Chemical Inactivation of Soybean Protease Inhibitors[1]

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This study aimed to optimize chemical treatments to destroy Kunitz (KTI) and Bowman-Birk (BBI) type protease inhibitors in model systems, and to destroy total trypsin inhibitor activity in soy flour. Time, temperature, and reagent concentration were studied and 40 to more than 85% inactivation of KTI and BBI were observed by treatment with 0.6 mM $Na_2S_2O_5$, 10 mM ascorbic acid + 0.8 mM $CuSO_4$ or 20 mM H_2O_2 + 0.8 mM $CuSO_4$ at 65–90°C for 0.5–1 hr. Upon treatment with $Na_2S_2O_5$, KTI and BBI amino acid composition had no significant change. In contrast, AH + CU^{2+} treatment of both KTI and BBI markedly increased aspartic acid + asparagine and glutamic acid + glutamine contents, and significantly decreased histidine, tyrosine, and methionine. With soy flour, only $Na_2S_2O_5$ effectively inactivated both protease inhibitors. Steeping soybean flour in 50 mM $Na_2S_2O_5$ at 65°C for 1 hr inactivated about 98% BBI and 95% KTI. The information conveyed is basic to developing chemical methodology needed to inactivate both KTI and BBI protease inhibitors in soy protein products.

KEY WORDS: Chemical inactivation, Kunitz, Bowman-Birk inhibitors, soybean, trypsin inhibitors.

Proteins of raw legumes have low nutritive value unless heated (1). Nutritional quality improves after heating because of destruction of heat-labile biological factors and conversion of raw native proteins to more digestible denatured forms. Excessive heat, however, markedly impairs soybeans' nutritive value (2), reduces protein efficiency ratio by destroying essential amino acids such as cysteine, methionine, and lysine, and can adversely affect functional properties of soy proteins.

Many have studied heat inactivation of protease inhibitors in foods. Rackis *et al.* (3) reviewed factors that affect protease inhibitor inactivation, and methods of inactivation. All commercially toasted soy products retain 5–20% of the trypsin inhibitor (TI) activity of the soybeans from which they were prepared (3). Sessa and Bietz (4) isolated and characterized proteins with residual TI activity in commercially toasted soybean flour. Kunitz TI (KTI) and Bowman-Birk inhibitors (BBI) accounted for most TI activity of toasted flour.

KTI has a molecular weight of 20,083 daltons, 181 amino acid residues, and two disulfide bridges (5). Its one-active site (arginine at residue 63 and isoleucine at residue 64) complexes with trypsin (5). KTI activity can be destroyed by modifying arginine residues with 1,2-cyclodexanedione (6). Enzymatic conversion of arginine to citrulline with peptidylarginine deiminase also rapidly destroys activity (7), as does cleavage of disulfides (8). Reducing agents, such as cysteine, N-acetylcysteine, mercaptoethanol, and reduced glutathione, enhance heat inactivation of soy TIs (9–12). Sessa and Ghantous (13) inactivated KTI in model systems and TIs in soy meal extracts with reducing salt sodium metabisulfite ($Na_2S_2O_5$), and the crosslinking agent, glutaraldehyde. In model systems, KTI also can be effectively inactivated with ascorbic acid + copper ion (14). Modification of tryptophan, tyrosine, or histidine residues in soy KTI also decreases inhibitory activity (15).

BBI, with a molecular weight of 7848 daltons, has 71 amino acid residues and 7 disulfide bridges (16). BBI has two active sites, a serine-lysine site that complexes with trypsin and a leucine-serine site that complexes with chymotrypsin (16). Birk (17) reviewed four decades of studies on the isolation, characterization, properties, structure, function, and possible uses of BBI from soybeans. Stahlhut and Hymowitz (18) and Tan-Wilson *et al.* (19) reported up to 5–10 Bowman-Birk isoinhibitors. Cleavage of four of BBIs seven disulfides inactivates the inhibitor (20). Preferential acetylation of the two tyrosines in the vicinity of either the trypsin or chymotrypsin active site was accompanied by loss of one inhibitory activity without affecting the other (21). In its purified state, BBI is highly heat stable (22). However, purified BBI heated in a soy flour matrix was inactivated more readily than similarly treated KTI (23). DiPietro and Liener (23) suggested that the microenvironment in soy flour appears to affect heat inactivation of BBI more than that of KTI.

Other than the effects of disulfide cleavage (20) and tyrosine acetylation (21), little research has been done on chemical inactivation of BBIs. The aim of this study was to develop and optimize methods by using the chemical treatments of Sessa and Ghantous (13) and Sessa *et al.* (14) for destruction of BBI and KTI in model systems. These methods were also used selectively to destroy TIs in soy flour.

