JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Comparative Studies on Sulfhydryl Determination of Soy Protein Using Two Aromatic Disulfide Reagents and Two Fluorescent Reagents

Qijun Ruan, Yeming Chen, Xiangzhen Kong, and Yufei Hua*

State Key Laboratory of Food Science and Technology and School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, Jiangsu Province, People's Republic of China

ABSTRACT: In this study, the sulfhydryl (SH) contents of unheated and heated (90 °C, 5 min) soy protein were detected under different conditions (pH, reagent addition order, SDS/GuHCl concentration, EDTA) using two aromatic disulfide reagents: 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 4,4′-dithiodipyridine (DPS). Two fluorescent alkylating reagents, monobromobimane (mBBr) and *N*-(1-pyrenyl)maleimide (NPM), were chosen due to their high sensitivity and were also used. Amino acid analysis was used to detect the SH (cysteine) contents of unheated (7.51 ± 0.45 μmol SH/g protein) and heated (1.47 ± 0.10 μmol SH/g protein) soy protein, and similar results were obtained using enzymatic hydrolysis-assisted DPS. The SH content detected by DTNB was affected by pH, denaturant species, and denaturant concentration, and the best results were obtained at pH 7.0 when 6 M GuHCl was added after DTNB. These results were lower than that of the amino acid analysis, however. The SH detected by DPS was not as affected as that of DTNB by pH, denaturant species, and denaturant concentration. Additionally, the results of the amino acid analysis were similar to that of DPS at pH 7.0 in 2% SDS and 4−6 M GuHCl when SDS and GuHCl were added after DPS. EDTA did not have a significant effect on SH detection when DTNB and DPS were added before SDS and GuHCl. Finally, although mBBr and NPM can detect SH in low protein concentrations ($^{1}/_{10}$ of that required for DTNB and DPS), mBBr and NPM overestimated the SH content of soy protein. Therefore, using DPS at pH 7.0 when it is added before SDS and GuHCl is the most reliable method for detecting the SH content of soy protein.

KEYWORDS: *soy protein, sulfhydryl (SH), cysteine, DTNB, DPS, mBBr, NPM*

■ INTRODUCTION

Sulfhydryl (SH) groups and disulfide bonds (SS) are two important functional groups of soy protein due to their influence on protein aggregation,[1,2] gel strength,[3,4] surface functional properties,[5] protein digestibility,[6] and soy allergy.[7] Consequently, it is important to detect SH groups and SS in soy protein under both nonreducing and reducing conditions. In this study, we measured SH in soy protein under nonreducing conditions.

Traditionally, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is used to detect the SH content of soy protein. DTNB is reacted with thiolate anion (S^-) to form 2-nitro-5-thiobenzoic acid (NTB) (Figure 1), which is detected at an absorbance of 412 nm.[8] Studies have previously reported a wide range for the SH content of unheated soy protein (2−8 μmol SH/g protein).[1,2,9−13] This variation is likely explained by the use of different soybean varieties and different methods of soy protein preparation. The different conditions used for detecting SH may also be a contributing factor, however. In this study, we examined the effects of pH, DTNB, and denaturant (SDS/GuHCl) addition order, denaturant concentration, and EDTA on the measured SH content of soy protein. The studies above used different denaturants (1% SDS, 9 M urea, 8 M urea, a mixture of 8 M urea and 0.5% SDS, and a mixture of 6 M urea and 0.5% SDS), different DTNB and denaturant addition orders,[13] and different EDTA concentrations (1−6 mM). Urea has been shown to contain varying amounts of cyanate, which can react with SH to form thiocarbamates, thereby decreasing the amount of SH detected.[15] Additionally, because the thiolate anion (S^-) is intrinsically one of the strongest biological nucleophiles, the SH group of cysteine is one of the most reactive functional groups found in proteins. Therefore, adding a denaturant before DTNB can unfold the soy protein, and this can result in unexpected SH oxidation before DTNB is added. A low concentration of EDTA (0.2 mM), which inhibits SH oxidation by chelating metal ions, may also be the ideal concentration for SH detection.[16] Finally, the studies above used a single alkaline pH of 8.0. Thurlkill reported that the pK_a of cysteine residues is 8.55 ± 0.03,[17] and high pH has been closely correlated with high DTNB hydrolysis[16] and increased SH to SS oxidation.[18]

Heat treatment is an important process for soy products. It induces protein aggregation,[1,19] resulting in heated soy protein possessing a different structure from that of unheated soy protein. Additionally, SH/SS exchange,[2] SH oxidation,[6] and SS β-elimination[20] may also all occur when heated. Woodward et al. indicated that *N*-(1-pyrenyl)maleimide (NPM) was better than DTNB for SH detection in reduced cellobiohydrolase,[21] and Riener et al. showed that the SH of many proteins had incomplete reactions with DTNB.[16] Therefore, it is doubtful whether DTNB is suitable for detecting SH of both unheated and heated soy protein. An alternative to DTNB is 4,4′-dithiodipyridine (DPS). DPS reacts with SH to form 4-

Received: July 12, 2012
Revised: February 20, 2013
Accepted: February 24, 2013
Published: February 25, 2013

