Chemical Inactivation of Soybean Trypsin Inhibitors[1]

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Sodium metabisulfite and glutaraldehyde were used alone and in combination to inactive Kunitz trypsin inhibitor (TI) in model systems and TI in lyophilized alkaline soy meal extract. Reaction of glutaraldehyde (0.1 to 3.0%, based on total volume of the reaction mixture) with Kunitz TI (3 mg/ml buffer) at 25 C resulted in 60–75% reduction in activity. Treatment of soy meal extract containing 0.08 mg TI/mg at a concentration of 10 mg sample/ml buffer under similar reaction conditions reduced TI activity only 40%, however. A reaction temperature of 75 C had little additional effect on TI inactivation. On the other hand, sodium metabisulfite (0.05 to 1.0 mM) inactivated >96% Kunitz TI within 1 hr at 75 C and 75–94% of the TI in soy meal extract. The combination of 0.6 mM metabisulfite and 0.5% glutaraldehyde at 75 C inactivated >99% Kunitz TI, and combination of 0.6 mM metabisulfite and up to 3% glutaraldehyde inactivated 88% of TI in soy meal extract vs 91% inactivation with metabisulfite alone. Thus, metabisulfite plus glutaraldehyde inactivated Kunitz TI better than either one alone, while bisulfite alone best inactivated TI in soy meal extract. When the reactions were performed at neutral pH, protein solubility of 80% or better was retained.

Protease inhibitors are found in a wide variety of foods, including cereals, dairy products, legumes, oilseeds, meats, fruits and vegetables (1). Trypsin inhibitors (TI) in raw soybeans cause growth inhibition, pancreatic hypertrophy and hyperplasia in experimental animals (2–4). In a 2-yr feeding study, pancreatic lesions (nodular hyperplasia and acinar adenoma) were prevalent in Wistar rats fed diets in which soybean flour or protein isolate were primary sources of protein (5–8). Incidence of pancreatic lesions was directly correlated with the dietary level of TI in raw and toasted soy products.

All commercially processed soy products retain some TI activity (9). To use soy in processed products, thermal treatment must be sufficient to destroy most TI activity without damaging nutritive value or functional properties of the proteins. Thus, most commercial edible-grade soy protein products retain 5–20% of the TI activity present in the soybeans from which they were prepared.

The residual TI activity in the toasted soybean flour used in a 2-yr rat feeding study (5) was sufficient to cause physiological effects (8). This activity resided mainly in Kunitz and Bowman-Birk inhibitors (10,11). These inhibitors can be inactivated by cleavage of the two disulfide bridges in Kunitz TI (12) and four of the seven disulfide bonds in Bowman-Birk inhibitors (13). Reducing agents (cysteine, N-acetylcysteine, mercaptoethanol and reduced glutathione) enhance heat inactivation of soy TI (14–18). Chemical modification of other amino acids may also affect Kunitz TI activity. TI activity can be destroyed by modification of arginyl residues with 1,2-cyclohexanedione (19), and enzymatic conversion of arginine to citrulline with peptidylarginine deiminase rapidly abolishes activity (20). Modification of tryptophan, tyrosine or histidine residues of soy Kunitz TI also results in loss of inhibitory activity, whereas guanidination of lysine groups has little effect (21).

In this study our objective was to maximize destruction of soy Kunitz TI as a model system via chemical modification with either sulfiting and/or cross-linking agents, while preserving and/or enhancing protein solubilities. Sulfiting agents cleave disulfide bonds to form thiols and S-sulfonic acid derivatives (22), which should destroy TI activity (12,13). Glutaraldehyde, a protein cross-linking agent, can react with α-amino groups of amino acids, the ϵ-amino group of lysine, and with nucleophilic protein functional groups, such as the sulfhydryl of cysteine, the imidazole ring of histidine and the phenolic hydroxyl group of tyrosine (23). It has already been established that Kunitz TI activity can be eliminated by modification of tyrosines and partially destroyed by modification of histidines (21). Therefore, glutaraldehyde should also be able to inactivate soy Kunitz TI. Conditions established for this model system were then also applied to TI in soy protein isolate.

