Intrinsic Sulfite Content of Isolated Soy Proteins

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ABSTRACT: Commercial isolated soy proteins (ISP) contain 22 and 31 ppm sulfite as measured by the optimized Monier-Williams method (Sulfites in Foods, *Official Methods of Analysis*, 16th edn., AOAC, Washington, DC, 1995, Official Method 990.28). A method was developed to cryogenically trap and quantify the sulfur dioxide produced by this method using GC–MS. The same commercial ISP samples were found to contain 17 and 26 ppm sulfite, respectively, with GC–MS. ISP prepared in the laboratory contained 33 ppm sulfur dioxide, and defatted soybean flakes contained only a trace. Adding dithiothreitol after beginning the boiling step of the Monier-Williams assay had no significant effect on the sulfite content of a commercial ISP, whereas adding dithiothreitol prior to bringing the sample to a boil reduced the sulfite content from 17 to about 1 ppm.

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KEY WORDS: GC–MS, methanethiol, Monier-Williams assay, soy protein, sulfites.

A major impediment to the expanded use of soy protein products in human foods is their characteristic flavor. Methanethiol is among the most potent odorants found in aqueous slurries of isolated soy proteins (ISP), concentrates, and soy milk. A proposed mechanism for the synthesis of methanethiol in soy proteins (1) involves residual components of a sulfur assimilation pathway (Fig. 1) responsible for cysteine synthesis during soybean germination (2,3). For this to be a viable mechanism of methanethiol synthesis in soy protein products, the amount of endogenous sulfite must be sufficient to act as a substrate.

A variety of methods are available to quantify sulfites in foods (4,5). The most widely used is the optimized Monier-Williams method (AOAC Official Method 990.28; Ref. 6). This method converts free and reproducible amounts of bound oxosulfur(IV) anions in foods to sulfur dioxide, which is distilled and oxidized to sulfuric acid in a hydrogen peroxide trap. The acid is then determined by titration. Several HPLC methods are useful for quantifying sulfites. Some analyze the sulfur distilled from the Monier-Williams procedure as sulfate ions (7,8), whereas others avoid the distillation step. Warner *et al*. (9) treated samples with formaldehyde to convert sulfites to hydroxymethylsulfonate. Other HPLC methods measure sulfite directly using electrochemical detection (10) or direct UV detection (11).

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Madl (12) found that ISP prepared without added sulfite contained 20–30 ppm sulfite by the Monier-Williams method. However, Warner *et al.* (9) found only 2 ppm sulfite in ISP using an HPLC method. The discrepancies in sulfite contents among methods, and the potential for volatile compounds besides sulfur dioxide to contribute to overestimation with the Monier-Williams assay (7,8,11), bring into question the actual sulfite content in ISP.

This investigation was undertaken to develop a method of cryogenically trapping the distillate from a Monier-Williams-type distillation and quantifying the amount of sulfite (as sulfur dioxide) in ISP and defatted flakes (DF). The effect of dithiothreitol (DTT) on sulfite levels was also examined.

MATERIALS AND METHODS

Hydroxymethylsulfonate (HMS) and DTT were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Sodium sulfite was obtained from Spectrum Laboratory Products (Gardena, CA). ISP, designated Supro 500E, were provided by DuPont's Protein Technologies International (PTI, St. Louis, MO) and by the Archer Daniels Midland Company (ADM, Decatur, IL). ISP samples were stored at 4°C. The ADM ISP without any additives was collected from the process line immediately after drying; no sulfites had been added during processing. Hexane-defatted (white) flakes (DF) were provided by ADM and stored at 4°C.

ISP was prepared in the laboratory by dispersing hexane-defatted soybean flakes (obtained from ADM) in water (1 part flakes to 10 parts water) at 22°C, followed by additions of 1 N sodium hydroxide, as needed, until a pH of 9 was achieved and maintained for 15 min (13). After centrifugation at $1500 \times g$ for 10 min, the supernatant was adjusted to a pH of 4.5 with 1 N HCl to precipitate the proteins. Following centrifugation at $1500 \times g$ for 10 min, the precipitate was washed twice with water, and the protein isolate was adjusted to pH 7 with 1 N NaOH. The resulting slurry was immediately transferred into a glass flask and placed in a boiling water bath. The protein slurry was stirred and brought to 77°C within 4 min, where it was held for 15 s, then cooled to 40°C in an ice bath and freeze-dried.

