent, the involvement of cysteine is likely. O_2 appears to promote reoxidation of thiols to disulfide bonds, thus causing some of the solubilized protein to precipitate.

The direct colorimetric technique was used to determine the effect of twin-screw extrusion on the thiol and disulfide content of corn meal. Table III suggests that a small amount of extrusion-induced rupturing of disulfide bonds occurred; however, this was statistically insignificant at the $P = 0.1$ level, with 5%, at most, of the cysteine content affected. These results are in accordance with Schofield et al (1983), who found that the free thiol group content was unaffected by heating at 100°C. In contrast, our results differ from the findings of Burgess and Stanley (1976) and Hager (1984), who found slight decreases in disulfide content but major increases in thiol groups in soy concentrate (70% protein on a dry basis) extruded at >180 and <150°C, respectively. Since the direct colorimetric technique does not measure disulfide bond rearrangements, the extent to which these may occur cannot be ascertained.

The unexpected but significant decrease in reactivity of both thiol and disulfide groups of extruded corn meal upon addition of SDS to the reaction mixtures (Fig. 5) suggests that extrusion-induced conformational changes in protein structure may inhibit access of colorimetric reagents when SDS is present. One possible explanation for this finding is that conformational changes in protein structure inhibit access of these reagents when SDS is present. Alternatively, the cysteine in extrudates is more prone to oxidation after extrusion. The decrease in total cysteine (197 vs. 179 nmol/mg of protein in native and extruded corn meal, respectively; Table III) is consistent with this possibility.

Although this study focused specifically on corn meal, the direct colorimetric assay should be applicable to a wide range of protein-containing products.

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