EXPERIMENTAL PROCEDURES

Materials. Commercial soy Kunitz TI, type 1-S (Sigma Chemical Co., St. Louis, MO), was used without further purification. Discontinuous polyacrylamide gel electrophoresis (13% T gels stained with Coomassie Brillant Blue R-250) revealed six protein bands in this preparation, five having antigenic similarity to Kunitz TI upon Western blot electrophoretic transfer (10). For this preparation, TI activity was 80% based on assays of 11 replicates, which gave 0.79±0.04 mg TI/mg sample and 0.99±0.06 mg protein/mg sample. Trypsin (type XIII from bovine pancreas) for use in TI assay was purchased from Sigma, and 50% aqueous glutaraldehyde (biological grade) from Polysciences Inc. (Warrington, PA). All other chemicals were reagent grade.

Soy meal extracts containing TI were prepared from raw, dehulled, hexane-defatted soy flakes by extraction of 10 g flakes first with 100 ml 0.01 N NaOH adjusted to pH 8.5 with 1 N NaOH, centrifugation and then extraction of the residue with 50 ml 0.01 N NaOH. The pooled extracts were dialyzed against distilled water and the retentates were lyophilized. For this preparation, TI activity was 8.3% based on assays of 11 replicates, which gave 0.08±0.01 mg TI/mg sample and 1.01±0.07 mg protein/mg sample.

Glutaraldehyde treatment. In a series of experi-

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ments, glutaraldehyde was added to Kunitz TI (3 mg/ml in 0.1 M sodium phosphate buffer, pH 6) to a concentration varying from 0.1 to 3.0% based on total volume of reaction mixture. The reaction mixture was stirred with a magnetic stirrer at 25 C for 5-120 min, and the reaction was then stopped by adding sodium metabisulfite (24); aliquots of the reaction mixture were extensively dialyzed against distilled water. Dialyzed samples were then assayed for protein and TI activity. Similar reactions were performed in 0.1 M sodium acetate buffer (pH 5), 0.1 M sodium phosphate buffer (pH 7) and 0.1 M TRIS HC1 buffer (pH 8) to determine the effect of pH on glutaraldehyde inactivation of Kunitz TI. TI in the lyophilized alkaline soy meal extract (10 mg/ml buffer) were treated similarly and also subjected to 3% glutaraldehyde at 75 C. Most specific reaction conditions were tested only once, since the similarity of the numerous reactions established the overall trend. Where replicates were performed, means are given with standard deviations.

Sulfiting treatment. Sodium metabisulfite, sodium dithionite and sodium sulfite (0.03 mM) were each reacted for 30 min at 75 C with Kunitz TI (3 mg/ml 0.1 M sodium phosphate buffer, pH 7) with magnetic stirring to establish their effectiveness as TI inactivators. Of these salts, sodium metabisulfite ($Na_2S_2O_5$) was selected (as discussed later). Variables assessed with $Na_2S_2O_5$ treatments of Kunitz TI and TI in lyophilized alkaline soy meal extract included reaction temperature (25-90 C), reaction time (0-60 min) and salt concentration (0.01-1.0 mM); for Kunitz TI the concentration ranged from 1-7 mg/ml, and for alkaline soy meal extract the concentrations were 3-40 mg/ml buffer. Sample solutions in 0.1 M sodium phosphate buffer, pH 7, were subjected to each variable specified above. Salt treatments were timed from the moment of salt addition, while temperature treatments were timed from the moment the reaction system was subjected to the specified water bath temperatures. Aliquots (3 ml) were taken at each specified time or temperature, cooled immediately in ice, and dialyzed against distilled water. Retentates were assayed for TI activity and protein.

$Na_2S_2O_5$/Glutaraldehyde treatment. Reaction mixtures of Kunitz TI (3 mg/ml 0.1 M sodium phosphate buffer, pH 7), alone or in combination with 0.01 or 0.6 mM $Na_2S_2O_5$, were heated for 1 hr at 75 C. In a second series, incubation of protein with $Na_2S_2O_5$ for ½ hr at 75 C was followed by addition of glutaraldehyde (0.5-3.0%) at 75 C for 30 min. Reaction mixtures were then chilled in ice to quench the reaction and dialyzed; retentates were assayed for TI activity and protein.

