Inactivation of Soybean Trypsin Inhibitors with Ascorbic Acid plus Copper

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L-Ascorbic acid plus cupric sulfate inactivates soybean Kunitz trypsin inhibitor (KTI) in model systems containing purified KTI. KTI at 3 mg/mL in 0.1 M sodium phosphate buffer, pH 6.5, was treated for 1–20 h with 0.0–20.0 mM ascorbic acid and 0.8 mM cupric sulfate at 27–65 °C. At 27 °C, 10 mM ascorbic acid plus 0.8 mM $CuSO_4$ inactivates more than 40% of the original KTI within 1 h. At 65 °C, under the same conditions, over 90% KTI was inactivated. For soybean Bowman–Birk inhibitors, these conditions led to 70% TI inactivation. Thus, ascorbic acid plus $CuSO_4$, especially with moderate heat, effectively inactivates soybean trypsin inhibitors in model systems. However, when soy flour is treated with ascorbic acid plus $CuSO_4$ under similar conditions, TI activity is preserved in spite of heat treatment. This preservation effect may reflect the ability of ascorbic acid to act as an oxidant of a component other than TI in a soy flour slurry. Inactivation of purified TIs may occur as a result of prooxidant action of ascorbic acid plus $CuSO_4$ acting directly on the TIs.

INTRODUCTION

Proteinase inhibitors of legumes, particularly soybeans, have been studied extensively because of their deleterious effects in animal nutrition (Liener and Kakade, 1980). In in vivo human experiments, proteinase inhibitors reduced protein absorption and can, therefore, adversely affect human nutrition (Linscheer et al., 1980). In a recent in vivo evaluation of the effects of soybean meals with high and low inhibitor levels on human duodenal enzymes and blood hormones, Holm et al. (1988) reported that infusions of raw soybean meal with high trypsin inhibitor (TI) activity initially reduced tryptic activity to 55% of the basal level in duodenal fluids. Then, within 1 h, tryptic activity increased 130%. Soybean meal with low TI activation when ingested caused an immediate increase in tryptic activity. Their findings corroborate those of Linscheer et al. (1980). Inclusion of soybean proteinase inhibitors in the human diet severely restricts efficient digestion of proteins.

Because of the deleterious effects of trypsin inhibitors in animal nutrition and the restrictive effect of trypsin inhibitors on protein digestion in humans, we need technologies to eliminate TI activity from processed foods and feeds to better use soy proteins. Rackis et al. (1986) reviewed protease inhibitors of plant foods and processes for their inactivation. Moist heat treatment of soybeans at 100 °C for 20 min generally inactivates 90% of the TIs. After processing by most commercial methods, however, residual TI activity remains. Previous chemical methods can inactivate >90% TI in soy meal extracts by using moderate heat treatment (Sessa and Ghantous, 1987). Similar inactivation was accomplished in both whole and cracked soybeans (Sessa et al., 1988). With moderate heat at 75 °C, sodium metabisulfite alone or with glutaraldehyde is an effective inactivator of soybean TIs. Because of the current investigative status on the use of sulfiting agents (see *Chem. Eng. News* **1987**, Feb 9, 21) and glutaraldehyde (see *Food Chem. News* **1987**, June 8, 16) in food products, we sought an alternative but similar method.

L-Ascorbic acid (AA) is ubiquitous in plants. For example, it is a natural constituent of immature and mature (40 mg/100 g) soybeans (Reddy and Kumari, 1988). AA, especially in the presence of trace amounts of metals such as copper, can function as a redox system (Sapper et al., 1982). Recently, Liao and Seib (1988) reviewed reactions of AA in foods. Cupric or ferric ion will catalyze the oxidation of AA by molecular oxygen (Khan and Martell, 1967). As a prooxidant AA will inhibit enzymes such as wheat lipoxygenase (Walsh et al., 1970), mammalian β-glucuronidase (Levvy and Marsh, 1957), catalase (Davison et al., 1986; Orr, 1967a,b), and mushroom polyphenol oxidase (Golan-Goldhirsh and Whitaker, 1984).

In this paper we report the effects of AA plus Cu^{2+} on soybean Kunitz trypsin inhibitor (KTI) inactivation and evaluate its effects on soybean Bowman–Birk inhibitors (BBI) and TIs of soybean flour.