EXPERIMENTAL PROCEDURES

Materials. Commercial soy KTI, type 1-S (Sigma Chemical Co., St. Louis, MO) was used without further purification. Crude BBI was prepared from defatted soy flour (Century variety) by alcohol extraction and acetone precipitation (24). Crude BBI was purified by anhydrotrypsin affinity column chromatography (25) by using methods described by Sessa *et al.* (14). KTI was 85.2% active (based on mg active TI/mg protein), while BBI was 88.5% active. Trypsin (type XIII from bovine pancreas) and chymotrypsin (type II from bovine pancreas) used in TI and chymotrypsin inhibitor (CTI) assays were from Sigma. All other chemicals were reagent grade.

Model system. Model systems contained 3 mg KTI/mL or 3 mg BBI/mL in 0.05 M sodium phosphate buffer, pH 6.5, and with one of the following: 0–10 mM ascorbic acid (AH) + 0.8 mM $CuSO_4$; 0–20 mM hydrogen peroxide

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[2]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.
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(H_2O_2) + 0.8 mM $CuSO_4$; or 0-1 mM sodium metabisulfite ($Na_2S_2O_5$). Samples were shaken at 80 shakes/min in a shaking water bath set at the desired temperature. Reaction times, temperatures and reagent concentrations were varied. Copper-catalyzed reactions were stopped by adding 1 mM ethylenediaminetetraacetic acid (EDTA). Sodium metabisulfite treatments were stopped by cooling the reaction vessel in ice water. Salt was removed by chromatography on PD-10 Sephadex G-25 columns (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) or dialysis against distilled water for two days with a Spectra/Por membrane (molecular weight cutoff 3500 daltons).

Soy treatments. Defatted soy flour was treated by dispersing 100 mg/mL sodium phosphate buffer containing 10-100 mM AH + 0.8 mM $CuSO_4$, H_2O_2 + 0.8 mM $CuSO_4$ or $Na_2S_2O_5$. Samples were shaken at 100 shakes/min in a shaking water bath set at 65°C for 1 hr. Treated samples were desalted by dialysis as described above.

Assays. Protein contents were estimated in triplicate by using the Lowry method (26) with bovine serum albumin as a standard. TI activity was determined by the method of Hamerstrand *et al.* (27). CTI activity was determined by the method of Erlanger *et al.* (28) with glutaryl-L-phenylalanine *p*-nitroanilide as substrate. Calculations were based on the percent residual active inhibitor compared to a room temperature control with no chemical treatment.

Amino acid analyses. Amino acid analysis was done by using pre-column derivatization and analysis on a Waters Pico Tag amino acid analyzer (Waters Associates, Milford, MA) (29). As described in this reference, treated samples were dialyzed, freeze dried, and hydrolyzed with 6M HCl in vapor phase for 24 hr at 110°C. Hydrolyzed samples were stripped of HCl under vacuum, dried, and derivatized with phenylisothiocyanate.

Statistical analysis. Linear, exponential, power, and reciprocal functions were fit to the data and best fit was determined by maximum R^2 with no bias in residual active inhibitor that remained after treatments.

RESULTS AND DISCUSSION

Variation of reaction parameters in model systems. Sessa *et al.* (14) optimized concentrations of AH and $CuSO_4$ needed to inactivate KTI and BBI. In model systems, 10 mM AH + 0.8 mM $CuSO_4$ inactivated 95.5% KTI and 67.4% BBI during 1 hr at 65°C. Cupric ion, at the same concentration used in the AH treatment, was also essential to H_2O_2 inactivation of KTI and BBI. Both BBI and KTI percent residual active inhibitor responses to increased H_2O_2 concentrations could be described by negative exponential function equations. Equations (± standard error) used to plot data points in Figure 1 were:

$$\ln(\text{BBI, \% residual active inhibitor}) = 4.461\ (\pm\ 0.027) - 0.0406\ (\pm\ 0.0026) \times \text{Concentration},\ R^2 = 0.98 \quad [1]$$

$$\ln(\text{KTI, \% residual active inhibitor}) = 3.271\ (\pm\ 0.157) - 0.1234\ (\pm\ 0.0165) \times \text{Concentration},\ R^2 = 0.93$$

As H_2O_2 concentration increased, the KTI percent residual active inhibitor decreased significantly faster ($P < .01$ by *t*-test) than did the BBI response. A bias can be observed for the KTI response in Figure 1. The equation KTI, % residual active inhibitor = 3.784 (± 1.159) + 32.838 (± 2.514) × concentration^{-1}, $R^2 = .98$, actually gave a better fit. However, the exponential function was better in fitting both BBI and KTI and thus was more useful for comparisons.

