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An improved method to determine SH and –S–S– group content in soymilk protein

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Abstract

Ellman's method is a simple, rapid and direct method to determine the SH and –S–S– group content in proteins. However, this method has difficulties in directly determining SH and –S–S– group content in soymilk because of the high turbidity and low SH group content of soymilk. In this experiment, low-turbidity protein solutions were prepared by separating the proteins from soymilk by acetone precipitation, the proteins were then dissolved in Tris–glycine buffer (pH = 8.0) containing or not containing 8 mol/L of urea. The content of SH and –S–S– groups in the separated protein was determined by a spectrophotometric method. Tests of soy protein isolates proved that the contents of SH and –S–S– groups were not significantly affected by the acetone treatment.

It can be concluded that acetone treatment is an effective method to determine the SH and –S–S– groups content of proteins in high turbidity solutions such as milk, soymilk, etc.

Keywords: Protein; Turbidity; Sulfhydryl

1. Introduction

Because of their high reactive activity, –SH and –S–S– groups in proteins play an important role in improving food functional properties (Friedman, 1994). The formation of gluten, gelatin and protein-based edible films are all related to the transformation of –SH groups to –S–S– (Fennema, 1996; Handa, Genndios, & Hanna, 1999; Koehler, 2003; Lee & Sherbon, 2002; Shimada & Cheftel, 1988). Furthermore, some food processing treatments such as heat or oxidizing and reducing agents are likely to result in transformation between –SH and –S–S– groups (Fennema, 1996; Guingamp & Humbert, 1999; Handa et al., 1999; Nabi, Britten, & Paquin, 2000; Santiago, Bonaldo, & Gonzalez, 1999). Thus, the content of –SH and –S–S– groups and their changes are often assayed when exploring the properties of proteins in foods. At present, the most widely used method is Ellman's method (Ellman, 1959), in which 5,5′-dithio-2-nitrobenzoate (DTNB) is used to react with SH groups to produce a yellow substance with a maximum absorbance at 412 nm. This method is simple, fast, direct, and an ideal method for determining the content of –SH in pure protein solution with low turbidity. However, if it is used to directly determine the content of –SH groups in turbid solutions such as milk or soymilk, the results will deviate from the real content because of the high turbidity of these solutions. This is a common problem of all spectrophotometric methods including ultraviolet spectrophotometry. In this experiment, acetone was used to destroy the emulsion system by precipitating the proteins, then the proteins were dissolved in tris-glycine buffer containing denaturant (urea) and the content of free sulfhydryl groups was determined. The experimental results proved that this is a satisfactory method to determine –SH and –S–S– group content of proteins in solutions with high turbidity.

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2. Materials and methods

2.1. Preparation of soymilk

Soymilk was prepared according to Kwok, Basker, and Niranjan (2000). Soybeans (*Glycine max*) were soaked in deionized water (bean to water, 1:10) for 12 h at 5 °C. The soaked soybeans along with the soaked water were blended in a Waring blender at low speed for 5 min. The slurries were filtered through 200-mesh nylon filter bag. The filtrate containing about 65 mg/ml total solids (with protein content about 3.5%) was used in the experiments.

2.2. Reagents

Three tris buffers were prepared. Buffer 1 contained 10.4 g Tris (trihydroxymethyl aminomethane), 6.9 g Glycine and 1.2 g EDTA in one liter of deionized water at pH 8.0. Buffer 2 contained 10.4 g Tris (trihydroxymethyl aminomethane), 6.9 g Glycine, 1.2 g EDTA and 480 g urea in one liter of deionized water at pH 8.0. Buffer 3 contained 10.4 g Tris, 6.9 g Glycine, 1.2 g EDTA and 600 g urea per liter in one liter of deionized water at pH 8.0. Ellman's reagent was prepared by dissolving 0.2 g of DTNB (5,5′-dithio-2-nitrobenzoate) in 50 ml of Buffer 1. The 12% TCA solution used to precipitate protein contained 120 g trichloroacetic acid per liter of deionized water.

2.3. Heat-treatment of soymilk

Heat treatment was carried out in a water bath. Test tubes containing 10 ml of soymilk were tightly stoppered and placed in a water-bath at 95 °C for 45, 90, 135 and 180 min, respectively.