Sulfite analyses. The amount of sulfite in the ISP and defatted flakes was determined using the optimized Monier-Williams method (6) but substituting a 400-mm Davies-type condenser (Ace Glass Co., Louisville, KY) for the 300-mm Allihn condenser. The condenser temperature was maintained at 4°C. Five-gram portions of ISP and of defatted flakes were used for sulfite analysis. Each sample was analyzed three times.

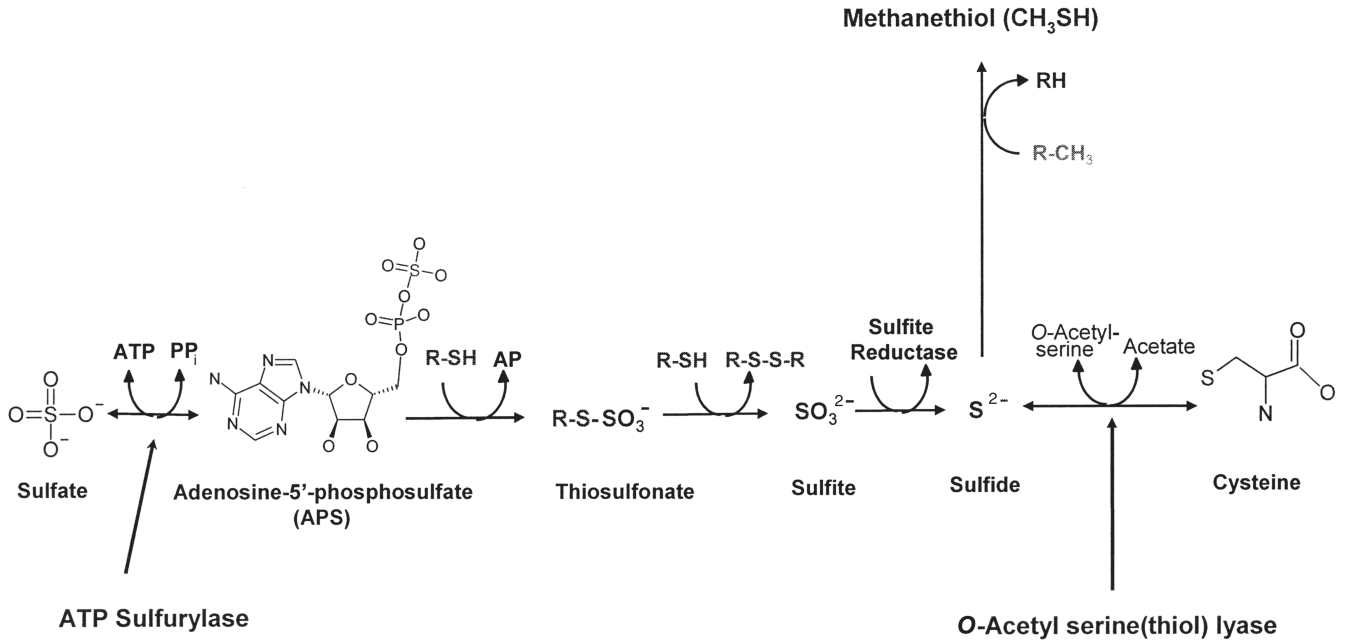

FIG. 1. Proposed metabolic pathway of sulfur assimilation in plants from sulfate to cysteine, with the added diversion of sulfide to methanethiol.