When comparing the AH and $CuSO_4$ system of Sessa *et al.* (14) and the H_2O_2 and $CuSO_4$ system just described, we observe that with either treatment, KTI inactivation was significantly greater than BBI inactivation. Part of this was due to thermal KTI inactivation. At 65°C, 22%

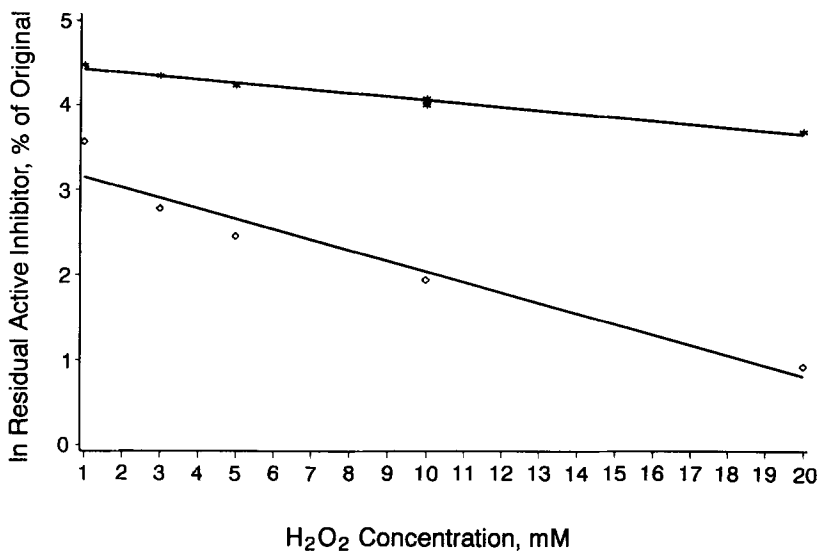

FIG. 1. Effect of H_2O_2 concentration on soy KTI and BBI (◇, KTI; *, BBI). Reaction conditions: 3 mg KTI or BBI/mL 0.05M sodium phosphate buffer, pH 6.5, and heated at 65°C for 30 min.

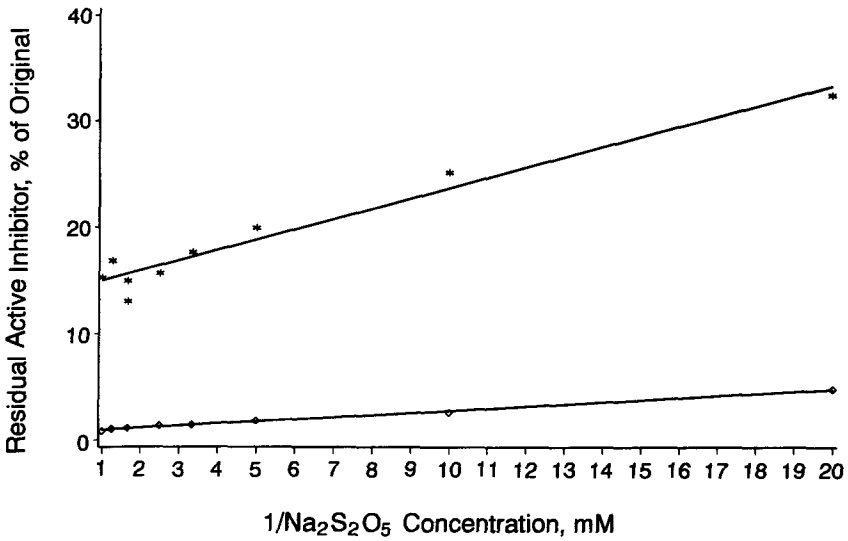

FIG. 2. Effect of $Na_2S_2O_5$ concentration on soy KTI and BBI (*, BBI; ◇, KTI). Reaction conditions: 3 mg KTI or BBI/mL 0.05M sodium phosphate buffer, pH 6.5, and heated at 75°C for 1 hr.

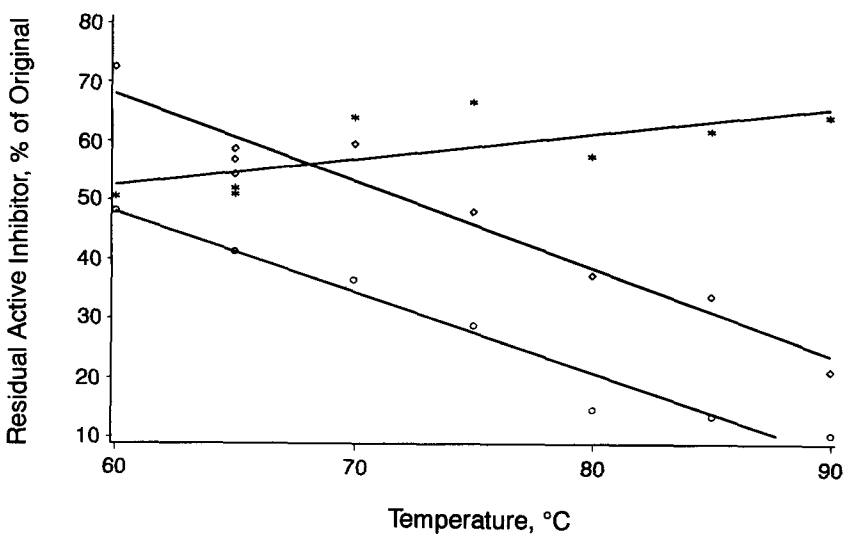

FIG. 3. Effect of temperature on inactivation of soy BBI. reaction conditions: 3 mg BBI/mL 0.05M sodium phosphate buffer, pH 6.5, heated 30 min in the presence of *, 10 mM ascorbic acid + 0.8 mM $CuSO_4$; ◇, 10 mM H_2O_2 + 0.8 mM $CuSO_4$; and ○, 0.6 mM sodium metabisulfite (MB).