ACS Publications © 2013 American Chemical Society

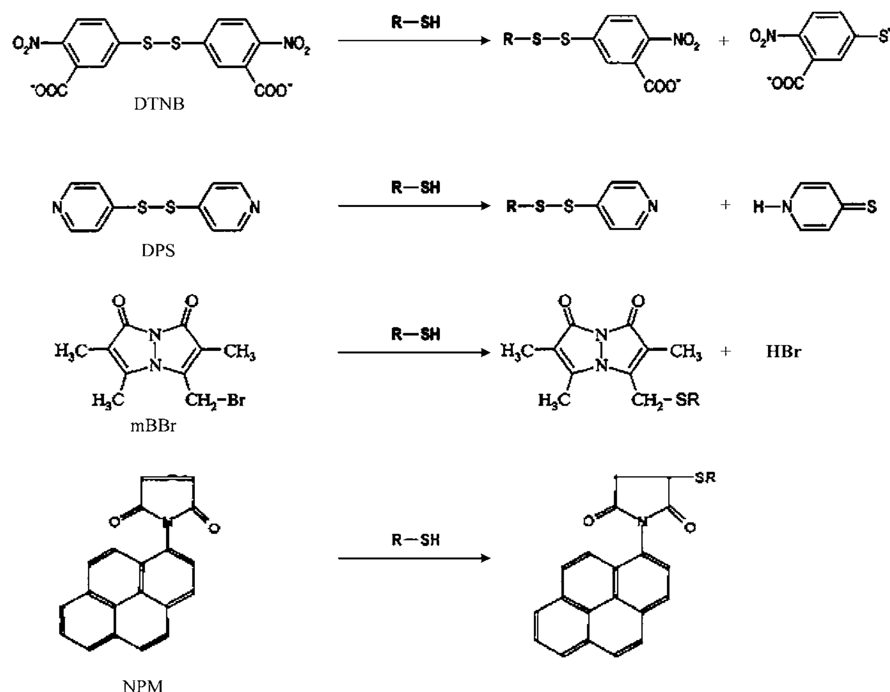

Figure 1. Molecular structures of four SH-detecting reagents and their reactions with SH.

thiopyridone (4-TP), which has an absorbance of 324 nm (Figure 1).[22] Because of its amphiphilic nature, small size, and lack of charge, DPS is preferable to DTNB.[16] DPS is rarely used with soy protein, however. Therefore, we also compared the SH detected by DPS to that of DTNB while testing the different factors above.

We used two fluorescent reagents with high sensitivity to detect the SH of soy protein. Fluorescent labeling of SH, combined with chromatographic or electrophoretic separation, is now a sensitive method for identifying SH-containing proteins or polypeptides.[26,27] Monobromobimane (mBBr) is a lipophilic fluorescent reagent that reacts with protein SH in a nucleophilic substitution reaction (Figure 1).[23] The resulting mBBr-protein derivative can be detected and quantified using fluorescence spectroscopy.[24] We also used NPM, which can spectrophotometrically detect SH in monoclonal antibodies.[25] Few studies have been reported using these fluorescent reagents with food proteins, however.

An accurate SH content is crucial to understanding and controlling functional properties of soy protein in food systems. In this study, we examined the effects of pH, DTNB/DPS, and denaturant (SDS/GuHCl) addition order, denaturant concentration, and EDTA on SH detection. mBBr and NPM were also used to detect SH content using fluorescence spectroscopy. These results were compared with the cysteine residues of soy protein, as determined by amino acid analysis, to identify the best reagent for detecting SH as well as the best conditions for detection.

■ MATERIALS AND METHODS

Materials. The following chemicals were purchased from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China): 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB); 4,4′-dithiodipyridine (DPS); *N*-(1-pyrenyl)maleimide (NPM); sodium dodecyl sulfate (SDS); monobromobimane (mBBr); and *N*-ethylmaleimide (NEM) All other chemicals were of analytical reagent grade.

Soy Protein Preparation. Dehulled and milled soybean flour was treated five times with *n*-hexane to remove any oil. The defatted flour was then mixed with hexane/ethanol (1:2, v/v) for 1 h at 4 °C. The slurry was vacuum filtered, and the filter cake was mixed with 95% (v/v) ethanol for 1.0 h at 4 °C. After drying, the meal was dispersed in distilled water (1/10, w/v) and adjusted to pH 7.0 by 2 M NaOH. The suspension was stirred for 1 h at 20 °C and then centrifuged (15800*g*, 30 min) at 4 °C. The resulting supernatant was adjusted to pH 4.5 by 2 M HCl and centrifuged (6000*g*, 30 min) at 4 °C. The pellet was washed twice with distilled water, resuspended in 5 times (w/w) the distilled water, and then adjusted to pH 7.0 by 2 M NaOH. This mixture was centrifuged (15000*g*, 30 min) at 4 °C, and the supernatant was then freeze-dried and stored at 4 °C. Protein content of the prepared soy protein was 90% (w/w) as determined by the micro-Kjeldahl method.

Heat Treatment of Soy Protein. Soy protein was dispersed in 0.01 M sodium phosphate buffer (PBS buffer, pH 7.0) at 30 g/L and magnetically stirred. The suspensions were centrifuged (40000*g*, 30 min), and the supernatant was then transferred into screw-capped test tubes. The tubes were heated in a 90 °C water bath for 5 min and immediately cooled in an ice water bath. Protein content was determined according to the Bradford method.

Preparation of Denaturant Buffers. Guanidine hydrochloride (GuHCl) was dissolved in 0.1 M PBS buffer, equilibrated at 25 °C, and adjusted to different pH values (6.0, 7.0, 7.5, and 8.0) by HCl or NaOH as needed. The final GuHCl concentration was 6.8 M (designated 6.8 M GuHCl buffer). GuHCl buffers with different concentrations (1.13, 2.26, 2.83, 3.4, 4.52, and 5.66 M) and pH 7.0 were also prepared as described above.

SDS was dissolved in 0.1 M PBS buffer and adjusted to different pH values (6.0, 7.0, 7.5, and 8.0) by HCl or NaOH as needed. The final SDS concentration was 12% (w/v) (designated 12% SDS buffer). SDS buffers with different concentrations (1.5 and 3%) and pH 8.0 were also prepared in 0.1 M PBS buffer.

Molar Extinction Coefficients of NTB or 4-TP under Different Conditions. The molar extinction coefficients of NTB or 4-TP under different conditions were determined according to the method of Riener.[13] About 50 mL of water and 0.42 mL of concentrated HCl were added to 181.1 mg of cysteine and stirred until the cysteine dissolved. The solution was then diluted to 500 mL with water (3 mM cysteine) and used within 0.5−1 h. For the DTNB and DPS methods,

3 mL of buffers was mixed with 0, 10, 20, 30, or 40 μL of 3 mM cysteine, and DTNB or DPS (125 μL, 4 mM in 12 mM HCl) was added. Absorbances were recorded at 412 nm (DTNB) or 324 nm (DPS) and plotted against cysteine concentration of the final assay volumes to obtain molar extinction coefficients of NTB or 4-TP. The molar extinction coefficients of NTB and 4-TP under different conditions are shown in Table 1.