The Monier-Williams method was modified by incorporating 1.5 m of 7.9-mm o.d. (6.4 mm i.d.) fluorinated ethylene propylene (FEP) tubing with two 13-cm diameter loops between the condenser and the 3% H_2O_2 trap. After the entire apparatus was flushed with nitrogen for 15 min, the tubing loops were submerged in liquid nitrogen to trap the sulfur dioxide cryogenically. Each end of the FEP tubing was fitted with a Teflon shutoff valve that connected the cryogenic trap to the condenser and the glass tubing leading to the 3% H_2O_2 solution. At the end of the distillation process, the shutoff valves were closed and detached from the rest of the apparatus. A 2-L Tedlar bag was connected to the downstream end of the FEP tubing, and the valve between the Tedlar bag and the FEP tubing was opened. The FEP loop was then removed from the liquid nitrogen and allowed to stand at room temperature for 15 min. A septum was then attached to the closed shutoff valve, the valve was opened, and nitrogen gas was introduced through the septum with a 20-gauge needle to flush the sulfur dioxide into the Tedlar bag. This step was continued until the 2-L Tedlar bag was filled (about 20 s). The valve between the Tedlar bag and the FEP tubing was closed, and the Tedlar bag was manually rotated at about 72 rpm for 30 s to facilitate mixing of the sulfur dioxide in the nitrogen gas. This rotation of the Tedlar bag was repeated prior to each injection. Five-milliliter portions of gas were removed from the septum of the Tedlar bag and injected into the GC–MS apparatus. A standard curve was prepared by adding varying amounts of HMS to the Monier-Williams apparatus (Fig. 2). The sulfur dioxide response from the samples was multiplied by 80/134 (the M.W. of sulfite divided by the M.W. of HMS) to calculate the amount of sulfite in ISP from the standard curve.

GC–MS. GC–MS was accomplished on a Hewlett-Packard Model 5890 Series II gas chromatograph with a 5971A mass spectrometer, an ms-NoVent system (SGE Intl., Ringwood, Australia), an indirect liquid nitrogen trap (SGE Intl.) at the beginning of the column to cryo-focus analytes, and a postcolumn splitter. The injection sequence began by bringing the liquid nitrogen trap to about –60°C. The headspace sample (5 mL) was injected at a rate of 5 mL/min followed by a 2-min wait. The flow of nitrogen to the cryogenic trap was stopped, and the

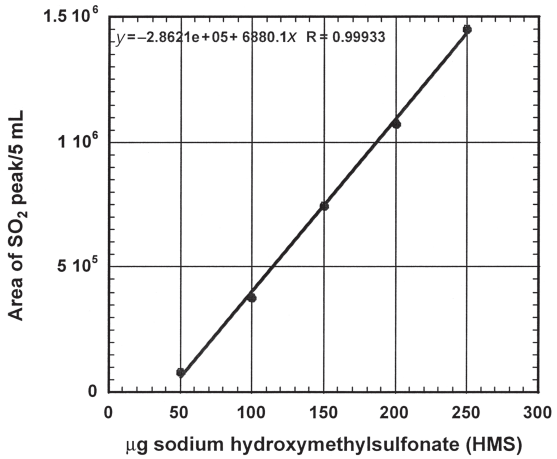

FIG. 2. GC–MS standard curve for quantifying sulfite as sulfur dioxide (*m/z* 48 and 64). Injection of 5 mL from a 2-L Tedlar gas sampling bag.

GC run was begun. The column was a DB-5MS capillary column (30 m × 0.53 mm i.d.) with 1.5-μm film thickness (Agilent Technologies, Palo Alto, CA). The helium flow rate through the columns was 3 mL/min, with 1 mL/min entering the mass spectrometer and 2 mL/min being vented during the run. The column temperature was held at 40°C for 2 min, then increased at 5°C/min to 165°C, where it was held for 5 min. The electron ionization detector was set to detect ions at *m/z* 48 and 64 for sulfur dioxide. The injection port temperature was maintained at 60°C. Sulfur dioxide was identified by comparison of mass spectra with a spectral database (NIST98; ChemSW, Inc., Fairfield, CA) and by comparison of retention times and mass spectra with an authentic standard.

Statistical evaluations were done using an SAS (14) software package with an ANOVA procedure. Least significant difference values were computed with an ANOVA procedure at $P \leq 0.05$, and comparisons between means were done using the Tukey–Kramer honestly significant differences test. Duplicate GC–MS analyses were performed on three separate Monier-Williams distillations for each commercial ISP sample and on two separate distillations for all other samples.