KTI was inactivated within 30 min and 38% within 1 hr. At this temperature, a maximum of only 3% BBI was inactivated.

With the $Na_2S_2O_5$ treatments, the metal ions CU^{2+} or Fe^{3+} did not catalyze inactivation of KTI or BBI beyond that of $Na_2S_2O_5$ alone. In the evaluation of the effect of $Na_2S_2O_5$ concentration on KTI and BBI inactivation (Fig. 2) the percent residual active inhibitor remaining with treatment was best described by a reciprocal function. As the reciprocal of concentration increased, the percent residual active inhibitor increased as a straight line according to the equations (± standard error):

$$\text{BBI, \% residual active inhibitor} = 14.073\ (\pm\ 0.647) + 0.9632\ (\pm\ 0.0828) \times (1/\text{concentration}),\ R^2 = 0.95 \quad [3]$$

$$\text{KTI, \% residual active inhibitor} = 0.821\ (\pm\ 0.043) + 0.2018\ (\pm\ 0.0053) \times (1/\text{concentration}),\ R^2 = 0.99 \quad [4]$$

The rate of increase was greater ($P < 01$ by t-test) for BBI inactivation than for KTI. In 1 hr at 75°C, 0.6 mM $Na_2S_2O_5$ inactivated about 87% BBI and 99% KTI. Temperature effects were evaluated for all treatments (Fig. 3). Here, the effects of temperature on percent residual

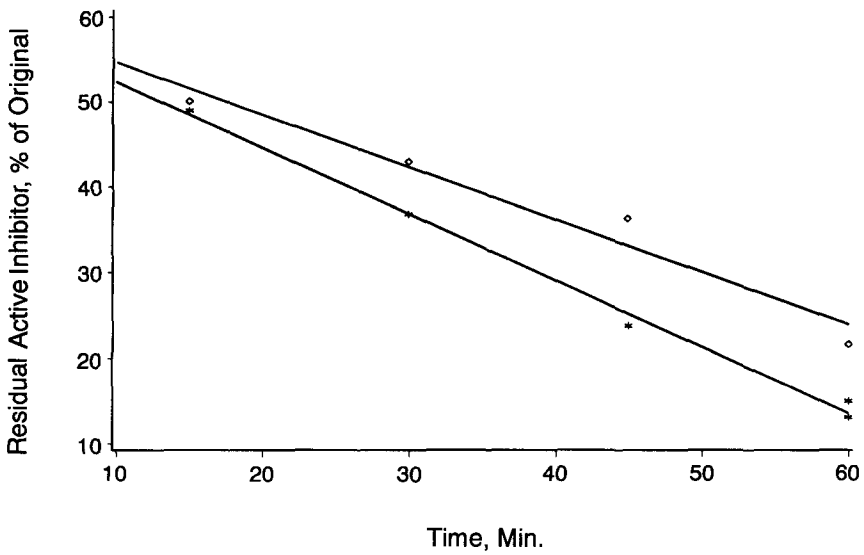

FIG. 4. Effect of heating time (65°C) on inactivation of soy BBI (◇, chymotrypsin activity; *, trypsin activity) (BBI). Reaction conditions: 3 mg BBI/mL 0.05M sodium phosphate buffer, pH 6.5, and containing 10 mM ascorbic acid + 0.8 mM $CuSO_4$.

inhibitor activity were best described by linear functions. Equations (± standard error) were:

$$AH+CuSO_4, \% \text{ residual active inhibitor} = 26.606 (\pm 13.365) + 0.4313 (\pm 0.1796) \times \text{temperature}, R^2 = 0.49 \quad [5]$$

$$H_2O_2+CuSO_4, \% \text{ residual active inhibitor} = 156.293 (\pm 11.035) - 1.4723 (\pm 0.1503) \times \text{temperature}, R^2 = 0.93 \quad [6]$$

$$Na_2S_2O_5, \% \text{ residual active inhibitor} = 129.536 (\pm 9.375) - 1.3586 (\pm 0.1239) \times \text{temperature}, R^2 = 0.96 \quad [7]$$

With the AH + $CuSO_4$ treatment the percent residual active inhibitor increased (P = .05) as temperature increased. This observation may be indicative of a free radical mode of inactivation. Evaluation of this system in the presence and absence of free radical scavengers is needed to establish this mode of inactivation. On the other hand, the H_2O_2 + $CuSO_4$ and $Na_2S_2O_5$ percent residual active inhibitor decreased at similar rates as temperatures increased. Although the H_2O_2 + $CuSO_4$ percent residual active inhibitor was consistently greater than $Na_2S_2O_5$ activity, where lines were parallel by similar (P >.01) slopes but different (P< .01) intercepts.