Lyophilized alkaline soy meal extract (10 mg/ml) was treated as above with 0.6 mM $Na_2S_2O_5$ and in combinations of $Na_2S_2O_5$ with glutaraldehyde (1-3%). To verify inactivation of TI in soy meal extract with metabisulfite plus glutaraldehyde, aliquots were periodically removed for analysis 2-30 min after addition of glutaraldehyde.

Assays for protein and TI activity. Protein content was estimated by the Lowry method (25) with bovine serum albumin as a standard.

TI activity was assayed and quantitated as described by Hamerstrand et al. (26) using α-N-benzoyl-DL-arginine-p-nitroanilide hydrochloride as substrate for trypsin.

RESULTS AND DISCUSSION

TI inactivation with glutaraldehyde. Jansen et al. (23) tested the effect of pH on glutaraldehyde insolubilization of soy Kunitz TI: the pH optimum was 4.8, at which lysine content dropped by one-half. These authors did not investigate loss of TI activity upon glutaraldehyde insolubilization of this protein. Since our objective was to retain protein solubility while optimizing TI inactivation, we evaluated glutaraldehyde reactions at pH 5, 6, 7 and 8; optimum solubility under all processing conditions was found at pH 6 or 7, where 70-90% of protein remained soluble. In Figure 1, a near-linear relationship between Kunitz TI activity and glutaraldehyde concentration occurred between 0.2 and 1.5% glutaraldehyde when reacted for 1 hr at pH 6 and 25 C.

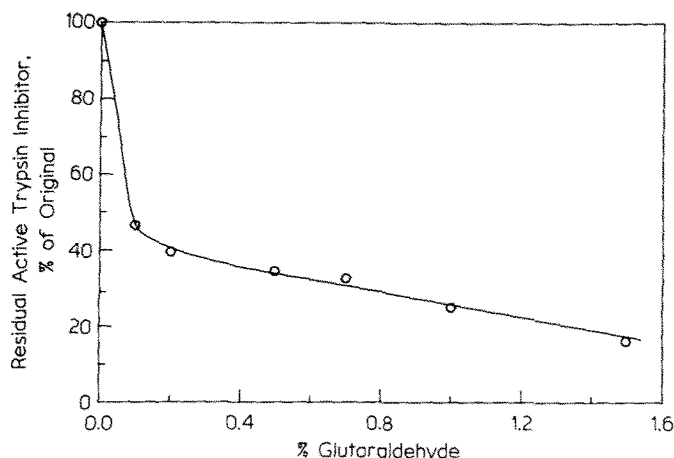

FIG. 1 Effect of glutaraldehyde concentration on soy Kunitz trypsin inhibitor (TI) inactivation. Reaction conditions: 3 mg Kunitz TI/ml 0.1 M sodium phosphate buffer, pH 6 at 25 C for 1 hr. The 100% value is for untreated inhibitor.

When processing times of Kunitz TI with 0.2% or 1.0% glutaraldehyde were varied at pH 6.0, curvilinear relationships resulted between the percentage of residual TI activity and reaction times (Fig. 2). A replicate run with 0.2% glutaraldehyde yielded a set of values for residual active TI with a standard deviation of ±5.72, demonstrating that reaction of Kunitz TI with glutaraldehyde at 25 C may not give highly reproducible results. Maximum (85%) TI inactivation was achieved at pH 6 with 1% glutaraldehyde for 2 hr at 25 C. No additional loss in TI activity was observed with 1.5 or 3.0% glutaraldehyde. These results show that, at best, only 85% TI inactivation can be achieved by glutaraldehyde treatment.

Lyophilized alkaline soy meal extract (10 mg/ml 0.1 M sodium acetate, pH 5) treated with 1.5% glutaraldehyde for 1 hr at 25 C retained 49.2±4.9% of its original activity, while only 21.7±5.3% of protein