RESULTS AND DISCUSSION

The sulfite contents found in the commercial ISP samples using the optimized Monier-Williams method (Table 1) were similar to values reported by Madl (12). To demonstrate that the Monier-Williams results were due to the oxosulfur(IV) anions and not interference from volatile compounds that managed to pass through the condenser (e.g., aldehydes that would be oxidized to acids in the hydrogen peroxide trap), we modified the Moiner-Williams method by cryogenically trapping the distillate after the condenser column and before the hydrogen peroxide trap (Fig. 3). We encountered several problems during the development of this method. When using nitrogen as the purge gas, too long a dwell time in the liquid nitrogen trap condensed the nitrogen. This caused excessive expansion of the material in the trap when it was brought to room temperature and loss of the trapped sulfur dioxide due to rupture of the Tedlar bag. The use of helium as the purge gas overcame this problem, but the specific gravity of gaseous helium at 1 atm is 0.138 and the specific gravity of gaseous sulfur dioxide is 2.26. This large difference is likely why we obtained very erratic results with helium as the purge gas. Eventually, we found that limiting the Teflon tubing trap submerged in the liquid nitrogen to two 13-cm diameter loops sufficiently trapped the sulfur dioxide but did not cause excessive expansion when it was removed from the liquid nitrogen. Attaching the 2-L Tedlar bag to the end of the tubing prior to removing it from the liquid nitrogen trap accommodated the minor expansion, and flushing the sulfur dioxide out of the Teflon tubing into the gas sampling bag separated the sulfur dioxide gas from the small amount (*ca.* 0.5 mL) of condensed liquid (possibly water) in the trap. Because sulfur dioxide is very soluble in water, this could lead to interference. Also, because the material that remained liquid at room temperature in the cryogenic trap was able to get past the condenser, it is possible that some volatile aldehydes could also have passed through and contributed to an artificially high result from the Monier-Williams titration (15). Examination of the volatiles from the Tedlar bag by GC–MS in the scan mode revealed acetaldehyde and hexanal peaks that were considerably larger than the sulfur dioxide peak. Because the titration end point of methyl red is 4.4 to 6.2, and the pK of acetic and hexanoic acids are 4.76 and 4.84, respectively, only a portion of these acids would be titrated. The sampling bag allowed for easy removal of 5-mL portions of gas without any restriction. The 3% H_2O_2 trap was used for each of the modified sulfite assays without any color change.

By using the Monier-Williams method modified to trap the sulfur dioxide cryogenically for analysis by GC–MS, we found a lower content of sulfite in both commercial ISP samples examined (Table 1). This indicates that about 5 ppm of the sulfur dioxide reported by the official AOAC method for ISP is due to some other component being distilled along with sulfur dioxide. It also clearly demonstrates that ISP contain a substantial amount of endogenous sulfite. A laboratory-prepared ISP sample contained substantially more sulfite, 33 ppm. Using the same assay, hexane-defatted soybean flakes were found to contain only a trace of sulfite. Because hexane-defatted soybean flakes are relatively high in sulfate (12 μmol sulfate/g of defatted

TABLE 1
Sulfite Content[a] of Isolated Soy Proteins (ISP) Determined by the Optimized Monier-Williams Method[b] and by Quantifying SO_2 from the Monier-Williams Method

ISP	Monier-Williams	Quantification of SO_2
Laboratory-prepared ISP	ND	33.0 (0.8)[a]
Supro 500E (PTI)[c]	30.7 (0.0)[a]	26.3 (0.2)[b]
ADM ISP[d] (no additives)	22.0 (0.5)[b]	16.8 (0.2)[c]
ADM ISP (with DTT added before adding acid)	ND	<6.3 ppm[e]
ADM ISP (with DTT added after boiling)	ND	16.0 (0.5)[c]
Defatted (white) soybean flakes (ADM)[d]	ND	<6.3 ppm[e]

[a]In mg/kg. Values in parentheses are SE. Means within columns and within rows without common roman superscripts differ significantly ($P < 0.05$). ND, not determined.
[b]Reference 6.
[c]Acquired from DuPont's Protein Technologies International (PTI, St. Louis, MO).
[d]ISP and defatted (white) flakes (DF) acquired from Archer Daniels Midland Company (ADM, Decatur, IL).
[e]The peak area for adding dithiothreitol (DTT) before boiling was 11,511 (785); the peak area for DF was 4,034 (1,865). The minimum peak area of 78,081 on the standard curve corresponds to 6.3 ppm.