As can be observed in Figures 4-6, both active sites of BBI were affected by the chemical treatments with AH + $CuSO_4$, H_2O_2 + $CuSO_4$, and $Na_2S_2O_5$. In Figure 4, the rate of decline in percent residual active inhibitor was best described by a negative exponential function. Equations (± standard error) were:

$$\ln(\text{trypsin, \% residual active inhibitor}) = 4.440 (\pm 0.052) - 0.0068 (\pm 0.00077) \times (\text{time in min}), R^2 = 0.94 \quad [8]$$

$$\ln(\text{chymotrypsin, \% residual active inhibitor}) = 4.506 (\pm 0.054) - 0.00575 (\pm 0.00077) \times (\text{time in min}), R^2 = 0.93 \quad [9]$$

The two equations (and slopes) could not be shown to be other than variations of the same line (P = .18 by *t*-tests).

In Figure 5, the rate of decline in percent residual active inhibitor was best described as declining linearly with the log of time. Equations (± standard error) were:

$$\text{Trypsin, \% residual active inhibitor} = 99.493 (\pm 5.559) - 12.6195 (\pm 1.4570) \times \ln(\text{time in min}), R^2 = 0.93 \quad [10]$$

$$\text{Chymotrypsin, \% residual active inhibitor} = 102.798 (\pm 7.187) - 13.5417 (\pm 1.8224) \times \ln(\text{time in min}), R = 0.93 \quad [11]$$

The two equations could not be shown to be other than variations of the same line (P = .50 by *t*-test).

In Figure 6, the effect of time on residual activity is best described by a straight line. The Equations (± standard error) were:

$$\text{Trypsin, \% residual active inhibitor} = 60.126 (\pm 1.441) - 0.7757 (\pm 0.0317) \times (\text{time in min}), R^2 = 0.99 \quad [12]$$

$$\text{Chymotrypsin, \% residual active inhibitor} = 60.800 (\pm 3.671) - 0.6147 (\pm 0.0894) \times (\text{time in min}), R^2 = 0.96 \quad [13]$$

When the slopes were compared by *t*-test, the trypsin, % residual active inhibitor had a tendency (P = .09) to decline at a faster rate than the chymotrypsin residual.

With time, temperature and reagent concentration as variables, we found that treatments of KTI and BBI with 0.6 mM $Na_2S_2O_5$, 10 mM AH + 0.8 mM $CuSO_4$ or 20

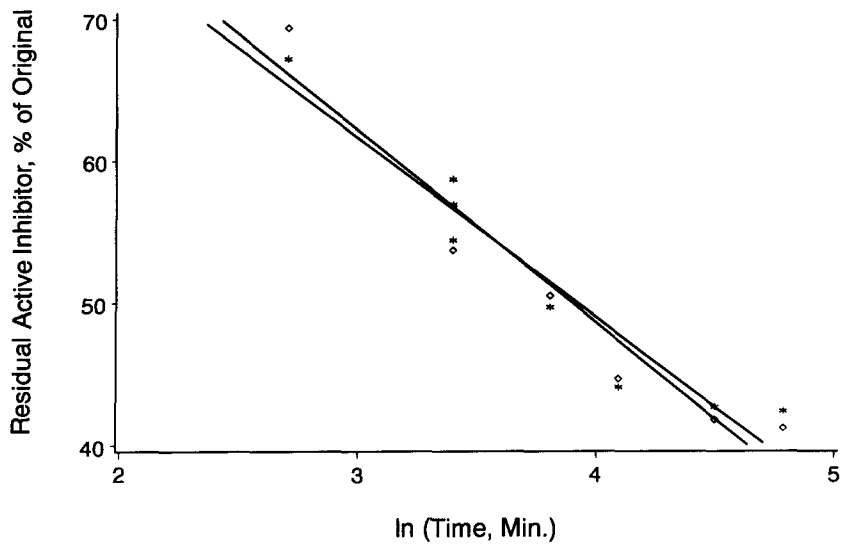

FIG. 5. Effect of heating time (65°C) on inactivation of soy BBI (◇, chymotrypsin activity; *, trypsin activity) (BBI). Reaction conditions: 3 mg BBI/mL 0.05M sodium phosphate buffer, pH 6.5, and containing 10 mM H_2O_2 + 0.8 mM $CuSO_4$.