Table 1. Molar Extinction Coefficients of NTB and 4-TP in the Absence of Denaturants, in the Presence of SDS, and in the Presence of GuHCl[a]

	DTNB ($\pm 100\ M^{-1}\ cm^{-1}$)	DPS ($\pm 100\ M^{-1}\ cm^{-1}$)
none	14300	21500
2% SDS, pH 6.0	13200	21300
2% SDS, pH 7.0	13900	21000
2% SDS, pH 7.5	14200	19800
2% SDS, pH 8.0	14000	18800
6 M GuHCl, pH 6.0	12300	20200
6 M GuHCl, pH 7.0	12400	20200
6 M GuHCl, pH 7.5	13300	20900
6 M GuHCl, pH 8.0	13100	20800
0.245% SDS, pH 7.0		21400
0.49% SDS, pH 7.0		21300
0.245% SDS, pH 8.0	14200	
0.49% SDS, pH 8.0	14200	
1 M GuHCl, pH 7.0	13200	20900
2 M GuHCl, pH 7.0	12900	20900
2.5 M GuHCl, pH 7.0	13300	20600
3 M GuHCl, pH 7.0	13200	20700
4 M GuHCl, pH 7.0	13000	20500
5 M GuHCl, pH 7.0	12200	20400

[a]Standard deviations were all $\leq 100\ M^{-1}\ cm^{-1}$.

SH Determination Using DTNB. Aliquots of soy protein solution were diluted using the following three procedures. Procedure A: a 0.14 mL volume of soy protein solution was diluted to 1.2 mL with 0.1 M PBS buffer (pH 7.0). Procedure B: soy protein solutions were diluted to 1.2 mL with different concentrations of GuHCl buffer; final GuHCl concentrations were 1, 2, 2.5, 3, 4, 5, and 6 M. Procedure C, soy protein solutions were diluted to 1.2 mL with different concentrations of SDS buffer; final SDS concentrations were 0.245, 0.49, and 2% (w/v). The soy protein was diluted to a concentration of 3.5 g/L, and 50 μL of 4 mM DTNB was added to react with the SH. The samples were vortexed, and their absorbances were immediately measured against a buffer blank at 412 nm by a UV-2450 UV−vis spectrophotometer (Shimadzu, Kyoto, Japan) until a maximum absorbance was reached. For the parallel reagent blank, soy protein solution was replaced with 0.01 M PBS buffer (pH 7.0), mixed with 50 μL of DTNB, and incubated for the same amount of time. For the parallel protein blank, 1.2 mL of diluted soy protein solution was mixed with 50 μL of water. SH content was expressed as micromoles SH per gram protein.

SH Determination Using DPS. The same method used for DTNB was used with DPS except that DTNB was replaced by DPS, and the absorbance was detected at 324 nm.

SH Determination Using Enzymatic Hydrolysis-Assisted DPS. A 0.28 mL volume of soy protein solution was diluted to 2.4 mL (3.5 mg protein/mL) with 0.1 M PBS buffer (pH 7.0). A 100 μL volume of 4 mM DPS and then 3% (enzyme/substrate w/w) Neutrase (EC 3.4.24.28, Novozymes, Bagsvaerd, Denmark) were added, and the solution was incubated in a 25 °C water bath for 1 h. Then, SDS was added to a SDS concentration of 2%. Absorbance was measured against a buffer blank at 324 nm by a UV-2450 UV−vis spectrophotometer (Shimadzu). The parallel reagent blank was prepared without the addition of protein, and the soy protein hydrolysate without DPS was used for the parallel protein blank. SDS-PAGE was used to examine the enzymatic hydrolysis result.

SH Determination Using mBBr. Soy protein solutions were diluted to 0.9 mL (0.3 g protein/L) with 6.8 M GuHCl buffer (pH 7.0). The final GuHCl concentration was 6 M. The SH of the soy protein solutions was labeled by incubating overnight with a 10 times molar excess of mBBr in the dark at 4 °C. A 0.5 mL volume of dichloromethane was then added, and the solutions were vortexed. The organic phase containing excess mBBr was discarded after centrifugation (15000*g*, 15 min) at room temperature. These steps were conducted under dim lighting because mBBr is light sensitive. The fluorescence emission spectra of the supernatants were measured between 400 and 600 nm with excitation at 390 nm. The spectra were measured using a quartz microcuvette (internal dimensions 4 mm × 4 mm × 45 mm) and an F-7000 spectrofluorometer (Hitachi, Tokyo, Japan) set at 5 nm slit width (excitation/emission) with high sensitivity. The fluorescence intensities of the reagent and protein blanks were subtracted for each sample. Fluorescence intensity was converted to SH concentration using standard curves from pure L-cysteine.

SH Determination Using NPM. Soy protein solutions were diluted to 0.9 mL (0.3 mg protein/mL) with PBS or 12% SDS buffer (pH 7.0). The final SDS concentrations were 2% (w/v). The SH of the soy protein solutions was labeled by incubating overnight with a 10 times molar excess of NPM at 4 °C. The fluorescence emission spectra of the solutions were measured between 350 and 500 nm with excitation at 330 nm using an F-7000 spectrofluorometer (Hitachi). The fluorescence intensities of the reagent and protein blanks were subtracted for each sample. Fluorescence intensity was converted to SH concentration using standard curves from pure L-cysteine.

Cysteine Determination Using Amino Acid Analysis. The SH of soy protein was blocked by a procedure modified from that of Rogers et al.[28] Briefly, 2.4 mL of soy protein (30 g/L), containing ~0.58 μmol of SH (as measured by DPS with 2% SDS), was incubated in 2% SDS with a 125 times molar excess of NEM for 4 h. The SDS and excess NEM were removed using gel filtration on a 5 mL Sephadex G-25 (coarse) column (GE, HiTrap).

Then, 36 mg of soy protein and SH-blocked soy protein were vacuum-dried, and performic acid was added. The resulting mixture was incubated at 0 °C for 20 h. Both cysteine and cystine can be converted into cysteic acid by performic acid oxidation.[29] The NEM−cysteine thioether derivative cannot be converted into cysteic acid, however. Cysteic acid was separated from the other amino acids using a Hitachi L-8900 amino acid analyzer after digestion in 6 M HCl at 110 °C for 22−24 h. The total cysteine and cystine content of soy protein (C_1) and SH-blocked soy protein (C_2) were determined by the form of cysteic acid. The cysteine content of soy protein was calculated by subtracting C_2 from C_1.