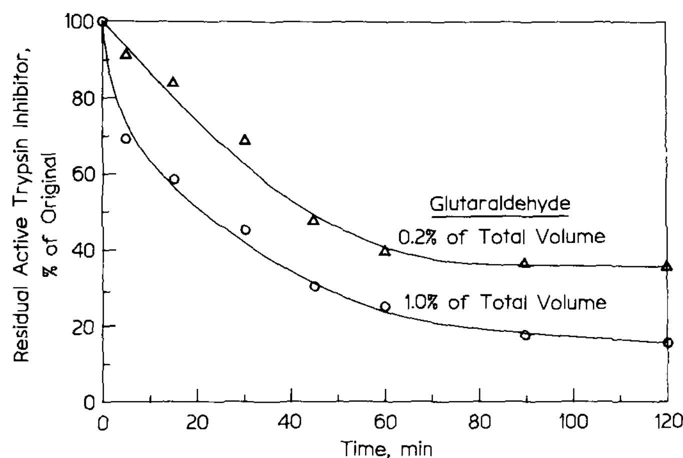

FIG. 2. Effect of processing time on glutaraldehyde-treated soy Kunitz trypsin inhibitor (TI) inactivation. Reaction conditions: 3 mg Kunitz TI/ml 0.1 M sodium phosphate buffer, pH 6, at 25 C. The 100% value is for untreated inhibitor.

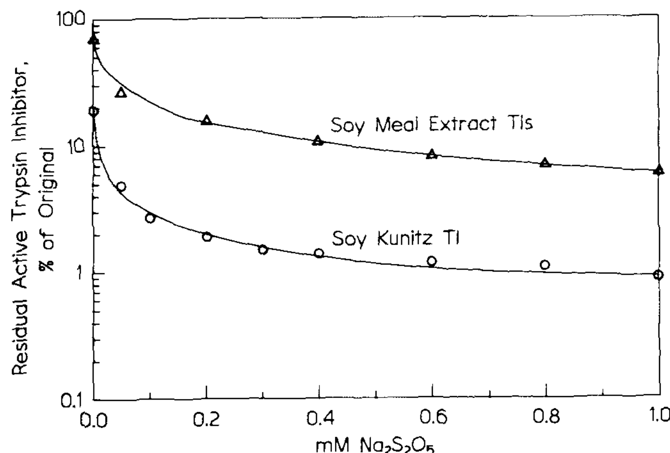

FIG. 3. Effect of sodium metabisulfite ($Na_2S_2O_2$) concentration on trypsin inhibitor (TI) inactivation. Reaction conditions: 3 mg soy Kunitz TI/ml 0.1 M sodium phosphate buffer, pH 7, and 10 mg lyophilized alkaline soy meal extract TI/ml buffer processed for 1 hr at 75 C, without and with varied amounts of $Na_2S_2O_5$.

remained soluble. At pH 6 or 7, 65–84% of the protein remained soluble after 2 hr reaction with 1.5 or 3.0% glutaraldehyde; however, a maximum of only 40% TI inactivation occurred. Increasing the temperature to 75 C during 1 hr of treatment of soy meal extract with 3% glutaraldehyde did not significantly decrease TI activity (i.e., 50% inactivation versus 40%). Thus, glutaraldehyde treatment is not as effective for inactivation of TI's in soy meal extract as for pure Kunitz inhibitor. Glutaraldehyde may preferentially cross-link amino acids on proteins other than TI, in the Kunitz TI model system, the only reactive amino acids are in the inhibitors themselves.

TI inactivation with sulfiting agents. Sodium metabisulfite, which converts to sodium bisulfite when heated in water, was added to glutaraldehyde reaction mixtures to quench the reaction (24). Upon this addition, a highly significant TI inactivation occurred in control samples (soy meal extract with $Na_2S_2O_5$ and no glutaraldehyde, heated at 75 C). Friedman et al. (18) alluded to use of sodium sulfite and other reducing salts to facilitate TI heat activation. We found that sodium metabisulfite, sodium dithionite and sodium sulfite (0.03 mM, pH 7, with ½ hr reaction at 75 C) inactivate soy Kunitz TI to the same extent while retaining good protein solubility (Table 1). $Na_2S_2O_5$ was selected for further study because of its better solubility in water, partial solubility in alcohol, low interaction with proteins to form S-sulfonate derivatives (22) and ability to react reversibly with aldehyde to form an aldehyde/bisulfite addition compound. These qualities might facilitate removal of it residues from processed soy foods.

Heat treatment alone (1 hr at 75 C) inactivated 80.5±0.6% Kunitz TI and 28.5±2% of the TI in soy meal extract; addition of $Na_2S_2O_5$ further enhanced inactivations (Fig. 3). With 0.6 mM $Na_2S_2O_5$, 99% Kunitz TI and 92% of the TI in soy meal extract were inactivated while retaining 91% of and 78% protein

TABLE 1.