2.4. Acetone treatment of soymilk

Mixtures of 1 ml soymilk and 9 ml anhydrous acetone were stirred and then held for 10 min before being centrifuged at 3000*g* for 15 min. The precipitate was twice resuspended in 5 ml acetone and centrifuged at 3000*g* for 15 min. The acetone was removed by evaporating the solvent from the centrifuge tubes with streams of cold air. The precipitate was dissolved in 5 ml of buffer 1 and buffer 2 to determine the content of sulfhydryl group on surface and total free sulfhydryl group, respectively. Mixtures of 1 ml soymilk and 4 ml buffer 1 or 4 ml buffer 2 were used to determine the content of sulfhydryl group on surface and total free sulfhydryl group, respectively, as controls.

2.5. Determination of –SH and –S–S– groups

2.5.1. Determination of free sulfhydryl group

Mixtures of 1 ml of pretreated soymilk (as described in 2.4) and 2.0 ml buffer 1 or 2 and 0.02 ml Ellman's reagent, were mixed and reacted at 25 °C for 5 min. The absorbance at 412 nm was then measured using a Hitachi U-1500 spectrophotometer. The reacted solutions with no soymilk as blank, soymilk with no Ellman's reagent for determination of the turbidity.

2.5.2. Determination of total cysteine content in protein

Mixtures of 0.2 ml of pretreated soymilk (2.4), 1 ml buffer 3 and 0.02 ml of 2-mercaptoethanol were mixed and held at 25 °C for 1 h, then 10 ml of 12% TCA was added and again held at 25 °C for 1 h, then centrifuged at 5000*g* for 15 min. The precipitate was twice resuspended in 5 ml of 12% TCA and centrifuged at 5000*g* for 10 min. The precipitate was then dissolved in 3.0 ml buffer 1, the color was developed with 0.05 ml Ellman's reagent, and the absorbance read at 412 nm.The preparation without addition of Ellman's reagent was used for determination of turbidity.

2.5.3. Calculation

The content of SH and cysteine were calculated according to the following formula (Beveridge, Toma, & Nakai, 1974):

$$\mu\text{mol SH/g} = (73.53 \times A_{412} \times D)/C,$$

where, A_{412} = difference of absorbance at 412 nm between with and without DTNB in color developing solutions. D is the dilution factor, for SH, $D = 3.02 \times 5 = 15.1$; for total cysteine, $D = (3.05/0.2) \times 5$; C is the total solids content of soymilk, 65 mg/ml.

2.6. Effect of acetone treatment on sulfhydryl group and total cysteine content

Soybean protein isolated (SPI) was soaked and stirred in anhydrous ethanol (weight to volume 1:10) for 0.5 h, filtered through Whatman filter paper, the residue was washed with anhydrous ethanol, and air-dried. Sulfhydryl group content or total cysteine was determined on samples of 1.5 g of ethanol-treated SPI dissolved in 50 ml of buffer 1, and treated with acetone as described in 2.4. Samples without acetone treatment were used as control.

2.7. Statistical analysis

Statistics on a completely randomized design with three replicates were determined with the SPSS 10.0 for Windows procedure.

3. Results and discussion

3.1. Direct determination with Ellman's method

Table 1 shows that there was hardly any difference in absorbance at 412 nm between the soymilk with or without addition of color developing agent DNTB when Ellman's method was used to directly determine the content of total sulfhydryl in soymilk. This is due to the high turbidity of soymilk, and the light scattering, which prevents the spectrophotometer from distinguishing the color differences in the reaction between DNTB and –SH group.

When determining the content of sulfhydryl groups, the free SH groups in protein are usually classified into two types, surface SH and buried SH. The former is determined by dissolving the proteins in a standard buffer, the content of buried SH is the difference between total SH and surface one. The content of total SH group is determined by dissolving proteins in a buffer added with denaturing reagents such as SDS and urea (Shimada & Cheftel, 1988).

In these experiments, the absorbance of soymilk when determining surface SH by using Tris–glycine buffer was higher than total SH by using the same buffer containing 8 mol/L of urea (Table 1). In addition, –SH cannot be determined by dilution as soybean contains low levels of cystine and cysteine. This meant that it is impossible to directly determine the free SH group in soymilk by Ellman's method because of the high turbidity of soymilk.