Statistical Analysis. Three separate soy samples were used, and each sample was run in triplicate. Data were subjected to an analysis of variance (ANOVA) with SAS 9.1 package (SAS 2005). Significant differences among variables were determined by LSD all-pairwise multiple comparisons ($p < 0.05$). Data were expressed as the mean ± SD ($n = 3$).

RESULTS AND DISCUSSION

SH Detection by the Two Aromatic Disulfide Reagents DTNB and DPS. *Effect of pH on SH Detection.* A pH of 8.0 is usually selected for detecting the SH content of soy protein when using DTNB.[1,2,9−13] This pH is used because DTNB is reacted with thiolate anion (S^-).[15] However, DPS has the advantage of reacting with SH at acidic pH values because the decrease in thiolate anion (S^-) concentration is partially compensated for by an increase in the protonation of the pyridine ring nitrogen.[15] Therefore, we tested four pH values (6.0, 7.0, 7.5, and 8.0) for SH detection. GuHCl and SDS were

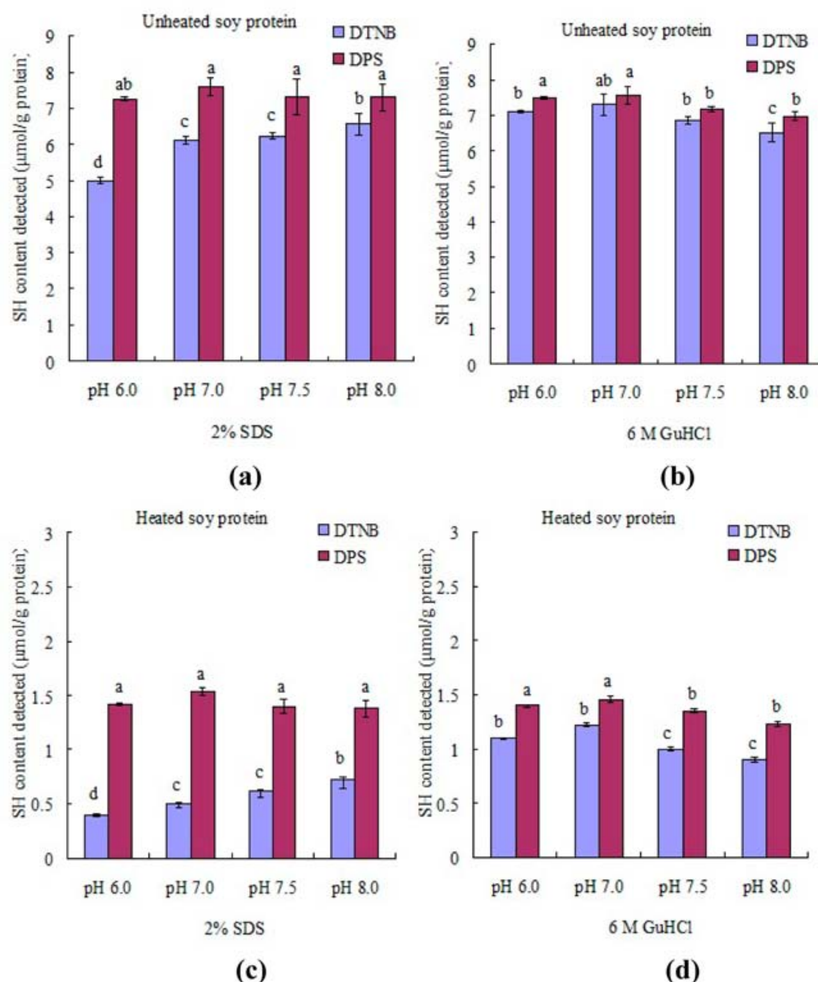

Figure 2. Effect of pH on the SH content determined using two aromatic disulfide reagents (DTNB and DPS). The SH contents of unheated and heated (90 °C, 5 min) soy protein were determined while using 2% SDS and 6 M GuHCl. Four different pH values (6.0, 7.0, 7.5, 8.0) were used. Means (n = 3) with different letters were significantly different ($p < 0.05$).

added after DTNB and DPS were mixed with the soy protein to weaken any unexpected SH oxidation caused by SH exposure to GuHCl and SDS.

One principal advantage of DTNB and DPS is their high specificity for SH.[15] The more SH detected by DTNB and DPS, the closer the value was to a reliable protein SH content. On this reasoning, the pH at which the highest SH content is detected should be considered the best pH for SH detection. Panels a and b of Figure 2 show that, for unheated soy protein, the highest SH content detected in 2% SDS was at pH 8.0 and pH 7.0 for DTNB and DPS, respectively, and at pH 7.0 in 6 M GuHCl for both DTNB and DPS. Panels c and d of Figure 2 show the SH content detected in heated soy protein follows the same trend as in unheated soy protein, although the detected SH is much lower than that of unheated soy protein. Thus, pH 7.0 was used for SDS and GuHCl with DPS, and pH 7.0 was used for GuHCl and pH 8.0 for SDS with DTNB.

Effect of Reagent Addition Order and Denaturant Incubation Time on SH Detection. After proteins are unfolded by denaturants, buried SH groups are exposed to the exterior, and unexpected SH oxidation may occur.[15] Therefore, we used the addition of SDS and GuHCl after DTNB and DPS as controls. When SDS and GuHCl were added before DTNB and DPS with no incubation time, the detected SH contents were similar to their corresponding controls. The SH detected decreased with incubation time, however, for every condition (Figure 3). A previous study reported that DTNB addition before GuHCl was important for SH detection,[14] and these data clearly indicated that DTNB and DPS should be added before SDS and GuHCl for the detection of SH content.

Effect of Denaturant Concentration and EDTA on SH Detection. Adding 1.4 g of SDS can bind, at most, 1 g of protein, and 6 M GuHCl is the typical concentration used for unfolding protein.[30,31] Consequently, we tested three ratios (0.5:1, 1.4:1, and 5:1, w/w) of SDS/protein and a concentration range of 1−6 M GuHCl for SH detection.