Effect of Reducing Salts (0.03 mM) on Heat-Inactivated Soy Kunitz Trypsin Inhibitor (TI)[a]

Salt	Residual active TI[b]	Soluble protein[b]
No salt	25.1	104.5
Sodium metabisulfite	15.4	92.9
Sodium dithionite	16.8	92.1
Sodium sulfite	16.9	91.8

[a]Conditions: 3 mg soy Kunitz TI with 0.79±0.04 mg TI/mg sample heated at 75 C for ½ hr in 1 ml 0.1 M sodium phosphate buffer, pH 7.
[b]Percent of original.

solubilities, respectively. Increasing $Na_2S_2O_5$ concentration to 1 mM inactivated slightly more TI, with no decrease in protein solubilities. With the salt/heat combinations used, better inactivation of TI was achieved with the purified Kunitz system. Other proteins in soy meal extract may protect TI from $Na_2S_2O_5$ inactivation.

A temperature above 60 C (with 0.6 mM $Na_2S_2O_5$ for 1 hr at pH 7) was essential to most effectively inactivate soy Kunitz TI and TI in soy meal extract (Fig. 4). A point of inflection occurred at ca. 65 C for both Kunitz TI and soy meal extract. Wu and Scheraga (27) established the transition temperature for thermal denaturation of soy Kunitz TI to be 63.8 C at pH 6.5 by measuring difference spectra at 298 nm. Thermal denaturation and inactivation of Kunitz TI appear synonymous; bisulfite enhances thermal inactivation without affecting transition temperature. $Na_2S_2O_5$ is a facilitator for thermal inactivation of TI. For example, heating Kunitz TI at 75 C with no bisulfite for 1 hr inactivated 80.5% TI vs 99.1% inactivation with 0.6 mM $Na_2S_2O_5$; for soy meal extract, how-

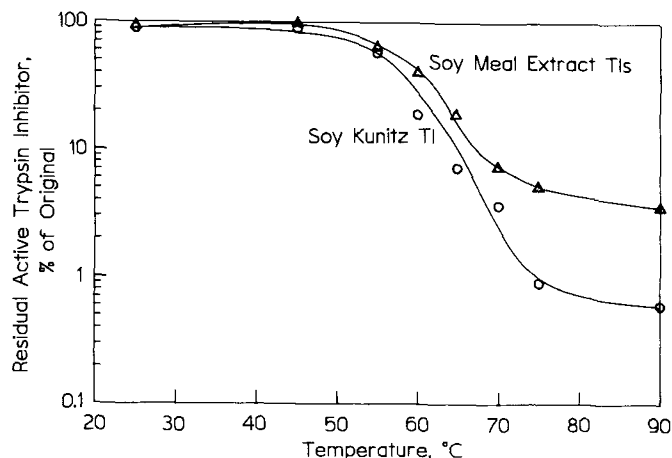

FIG. 4. Effect of temperature on trypsin inhibitor (TI) inactivation. Reaction conditions: 3 mg soy Kunits TI/ml 0.1 M sodium phosphate buffer, pH 7, and 10 mg lyophilized alkaline soy meal extract TI/ml buffer processed with 0.6 mM sodium metabisulfite for 1 hr.

ever, 28.5% inactivation occurred under similar conditions with no $Na_2S_2O_5$ vs 94.8% with $Na_2S_2O_5$. Here the proportion inactivated by added $Na_2S_2O_5$ is much higher for soy meal extract than for Kunitz TI.

When processing time was varied (Fig. 5), treatments of 30 min or longer with 0.6 mM $Na_2S_2O_5$ at 75 C were needed for optimal inactivation of Kunitz TI and soy meal extract. A 1-hr reaction inactivated 98.3% Kunitz TI and 93.9% of the TI in soy meal extract. Comparison of these results to Fig. 4 shows excellent reproducibility (98.7%±0.6% inactivation of Kunitz TI and 94.4±0.6% inactivation in soy meal extract). In all reactions using heat treatments up to 75 C and $Na_2S_2O_5$ concentrations to 1 mM, protein solubilities were 83% or better for both Kunitz TI and soy meal extract.