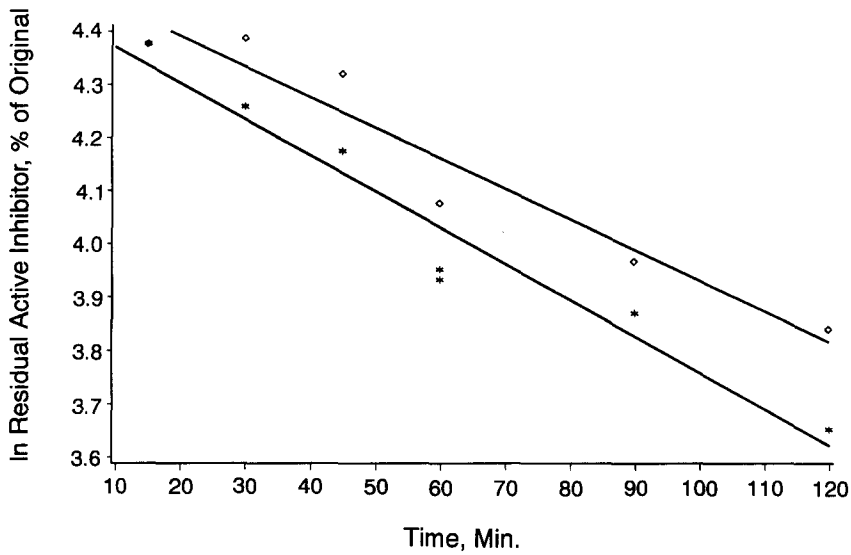

FIG. 6. Effect of heating time (80°C) on inactivation of soy BBI (◇, chymotrypsin activity; *, trypsin activity) (BBI). reaction conditions: 3 mg BBI/mL 0.05M sodium phosphate buffer, pH 6.5, and containing 0.6 mM $Na_2S_2O_5$.

mM H_2O_2 + 0.8 mM $CuSO_4$ for 0.5–1 hr at 65–90°C inactivated 40 to more than 85% of both inhibitors. Inactivation of BBI with 10 mM AH + 0.8 mM $CuSO_4$ did not increase with increased temperature (Fig. 3). At 65°C, over 90% KTI was inactivated (14). If we consider concentration of AH (14) and time as the variables (Fig. 4), 10 mM AH + 0.8 mM $CuSO_4$ treatment of either KTI or BBI for one or more hours will achieve optimum inactivation of either inhibitor. With H_2O_2 + $CuSO_4$ or $Na_2S_2O_5$ treatments, the highest degree of inactivation of BBI and KTI can be achieved with 20 mM H_2O_2 + 0.8 mM $CuSO_4$ or 0.6 mM $Na_2O_2O_5$ at temperatures 75°C or higher for at least 1 hr.

Effects of amino acid composition with treatment. Changes in amino acid composition of KTI and BBI by treatment with AH + Cu^{2+} were assessed. In Table 1 each amino acid was subjected to analysis of variance where protein (KTI *vs.* BBI) and method (theory, native, $Na_2S_2O_5$, and AH + Cu^2) were the main effects. Linear contrasts, within the method effect, made specific tests of native vs. theory, $Na_2S_2O_5$ and AH + Cu^{2+}.

Differences between the two proteins, KTI and BBI, were found for all amino acids except with met, thr, and tyr. Differences for these three amino acids were predicted

TABLE 1

Comparison of Amino Acid Compositions of Native and Chemically Modified Soy KTI and BBI

	Asx[a]	Glx[b]	Ala	Arg	Cys	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thy	Tyr	Val
Soy KTI																	
Theory	14.68	10.17	4.52	5.09	1.13	9.04	1.13	7.91	8.47	5.65	1.13	5.09	5.65	6.21	3.96	2.26	7.91
Native	15.18	10.21	5.45	5.66	1.38	8.44	1.49	7.57	8.01	5.82	1.55	4.68	6.87	4.69	3.75	2.11	7.13
$Na_2S_2O_5$	18.07	10.29	4.86	5.37	1.29	8.22	1.46	7.57	7.61	5.97	1.48	4.59	6.42	4.57	3.65	1.87	6.71
AH+Cu^2	19.40	10.73	5.45	5.51	.17	8.79	1.18	7.75	7.87	4.75	.86	4.46	6.34	4.62	3.76	1.20	7.16
Soy BBI																	
Theory	17.2	10.9	3.1	6.3	10.9	0	1.6	3.1	3.1	7.8	1.6	3.1	9.4	14.1	3.1	3.1	1.6
Native	17.36	11.03	3.54	5.82	5.94	1.38	1.69	3.70	4.40	8.33	1.92	3.43	11.00	11.14	4.43	2.84	2.05
$Na_2S_2O_5$	19.26	11.51	3.67	5.87	5.56	1.35	1.77	4.08	4.30	8.05	1.43	3.32	10.26	11.72	4.38	1.51	1.97
AH+Cu^{2+}	22.34	11.91	4.06	6.57	.20	1.81	1.26	3.55	4.39	7.84	0.66	3.59	11.64	13.03	4.40	.55	2.19
Comparison probabilities[c]																	
KTI vs. BBI	<.01	<.01	<.01	.06	.10	<.01	.05	<.01	<.01	<.01	ns	.01	<.01	<.01	ns	ns	<.01
Theory *vs.* native	ns	ns	.05	ns	ns	ns	.15	ns	ns	ns	ns	ns	.07	.03	ns	ns	ns
Native *vs.* $Na_2S_2O_5$	.02	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Native *vs.* AH+Cu^{2+}	<.01	.03	ns	ns	ns	ns	.05	ns	ns	.09	.02	ns	ns	ns	ns	.05	ns