With SDS, the SH detected by DTNB increased with SDS concentration for unheated soy protein. The SH detected by DPS increased between no SDS and 0.245% SDS, and higher SDS concentrations did not significantly affect SH detection (Figure 4a). In heated soy protein, the SH detected by DTNB also increased with SDS concentration, whereas the concentration of SDS did not significantly affect SH detection by DPS (Figure 4b). These results can be closely correlated with the increased extent of soy protein unfolding that occurs with increased SDS concentration.[30] Additionally, we found that DPS detected more SH than DTNB under all conditions. The highest SH contents detected for unheated (7.60 ± 0.26 μmol/

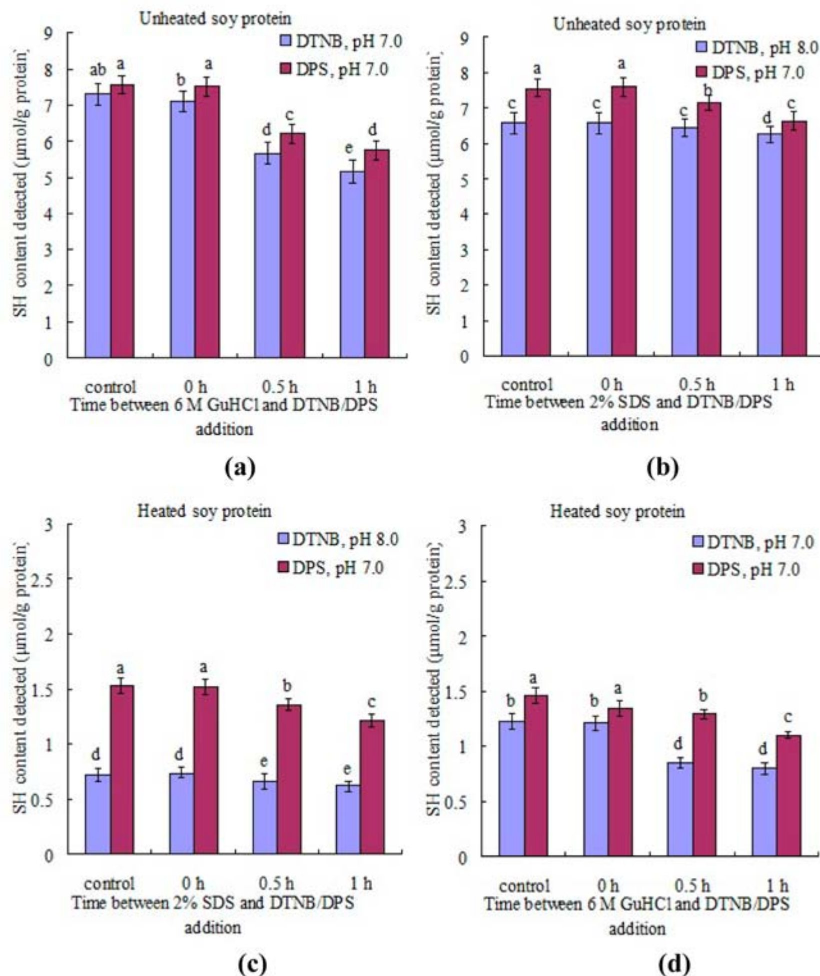

Figure 3. Effect of reagent addition order and denaturant incubation time on the SH content determined using DTNB and DPS. The SH contents of unheated and heated (90 °C, 5 min) soy protein were determined while using 2% SDS and 6 M GuHCl. Control, reagent added before SDS and GuHCl; 0 h, reagent added after SDS and GuHCl with no incubation time; 0.5 h, reagent added after the soy protein was incubated with SDS and GuHCl for 0.5 h; 1 h, reagent added after the soy protein was incubated with SDS and GuHCl for 1 h. Means (n = 3) with different letters were significantly different (p < 0.05).

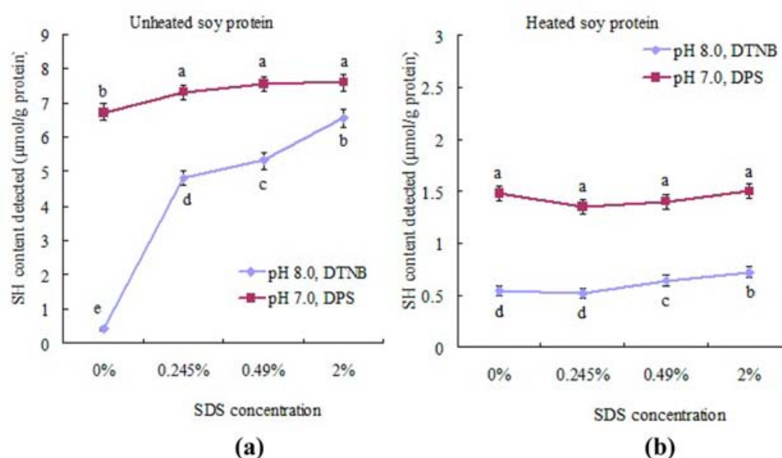

Figure 4. Effect of SDS concentration on the SH content determined using DTNB and DPS. Final SDS concentrations were 0.245, 0.49, and 2%. DTNB was used at pH 8.0, and DPS was used at pH 7.0. Means (n = 3) with different letters were significantly different (p < 0.05).

g protein) and heated (1.53 ± 0.04 μmol/g protein) soy protein were both detected using 2% SDS and DPS. These results agree with those reported by Hansen et al.[15] and Riener et al.[16] and are also closely correlated with the different properties of DTNB and DPS. DPS reacts with the SH in proteins by weakening the steric hindrance and avoiding electrostatic repulsion due to its smaller size, amphiphilic nature, and lack of charge.[15] Together, these data showed that

SDS was not a suitable denaturant for SH detection with DTNB, whereas 2% SDS was suitable for SH detection with DPS.