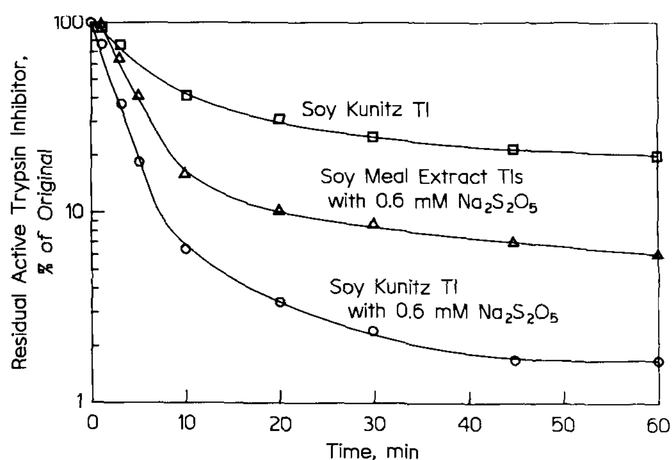

FIG. 5. Effect of processing time on trypsin inhibitor (TI) inactivation. Reaction conditions: 3 mg soy Kunitz TI/ml 0.1 M sodium phosphate buffer, pH 7, processed at 75 C; either 3 mg soy Kunitz TI or 10 mg lyophilized alkaline soy meal extract TI/ml 0.1 M sodium phosphate buffer, pH 7, each with 0.6 mM sodium metabisulfite processed at 75 C. The 100% value is for untreated inhibitor.

TI concentration may also be expected to influence inactivation. Kunitz TI (1, 3, 5 and 7 mg/ml buffer, pH 7) and TI in soy meal extract (5, 10, 20, 30 and 40 mg/ml buffer) were each reacted with 0.6 mM $Na_2S_2O_5$ at 75 C for 1 hr. Results (data not shown) indicated little difference in percentage of inactivation: the mean inactivation was 98.6 ± 0.3% for Kunitz TI and 93.1 ± 1% for soy meal extract. Since both values are within the range for replicates from Figures 4 and 5, chemical treatments with $Na_2S_2O_5$ will inactivate a wide range of TI concentrations.

Thus, studies using $Na_2S_2O_5$ to inactivate TI show excellent reproducibility and prove that $Na_2S_2O_5$ promotes soy TI inactivation with only mild heat treatment while retaining good protein solubility.

TI inactivation with $Na_2S_2O_5$ plus glutaraldehyde. The previous sections demonstrate that glutaraldehyde alone is not an effective TI inactivator, that moderate heat (>60 C) treatment is essential to inactivate TI with $Na_2S_2O_5$ and that $Na_2S_2O_5$ treatment for 1 hr at 75 C inactivates >98% Kunitz TI and >93% of TI in soy meal extract. $Na_2S_2O_5$ most likely cleaves TI disulfide bonds, thereby disrupting conformation integrity and resulting in inactivation. Glutaraldehyde, however, can react both with released sulfhydryls and with other susceptible amino acids to modify TI activity; glutaraldehyde also reacts with residual bisulfite to form aldehyde-bisulfite adducts. Because of the individual effectiveness of $Na_2S_2O_5$ and glutaraldehyde in inactivating TI, we next studied their use in combination.

Since disruption of disulfide bonds must precede their reaction with glutaraldehyde, we first treated TI with metabisulfite, and then with glutaraldehyde. Results (Table 2) for the model soy Kunitz TI system show that combined $Na_2S_2O_5$/glutaraldehyde treatment inactivated TI more completely than either compound alone. Glutaraldehyde treatment, however, lowered protein solubility by approximately 14% compared to heat-treated Kunitz TI. Glutaraldehyde treatment at 75 C (no. 5, Table 2) gave a 54% decrease in Kunitz TI activity beyond heat treatment alone

TABLE 2.