[a]Asx = Asp + Asn.

[b]Glx = Glu + Gln.

[c]Probability for hypothesis of no consistent difference. Probabilities of >.20 are listed as ns. KTI *vs.* BBI comparison was made as a main effect, the other three comparison between methods were made by linear contrasts, all within analysis of variance.

in theory, but the methods of analysis were not consistent in their calculated differences.

The theoretical value was smaller (Table 1) than the measured native value for ala, his, and pro. The theoretical value was larger than the native value for ser. The $Na_2S_2O_5$ treatment value was consistently larger than the native value only in the asx. Otherwise, $Na_2S_2O_5$ treatment of either KTI or BBI caused no significant changes in amino acid composition. The AH + Cu^{2+} treatment value was larger than the native in asx and glx. The AH + Cu^{2+} treatment value was smaller than the native value in his, lys, met, and tyr. Amino acids in which differences among the methods of analysis were insignificant or inconsistent were arg, cys, gly, ile, leu, phe, thr, and val. With the inconsistency of the cys analyses, the large apparent drop in its content for both KTI and BBI treated with AH + Cu^{2+} was not significant.

To explain some of the changes we osbserved with AH + Cu^{2+} treatments of KTI and BBI, Uchida and Kawakishi (30) observed selective oxidation of histidine and tryptophan when they treated bovine serum albumin with AH + Cu^{2+}. To assess the mechanism of action, a model system of N-benzoylhistidine treated with metal/ascorbate was evaluated. They found a free radical, site-specific mechanism (31) that generated many products, including N-benzoylasparagine and N-benzoylaspartate. The increased contents of aspartic acid + asparagine and decrease in histidine observed upon treatment of KTI with AH + Cu^{2+} could be the result of oxidative degradation of histidine. Uchida and Kawakishi (32) evaluated oxidation of histidine-containing peptides by AH + Cu^{2+}, and showed that histidine breakdown generates ammonia and trace amounts of aspartate, glutamate, and serine. In ascorbic acid-induced crosslinking of bovine lens protein (free of metal catalysts), Ortwerth and Olesen (33) showed significant loss of lysine, along with extensive protein crosslinking and considerable browning, attributed to a Maillard-type reaction. This may account for the apparent loss in lysine in our study. Fleming (34) showed that disulfides can complex directly with ascorbate, leading to a loss of cystine. Methionine loss may be caused by copper-catalyzed oxidation of methionine to a sulfone or sulfoxide.

Protease inhibitor inactivation in soy products. Table 2 summarizes results of various chemical treatment to soy flour. Analysis of variance results for Table 2 are shown in Table 3. Treatment, level, and treatment × level interaction were all significant sources of variation for both TI and CTI inactivation.

For TI inactivation, overall, the H_2O_2 treatment had a tendency (P = .09) to leave greater residual activity than did the AH; both H_2O_2 and AH left greater (P < .01) residual activity than did the $Na_2S_2O_5$-treated soy flour. Across all three treatments, adding 10 mM decreased (P < .01) % residual activities. Addition of 50 mM caused lower (P < .01) % residual activity than 10 mM but 50 mM and 100 mM had similar (P = .41) % residual activity.

Within each treatment, the response in % residual activity was a straight line when regression was run on the log values of the 10, 50, and 100 mM levels (ignore zero levels). Equations (± standard error) were:

$$\text{AH, \% residual activity} = 60.601\ (\pm.886) - 2.995\ (\pm.237) \times \ln \text{level},\ R^2 = .99 \quad [14]$$

$$H_2O_2\text{, \% residual activity} = 82.129\ (\pm2.876) - 7.517\ (\pm.770) \times \ln \text{level},\ R^2 = .99 \quad [15]$$

$$Na_2S_2O_5\text{, \% residual activity} = 24.537\ (\pm3.283) - 4.872\ (\pm.869) \times \ln \text{level},\ R^2 = .94 \quad [16]$$

Comparison of slopes (by *t*-test) differentiated between AH and H_2O_2 treatments and found $Na_2S_2O_5$

TABLE 2

Inactivation of Protease Inhibitors in Defatted Soy Flour When Steeping at 65°C for 1 hr

Treatment	Level (mM)	% Residual active inhibitor			
		n	TI[a]	n	CTI[b]
Heat only	0	4	63.24	4	51.62
AH[c]	10	2	53.82	1	54.27
	50	2	48.86	1	51.10
	100	1	47.03	1	56.37
H_2O_2	10	1	65.13	1	47.93
	50	1	51.70	2	43.95
	100	1	48.23	2	34.90
$Na_2S_2O_5$	10	1	13.90	1	23.30
	50	2	4.51	2	1.92
	100	1	3.45	1	1.86

[a]TI = Trypsin inhbitor.