The SH detected by DTNB increased with GuHCl concentration. The SH detected by DPS decreased between no GuHCl and 2.5 M GuHCl and increased between 2.5 and 6 M GuHCl for unheated soy protein. A similar trend was observed in heated soy protein (Figure 5). The DTNB results can be explained by the increased extent of soy protein unfolding that occurs with increased GuHCl concentration.[30] For DPS, the reason for the initial decrease and subsequent increase was unclear. We did not find any other studies examining the effect of GuHCl concentration on SH detection with DPS. The SH detected by DTNB was lower than that by DPS for all GuHCl concentrations. Again, this difference can be correlated to the different properties of DTNB and DPS as stated above. These data showed that GuHCl was also not a suitable denaturant for SH detection with DTNB, whereas a concentration between 4 and 6 M GuHCl was suitable for SH detection with DPS.

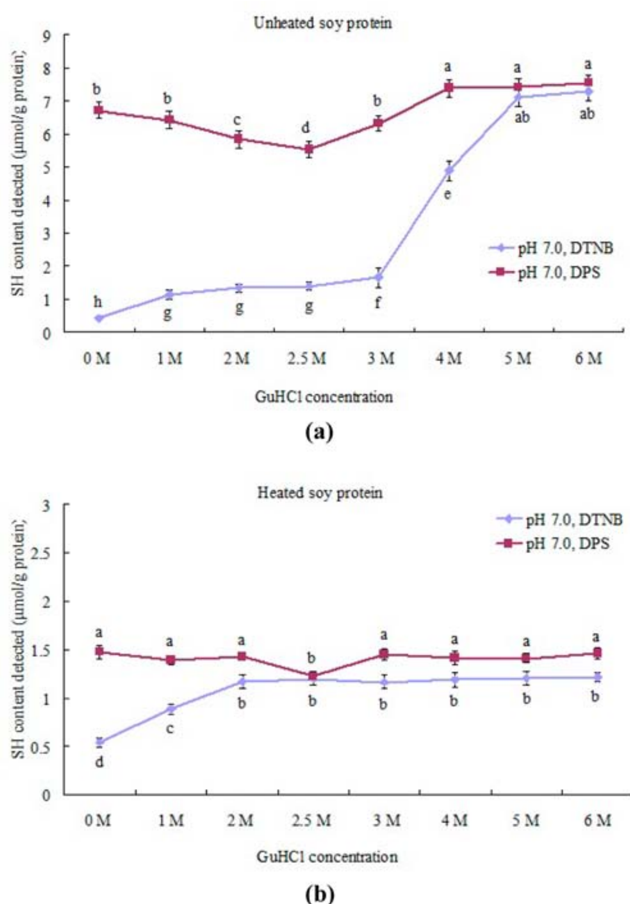

Figure 5. Effect of GuHCl concentration on the SH content determined using DTNB and DPS. Final GuHCl concentrations were 1, 2, 2.5, 3, 4, 5, and 6 M. Both DTNB and DPS were used at pH 7.0. Means ($n = 3$) with different letters were significantly different ($p < 0.05$).

EDTA is generally used in SH detection to chelate metal ions and suppress SH oxidation.[15] Additionally, it was reported that 0.2 mM EDTA was ideal for SH detection,[16] although a wide concentration range of 1−6 mM EDTA has been used for SH detection in soy protein.[9−13] We found that 0.2 mM EDTA does not have a significant effect on SH detection for all conditions (Figure 6). This was likely due to the addition of DTNB and DPS before SDS and GuHCl. Because DTNB and especially DPS react quickly with the SH in protein after SDS and GuHCl unfold it, very few SH groups can be oxidized.

Together, the results above revealed that it was better to detect the SH content of soy protein by using DPS at pH 7.0 in either 2% SDS or 4−6 M GuHCl and that DPS should be added before SDS and GuHCl.

SH Detection Using the Two Fluorescent Reagents mBBr and NPM. The fluorescent reagents mBBr and NPM were used in an attempt to improve the sensitivity of protein SH detection. mBBr reacts with SH to yield a fluorescent product with a maximal fluorescence emission spectra at 480 nm with excitation at 390 nm. The fluorescence intensities produced at 480 nm by the reactions of 1−5 μmol/L L-cysteine and mBBr were used to establish a standard curve of fluorescence intensity versus SH content ($R^2 = 0.99$). Because light exposure causes bromobimane photolysis and conversion of mBBr to a fluorescent bimane, unreacted mBBr must be extracted by dichloromethane. Consequently, if SDS were used, it would simultaneously be removed by dichloromethane, and the existing protein state would change. Therefore, we detected SH content using only GuHCl with mBBr.

NPM also fluoresces after reacting with SH, and unreacted NPM does not need to be removed after labeling. The fluorescence emission spectra were measured from 350 to 500 nm with excitation at 330 nm. The fluorescence emission spectra of NPM had two peaks, one at 378 nm and one at 395 nm. The first peak was used as an indicator for fluorescent protein−NPM.[26] The standard curve of fluorescent L-cysteine−NPM was linear between 1 and 10 μmol/L ($R^2 = 0.99$). The fluorescence emission spectra of L-cysteine−NPM in GuHCl showed a single peak at 389 nm, which was different from that of protein−NPM. Therefore, it appeared that the SH content of soy protein in GuHCl could not be quantified by the standard curve above. As a result, we detected SH content using only SDS with NPM.

The SH contents detected by mBBr in 6 M GuHCl were 8.33 ± 0.31 and 1.68 ± 0.07 μmol SH/g protein for unheated soy protein and heated soy protein, respectively. The corresponding contents detected by NPM in 2% SDS value were 9.48 ± 0.40 and 2.00 ± 0.10 μmol SH/g protein (Table 2). These results were higher than the highest value detected by DPS. The SH contents detected by mBBr and NPM may be overestimated, however. Therefore, we validated these results below.

SH Detection Using Enzymatic Hydrolysis-Assisted DPS and Amino Acid Analysis. To examine whether DPS reacted with SH completely, unheated and heated soy proteins were treated with proteolytic enzymes to eliminate any possible steric hindrances of the reaction between DPS and SH. Soy protein was hydrolyzed with Neutrase (EC 3.4.24.28, Novozymes) at neutral pH, and the enzymolysis was performed in the presence of DPS to prevent SH loss induced by SH oxidation during the enzymolysis process. SDS-PAGE (result not shown) showed that large protein molecules were hydrolyzed into small peptides. Table 2 shows that the SH detected by enzymatic proteolysis-assisted DPS is identical with that detected by DPS alone.