Heat Inactivation[a] of Soy Kunitz Trypsin Inhibitor (TI) in Presence of Sodium Metabisulfite (A) and Glutaraldehyde (B)

Treatment	Reaction time (min)	Residual active TI[b]	Soluble protein[b]
1. No A or B	60	16.0 ± 3.4	97.1 ± 1.6
2. 0.01 mM A	60	15.3	94.6
3. 0.01 mM A; 0.5% B	30; 30	3.3	79.5
4. 0.6 mM A	60	1.0 ± 0.1	90.3 ± 0.8
5. 3% B	60	8.7	81.2
6. 0.6 mM A; 3% B	30; 30	0.4	84.6
7. 0.6 mM A; 2% B	30; 30	0.4	81.0
8. 0.6 mM A; 1% B	30; 30	0.5	86.5
9. 0.6 mM A; 0.5% B	30; 30	0.5	87.1

[a]Conditions: 3 mg soy Kunitz TI with 0.79 ± 0.04 mg TI/mg sample heated at 75 C in 1 ml 0.1 M sodium phosphate buffer, pH 7.
[b]Percent of original.

TABLE 3.

Heat Inactivation[a] of Trypsin Inhibitors (TI) in Lyophilized Alkaline Soy Meal Extract in Presence of 0.6 mM Sodium Metabisulfite (A) and 3% Glutaraldehyde (B)

Treatment	Reaction time (min)	Residual active TI[b]
1. No A or B	60	71.5 ± 2.0
2. A	60	6.1
3. A	30	8.7
4. B	60	49.8
5. B	30	75.9
6. A; B	30; 2	8.8
7. A; B	30; 5	9.1
8. A; B	30; 10	11.0
9. A; B	30; 20	12.2
10. A; B	30; 30	12.2

[a]Conditions: 10 mg soy meal extract with 0.08 ± 0.01 mg TI/mg sample heated 75 C in 1 ml 0.1 M sodium phosphate buffer, pH 7.
[b]Percent of original.

(i.e., no. 1). This decrease in activity was similar to that found when Kunitz TI was treated with 3% glutaraldehyde at 25 C (46%, data not shown). Thus, chemical modification by glutaraldehyde does not seem to be heat dependent. Since glutaraldehyde also reacts with bisulfite, its effective concentration needed to promote further inactivation may be limited. However, no significant differences in TI inactivation were observed for 0.6 mM $Na_2S_2O_5$/glutaraldehyde treatments when glutaraldehyde concentrations ranged from 0.5–3%; 0.5% glutaraldehyde was sufficient.

When combined $Na_2S_2O_5$/glutaraldehyde treatment was applied to lyophilized alkaline soy meal extract, residual TI activity increased slightly upon heating for 2 to 20 min with glutaraldehyde, and was greater than that using $Na_2S_2O_5$ alone (Table 3). To verify this observed increase, replicate analyses were performed, which showed similar increased TI activity (standard deviation ± 0.5). Either partial reactivation may occur, or chemical modification may activate additional TI. No attempt was made to determine which mechanism occurs.

DISCUSSION

Our results demonstrate that sodium metabisulfite facilitates heat inactivation of purified Kunitz TI as well at TI in soy meal extract. Sulfiting agents can disrupt disulfide bridges (22), thereby destroying native conformations of proteins; this is presumably the mechanism of TI inactivation by $Na_2S_2O_5$ (12,13). Sulfiting agents have been used extensively in winemaking, processing fruits and vegetables, freezing and brining, and for preserving fruit juices, purees, syrups and condiments (28). However, since current legislation by the Food and Drug Administration calls for revoking Generally Recognized as Safe (GRAS) status for sulfiting agents on fruits and vegetables to be served or sold raw to consumers (29), any new food processing with sulfiting agents must receive close scrutiny.

Glutaraldehyde, a protein cross-linking agent, inactivates TI by a different mechanism which does not require heat. This reagent reacts with α and ϵ-amino groups, cysteine sulfhydryls, the imidazole ring in histidine and the phenolic hydroxyl group of tryosine (23); some of these interactions are known to inactivate TI (21). Glutaraldehyde also reacts with bisulfite to form aldehyde/bisulfite addition compounds. Therefore, when glutaraldehyde is used with bisulfite, some TI inactivation by bisulfite may be counteracted by glutaraldehyde. Glutaraldehyde has been used in drug synthesis, and GRAS status for use as a food ingredient for cross-linking collagen in sausage casings has been applied for (Custer, M., Food and Drug Administration, personal communication).

Because of the current investigative status for use of sulfiting agents and glutaraldehyde in food products, the results of this research should be assessed not solely in terms of potential food usage in processed products, but rather on the principles involving chemical inactivation of TI. Future research will involve determining effects of sulfites on Bowman-Birk trypsin inhibitors.

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