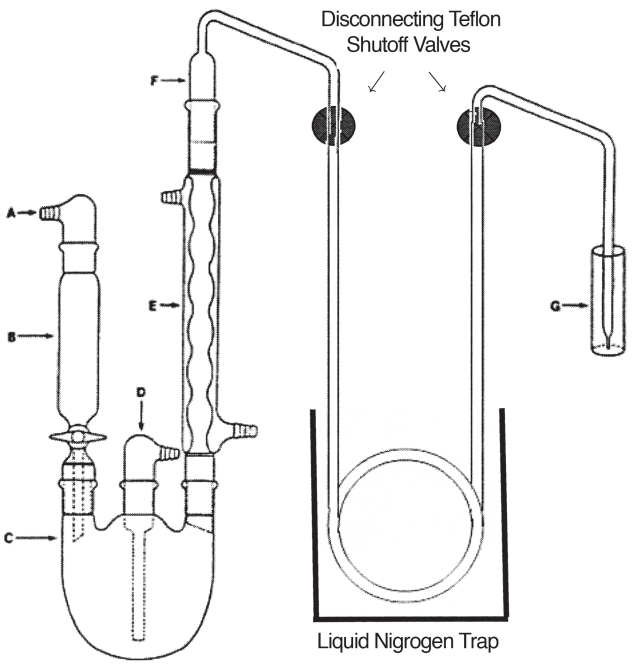

FIG. 3. Representation of the modified Monier-Williams apparatus with a cryogenic trap. For a description of the lettered notations, see Reference 6.

soy flakes, or 1,152 ppm) (16), sulfates may serve as a substrate for sulfite synthesis during the processing of ISP.

DTT has been used to liberate sulfite bound to proteins (17,18). When added to aqueous slurries of ISP, DTT causes large increases in headspace methanethiol (1). We examined the effect of adding 0.5 g of DTT prior to, and 10 min after, the boiling step of our modified Monier-Williams assay (Table 1). When DTT was added to the sealed Monier-Williams apparatus prior to adding the acid and boiling it, the ISP contained considerably less sulfite than the minimum point on the standard curve (6.3 ppm). With a peak area about one-sixth the minimum standard curve point, we estimate this value to be about 1 ppm. When DTT was added 10 min after boiling, the ISP sulfite content was not significantly different from ISP when no DTT was added. These findings indicate that when DTT is added to ISP that has not been denatured with heat, the sulfite is being converted to something else that is not being detected by the Monier-Williams-type assay. Methanethiol would not be detected by the Monier-Williams-type assay. After boiling, the DTT had little effect, indicating that the sulfite was converted rapidly into sulfur dioxide and/or that boiling inactivated whatever was acting on the sulfite. These results also demonstrate that no additional sulfite was being liberated with the addition of DTT.

One possible explanation for our observations is that residual components from the sulfate-to-cysteine reaction pathway present in mature soybeans are still active in ISP. This type of mechanism may convert the sulfate in defatted flakes into sulfites during ISP processing, and may contribute to methanethiol in aqueous solutions of ISP. DTT can act as a sulfur acceptor from adenosine-5′-phosphosulfate, liberate bound sulfites, and catalyze the reduction of sulfite to sulfide (19). The sulfur assimilation pathway in plants (Fig. 1) has received much less attention than the assimilation of carbon and nitrogen, and the mechanisms are still being deliberated, including whether the sulfite formed from the reduction of adenosine-5′-phosphosulfate remains bound or is free. Recent findings (20–22) indicate that free sulfite is the reaction product from adenosine-5′-phosphosulfate reductase. Acid hydrolysis of thiosulfonates reportedly produce a thiol and a hydrogen sulfate ion (23), which would not be detected by the Monier-Williams assay; thus, thiosulfonates would not be detected by this method.

These findings demonstrate that commercial and laboratory-prepared ISP contain between 17 and 33 ppm sulfite, whereas hexane-defatted soybean flakes contain only a trace of sulfite. Adding DTT prior to the Monier-Williams boiling step significantly reduces the sulfite content of ISP. Although not providing conclusive results, the amount of sulfite present in hexane-defatted flakes and ISP, and the disappearance of sulfite in ISP with the addition of DTT, coincide with the possible presence of components of a sulfur assimilation pathway. The procedure described in this paper is quite laborious and is recommended only when substantial amounts of interfering volatile compounds are co-distilled with sulfur dioxide. Laboratories with the appropriate instrumentation should be able to achieve similar results by measuring sulfate from the hydrogen peroxide trap when using either HPLC (5,6) or capillary electrophoresis (24).

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