The cysteine content of unheated and heated (90 °C, 5 min) soy protein was determined by amino acid analysis. The

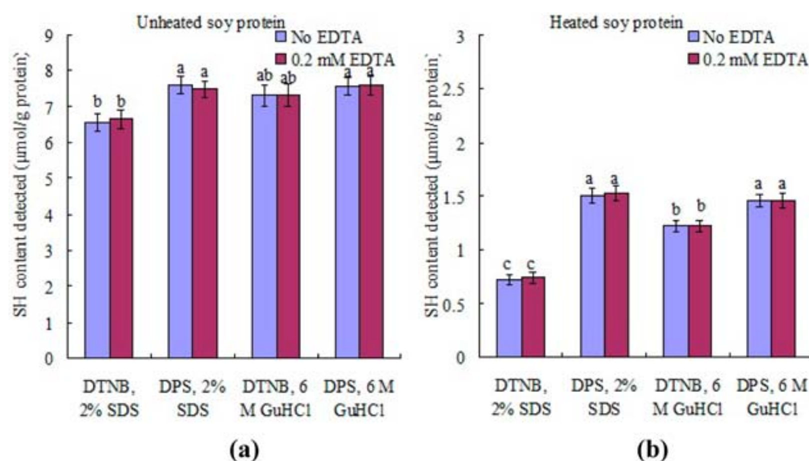

Figure 6. Effect of EDTA on the SH content determined using DTNB and DPS. Final EDTA concentration was 0.2 mM. Means (n = 3) with different letters were significantly different ($p < 0.05$).

Table 2. SH Contents of Unheated and Heated (90°C, 5 min) Soy Protein Determined Using DTNB, DPS, and NPM with 2% SDS and DTNB, DPS, and mBBr with 6 M GuHCl[a]

	unheated soy protein	heated soy protein
cysteine content using amino acid analysis	7.51 ± 0.45 C	1.47 ± 0.10 c
enzymatic hydrolysis-assisted DPS	7.70 ± 0.25 C	1.38 ± 0.04 c
DTNB + 2% SDS, pH 8.0	6.56 ± 0.30 D	0.72 ± 0.03 e
DTNB + 6 M GuHCl, pH 7.0	7.30 ± 0.30 CD	1.22 ± 0.02 d
DPS + 2% SDS, pH 7.0	7.60 ± 0.26 C	1.53 ± 0.04 c
DPS + 6 M GuHCl, pH 7.0	7.56 ± 0.26 C	1.46 ± 0.03 c
mBBr	8.33 ± 0.31 B	1.68 ± 0.07 b
NPM	9.48 ± 0.40 A	2.00 ± 0.10 a

[a]The cysteine content determined by amino acid analysis after performic acid oxidation and acid digestion is also shown. Means (n = 3) with different letters in the same columns were significantly different ($p < 0.05$).

cysteine content of unheated soy protein was 7.51 ± 0.45 μmol/g protein, which agreed with the SH detected by DPS in 2% SDS and 6 M GuHCl. The cysteine content of heated soy protein (90 °C, 5 min) was 1.47 ± 0.10 μmol/g protein (Table 2). This result also agreed with the SH detected by DPS. Therefore, the SH content detected by DPS was reliable, whereas those by mBBr and NPM overestimated the actual content in the presence of denaturants. This may be explained by the following reasons. First, in addition to the main reaction between mBBr/NPM and the SH in soy protein, mBBr may also react with the hydroxyl group of tyrosine, the e-amino group of lysine, the imidazole group of histidine, and the N-terminus of proteins, thereby increasing fluorescence intensity.[23] Similarly, Hansen and Winther reported that NPM could also react with protein amines to produce fluorescence.[15] The fluorescent chromophore of mBBr and NPM is also hydrophobic. This may be easily disturbed by the residual coiled protein structures in SDS and GuHCl, which would enhance fluorescence intensity.[32]

In summary, four reagents (DTNB, DPS, mBBr, and NPM) were used to detect the SH content of unheated and heated soy protein. Their results were compared to the SH detected by enzymatic hydrolysis-assisted DPS method, and the cysteine content as measured by amino acid analysis. The SH detected by DTNB was affected by pH, denaturant species, and denaturation concentration, and DTNB was not considered to be suitable to reliably detect SH in soy protein. Compared with DTNB, DPS was more suitable for reliably detecting the SH content of soy protein using SDS and GuHCl at pH 7.0. We also showed that DPS should be added before SDS and GuHCl. Finally, although mBBr and NPM could detect SH at low protein concentrations (0.35 g/L), both mBBr and NPM overestimated the SH content. This study identified a more reliable method of detecting the SH content of soy protein. We found that using DPS at pH 7.0 and adding it before SDS and GuHCl was the most reliable method.

■ AUTHOR INFORMATION

Corresponding Author

*Postal address: State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, Jiangsu Province, PR China. Phone: 0510-85329091. Fax: 0510-85329091. E-mail: yufeihuajiangnan@126.com; yfhua@jiangnan.edu.cn.

Funding

We gratefully acknowledge financial support received from the Natural Science Foundation of China (21146002), the Natural Science Foundation of Jiangsu Province, PR China (BK2011151), the Open Project Program of State Key Laboratory of Food Science and Technology, Jiangnan University (SKLF-ZZB-201202), and the Doctor Candidate Foundation of Jiangnan University (JUDCF10025).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

SH, sulfhydryl; SS, disulfide bond; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DPS, 4, 4′-dithiodipyridine; NPM, *N*-(1-pyrenyl)maleimide; mBBr, monobromobimane; NEM, *N*-ethylmaleimide; GuHCl, guanidinium hydrochloride; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid

■ REFERENCES

(1) Petruccelli, S.; Anon, M. C. Thermal aggregation of soy protein isolates. *J. Agric. Food Chem.* **1995**, *43*, 3035−3041.

(2) Shimada, K.; Cheftel, J. C. Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 147−153.