[b]CTI = Chymotrypsin inhibitor.

[c]AH and H_2O_2 treatments were catalyzed with 0.8 mM Cu^{2+}.

TABLE 3

Analysis of Variance for Data in Table 2

Source	dF	Mean squares	
		TI	CTI
Treatment	2	1834	1510
Level	3	660	343
T × L	6	292	234
Residual activity	4	9.5	6.3

intermediate. Thus, overall, $Na_2S_2O_5$ treatment had lowest % residual activity, H_2O_2 had greater % residual activity at the lower levels but declined faster than AH treatment when levels were increased so that H_2O_2 and AH treatments were similar at the 50 and 100 mM levels.

For CTI inactivation, overall, the AH treatment had higher levels of % residual activity than H_2O_2, which in turn had higher levels of % residual activity than $Na_2S_2O_5$. Over all treatments, the 10 mM levels had lower % residual activity than the 0 levels; the 50 mM level had lower % residual activity than the 10 mM but 50 and 100 mM levels were similar.

Within the AH treatment, the level of concentration had no effect on % residual activity. In the other two treatments (ignoring the zero level), % residual activity declined with the log value of concentration. Equations (± standard erros) were:

$$H_2O_2,\ \%\ \text{residual activity} = 62.787\ (\pm 7.905)$$

$$-6.084\ (\pm 2.118) \times \ln \text{level},\ R^2 = .89 \qquad [17]$$

$$Na_2S_2O_5,\ \%\ \text{residual activity} = 45.314\ (\pm 9.737)$$

$$-10.335\ (\pm 2.577) \times \ln \text{level},\ R^2 = .89 \qquad [18]$$

Heat alone inactivated more CTI in soy flour than observed in the model system. BBI in a soy flour matrix is inactivated by heat more readily than is purified BBI (23). Tanahashi *et al.* (35) established a protease inhibitor inactivation mechanism by which soy proteins affect heat inactivation. They found that KTI and BBI heated with purified soy 11S protein reduced TI activities and thermal stabilities of TIs. Two moles of thiols on 11S reduce disulfides of purified TIs. When 11S protein and TIs are heated at 100°C for 15 min, 65% of KTI and 47% of BBI were inactivated. These results do not explain why TI was more resistant to thermal inactivation than CTI in soy flour. This resistance to TI inactivation was reversed when defatted soy flour was treated with AH + Cu^{2+} (Table 2). Under conditions employed in this study, TI was more readily inactivated in the presence of AH + Cu^{2+}, while CTI inactivation was similar to that with heat alone.

A previous study (36) showed that steeping whole or cracked soybeans with 100 mM $Na_2S_2O_5$ at 65°C for 2 hr eliminated 94% or more TI activity, giving a product with better nitrogen solubility than that of beans treated with heat alone. In that paper more TI was inactivated by heat alone than in our purified KTI (Fig. 1) and also in our defatted soy flour (Table 2). Apparently, processing whole or cracked soybeans to a defatted flour makes it more difficult to inactivate TIs. Endogenous components that exist in the whole or cracked bean in addition to 11S proteins must facilitate heat inactivation of TIs, in much the same way as treatment with a reducing salt.

This study shows that $Na_2S_2O_2$ treatments of defatted soy flour with moderate heat (65°C) eliminates more than 90% TI activity. Sulfite treatments significantly improved the nutritional value of flour in rat-feeding studies (37). In addition, oxidative sulfitolysis by treatment of 11S protein with sodium sulfite will increase the solubility of this protein's basic and acidic polypeptides (38). These authors (38) found that surface hydrophobicity and digestibility increase in acidic subunits, but decrease for basic subunits, which aggregate through hydrophobic interactions. Thus, sulfite treatments of soy not only proved to be effective in inactivating antinutritional factors, but also are known to modify functional characteristics. On a small scale, processing techniques (such as dialysis) will eliminate sulfite residues almost completely from defatted soy flour when preparing a soy protein concentrate (39). Though dialysis is not amenable to large-scale

processing some type of membrane technique can certainly be developed to eliminate sulfite residues, so that sulfiting agents can be used to a greater extent in the future for food and feed processing.

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