As to the determination of the content of total cysteine, because a smaller amount of sample was tested and the protein was separated by precipitation in 12% TCA, the turbidity of the protein contributed very little to the absorption (Table 1). Thus, Ellman's method can be directly used to determine the total cysteine content of protein in high-turbidity food.

3.2. Determination with Ellman's method after acetone treatment

Acetone can separate protein from the emulsion system of the protein and fat by precipitation of protein

Table 1
A_{412} of soymilk before acetone treatment

Heat treatment	Total free SH group (μmol/g)		Surface SH (μmol/g)		Total content of cysteine (μmol/g)	
	+DTNB	−DTNB	+DTNB	−DTNB	+DTNB	−DTNB
Control	1.153 ± 0.012	1.150 ± 0.015	1.316 ± 0.022	1.308 ± 0.018	1.083 ± 0.014	0.012 ± 0.008
45 min	1.200 ± 0.015	1.155 ± 0.012	1.476 ± 0.025	1.484 ± 0.026	1.078 ± 0.015	0.123 ± 0.010
90 min	1.117 ± 0.020	1.144 ± 0.017	1.445 ± 0.018	1.392 ± 0.022	1.069 ± 0.020	0.122 ± 0.007
135 min	1.143 ± 0.016	1.114 ± 0.012	1.475 ± 0.026	1.472 ± 0.028	1.066 ± 0.018	0.132 ± 0.015
180 min	1.166 ± 0.023	1.170 ± 0.021	1.482 ± 0.023	1.496 ± 0.024	0.882 ± 0.012	0.154 ± 0.014

Table 2
A_{412} of soymilk after acetone treatment

Heat treatment	Total free SH group (μmol/g)		Surface SH (μmol/g)		Total content of cysteine (μmol/g)	
	+DTNB	−DTNB	+DTNB	−DTNB	+DTNB	−DTNB
Control	0.474 ± 0.010	0.155 ± 0.006	0.424 ± 0.009	0.235 ± 0.004	0.963 ± 0.012	0.054 ± 0.000
45 min	0.401 ± 0.008	0.228 ± 0.004	0.364 ± 0.010	0.208 ± 0.006	0.959 ± 0.015	0.023 ± 0.002
90 min	0.27 ± 0.006	0.107 ± 0.005	0.283 ± 0.006	0.158 ± 0.005	0.957 ± 0.018	0.018 ± 0.000
135 min	0.256 ± 0.011	0.083 ± 0.003	0.278 ± 0.007	0.136 ± 0.004	0.938 ± 0.009	0.024 ± 0.000
180 min	0.321 ± 0.012	0.169 ± 0.009	0.348 ± 0.008	0.204 ± 0.005	0.812 ± 0.012	0.124 ± 0.004

Table 3
Effect of acetone treatment on A_{412} and SH content of SPI

Acetone treatment	Total SH			Total content of cysteine		
	A_{412}		SH content (μmol/g)	A_{412}		Content of cysteine (μmol/g)
	+DTNB	−DTNB		+DTNB	−DTNB	
No	0.411 ± 0.008	0.116 ± 0.004	10.91 ± 0.23	0.621 ± 0.009	0.023 ± 0.007	111.8 ± 0.46
Yes	0.378 ± 0.008	0.088 ± 0.001	10.73 ± 0.18	0.608 ± 0.010	0.018 ± 0.004	110.3 ± 0.54

and significantly decrease the turbidity of soymilk (Table 2). Thus, Ellman's method can be used to determine both contents of total and surface free-SH group after soymilk was treated by acetone.

3.3. Effect of acetone treatment on the content of SH groups

As mentioned above, soymilk treated by acetone made it possible to determine SH groups by Ellman's method because acetone greatly decreased turbidity of soymilk. However, whether acetone treatment would affect the content of SH would need to be elucidated before the method could be used. Soybean protein isolate (SPI) treated by acetone treatment showed that it had no effect on the content of SH and cysteine in SPI (see Table 3).

4. Conclusion

Ellman's method can be employed to determine quite accurately the content of sulfhydryl group of protein in turbid solutions after proteins were precipitated by acetone.

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