(3) Renkema, J. M. S.; van Vliet, T. Heat-induced gel formation by soy proteins at neutral pH. *J. Agric. Food Chem.* **2002**, *50*, 1569−1573.
(4) Wagner, J. R.; Gueguen, J. Surface functional properties of native, acid-treated, and reduced soy glycinin. 1. Foaming properties. *J. Agric. Food Chem.* **1999**, *47*, 2173−2180.
(5) Faris, R. J.; Wang, H.; Wang, T. Improving digestibility of soy flour by reducing disulfide bonds with thioredoxin. *J. Agric. Food Chem.* **2008**, *56*, 7146−7150.
(6) Friedman, M.; Brandon, D. L. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **2001**, *49*, 1069−1086.
(7) Wilson, S.; Blaschek, K.; de Mejia, E. G. Allergenic proteins in soybean: processing and reduction of P34 allergenicity. *Nutr. Rev.* **2005**, *63*, 47−58.
(8) Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70−77.
(9) Wang, J.; Xia, N.; Yang, X.; Yin, S.; Qi, J.; He, X.; Yuan, D.; Wang, L. Adsorption and dilatational rheology of heat-treated soy protein at the oil−water interface: relationship to structural properties. *J. Agric. Food Chem.* **2012**, *60*, 3302−3310.
(10) Lakshmanan, R.; de Lamballerie, M.; Jung, S. Effect of soybean-to-water ratio and pH on pressurized soymilk properties. *J. Food Sci.* **2006**, *71*, 384−391.
(11) Hou, D. H. J.; Chang, S. K. C.. Structural characteristics of purified glycinin from soybeans stored under various conditions. *J. Agric. Food Chem.* **2004**, *52*, 3792−3800.
(12) Wu, W.; Zhang, C.; Kong, X.; Hua, Y. Oxidative modification of soy protein by peroxyl radicals. *Food Chem.* **2009**, *116*, 295−301.
(13) Boatright, W. L.; Hettiarachchy, N. S. Soy protein isolate solubility and surface hydrophobicity as affected by antioxidants. *J. Food Sci.* **1995**, *60*, 798−801.
(14) Cayot, P.; Rosier, H.; Roullier, L.; Haertle, T.; Tainturier, G.. Electrochemical modifications of proteins: disulfide bonds reduction. *Food Chem.* **2002**, *77*, 309−315.
(15) Hansen, R. E.; Winther, J. R. An introduction to methods for analyzing thiols and disulfides: Reactions, reagents, and practical considerations. *Anal. Biochem.* **2009**, *394*, 147−158.
(16) Riener, C. K.; Kada, G.; Gruber, H. J. Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4′-dithiodipyridine. *Anal. Bioanal. Chem.* **2002**, *373*, 266−276.
(17) Thurlkill, R. L.; Grimsley, G. R.; Scholtz, J. M.; Pace, C. N. p*K* values of the ionizable groups of proteins. *Protein Sci.* **2006**, *15*, 1214−1218.
(18) Monahan, F. J.; German, J. B.; Kinsella, J. E. Effect of pH and temperature on protein unfolding and thiol/disulfide interchange reactions during heat-induced gelation of whey proteins. *J. Agric. Food Chem.* **1995**, *43*, 46−52.
(19) Ono, T.; Choi, M. R.; Ikeda, A.; Odagiri, S. Changes in the composition and size distribution of soymilk protein particles by heating. *Agric. Biol. Chem.* **1991**, *55*, 2291−2297.
(20) Lagrain, B.; Rombouts, I.; Brijs, K.; Delcour, J. A. Kinetics of heat-induced polymerization of gliadin. *J. Agric. Food Chem.* **2011**, *59*, 2034−2039.
(21) Woodward, J.; Tate, J.; Herrmann, P. C.; Evans, B. R. Comparison of Ellman's reagent with *N*-(1-pyrenyl)maleimide for the determination of free sulfhydryl groups in reduced cellobiohydrolase I from *Trichoderma reesei*. *J. Biochem. Biophys. Methods* **1993**, *26*, 121−129.
(22) Egwim, I. O. C.; Gruber, H. J. Spectrophotometric measurement of mercaptans with 4,4′-dithiodipyridine. *Anal. Biochem.* **2001**, *288*, 188−194.
(23) Kosower, E. M.; Kosower, N. S. Bromobimane probes for thiols. *Methods Enzymol.* **1995**, *251*, 133−148.
(24) Wright, S. K.; Viola, R. E. Evaluation of methods for the quantitation of cysteines in proteins. *Anal. Biochem.* **1998**, *265*, 8−14.
(25) Zhang, W.; Czupryn, M. J. Free sulfhydryl in recombinant monoclonal antibodies. *Biotechnol. Prog.* **2002**, *18*, 509−513.
(26) Ercal, N.; Oztezcan, S.; Hammond, T. C.; Matthews, R. H.; Spitz, D. R. High-performance liquid chromatography assay for *N*-acetylcysteine in biological samples following derivatization with *N*-(1-pyrenyl)maleimide. *J. Chromatogr., B* **1996**, *685*, 329−334.
(27) Yano, H. Fluorescent labeling of disulfide proteins on 2D gel for screening allergens: a preliminary study. *Anal. Chem.* **2003**, *75*, 4682−4685.
(28) Rogers, L. K.; Leinweber, B. L.; Smith, C. V. Detection of reversible protein thiol modifications in tissues. *Anal. Biochem.* **2006**, *358*, 171−184.
(29) Synowieckit, J.; Shahidi, F. Heat-induced changes in sulfhydryl groups of harp seal muscle. *J. Agric. Food Chem.* **1991**, *39*, 2006−2009.
(30) Clarasze, K. W.; Kshirsaga, H. H.; Venkatachalam, M.; Sathe, S. K. A Circular dichroism and fluorescence spectrometric assessment of effects of selected chemical denaturants on soybean (*Glycine max* L.) storage proteins glycinin (11S) and *β*-conglycinin (7S). *J. Agric. Food Chem.* **2007**, *55*, 8745−8753.
(31) Reynolds, J. A.; Tanford, C. Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. *PANS* **1970**, *66*, 1002−1003.
(32) Lacy, E. R.; Baker, M.; Brigham-Burke, M. Free sulfhydryl measurement as an indicator of antibody stability. *Anal. Biochem.* **2008**, *382*, 66−68.