EXPERIMENTAL METHODS

Materials. Commercial soy KTI, type 1-S (Sigma Chemical Co., St. Louis, MO) was used without further purification. To defat soy flour, whole soybeans (var. Century, Kelly Seed Co., Peoria, IL) were cracked by passage through cracking rolls, winnowed to remove hulls, and flaked by passage through smooth rolls; flakes were hexane extracted batchwise at 30 °C, air-dried, and micromilled. BBI was prepared from this defatted soy flour by the alcohol extraction–acetone precipitation method of Frattali (1969). The crude BBI inhibitor preparation was then purified by passage through an anhydrotrypsin affinity column (Pusztai et al., 1988). The procedure involved synthesis of anhydrotrypsin (Ishii et al., 1983), a catalytically inactive trypsin derivative that binds inhibitors without altering them, as does trypsin (Hixson and Laskowski, 1970). Anhydrotrypsin was covalently attached to cyanogen bromide activated beaded agarose and used as a column packing. Crude BBI was dissolved in 0.05 M Tris-HCl, pH 8.0, to give a solution containing 25 mg/mL. This solution was injected onto a 16 × 50 mm anhydrotrypsin/agarose-packed column. A two-step elution program performed on the Pharmacia chromatography system package (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) consisted of 0.05 M Tris-HCl, pH 8.0, followed by 0.025 M citric acid containing 0.1 M KCl. Flow rate was 0.8 mL/min; eluates were monitored at 280 nm. Eluates representing the acidic peak were pooled and dialyzed 3 days in 18-mm casing with

3500 M_r cutoff against deionized, distilled water, changed twice daily; retentates were freeze-dried. Trypsin, type XIII from bovine pancreas, for use in TI assay was purchased from Sigma. All other chemicals were of reagent grade.

AA plus Cu^{2+} Treatment. The KTI model system consisted of 3 mg of KTI/mL in 0.1 M sodium phosphate buffer, pH 6.5, containing from 0 to 20 mM AA. The reaction was initiated by addition of 0–8.0 mM cupric sulfate dissolved in distilled water. Samples were shaken at 150 rpm on an incubator–shaker at 27, 50, or 65 °C. The reaction was stopped after 1, 2, 5, or 20 h by adding 1 mM EDTA and chilling the reaction vessel in ice water. Samples, chilled to room temperature, were desalted by passage through PD-10 Sephadex G-25 columns (Pharmacia) equilibrated for 1 h at 45 mL/h with 0.1 M sodium phosphate buffer, pH 6.5. Eluates were monitored at 280 nm. Two major peaks eluted: the peak eluting with the solvent front assayed positively for protein (see Assays for Protein and TI Activity). Protein eluates were pooled and assayed for TI activity.

The BBI model system consisted of 3 mg of BBI/mL of 0.1 M sodium phosphate buffer, pH 6.5, with 0–10 mM AA plus 0.8 mM $CuSO_4$. A control without AA plus $CuSO_4$ and treated samples were each heated 1 h at 65 °C and analyzed similarly to the KTI model system. Three replicates were analyzed in triplicate.

Soy flour was treated by dispersing 10 mg of soy flour/mL in either distilled water, 0.1 M sodium phosphate buffer, pH 6.5, or 0.2 M Tris buffer, pH 8.5, containing 10 mM AA with 0 or 0.8 mM $CuSO_4$. Slurries were shaken at 150 rpm at 65 °C for 1 h in Parafilm-sealed Erlenmeyer flasks in an incubator–shaker. Reactions were stopped by adding 1 mM EDTA and chilling flasks in ice water. Contents of each flask were centrifuged at 14900*g* for 20 min at 5 °C. Solid ammonium sulfate was added to the supernatants to bring them to 70% saturation, and the suspensions were then recentrifuged at 14900*g* for 20 min at 5 °C. The resulting precipitates were dispersed in 0.1 M sodium phosphate buffer, pH 6.5, and dialyzed for 3 days against distilled water with twice daily changes of water. Retentates were freeze-dried, and each freeze-dried sample was stored under refrigeration until assayed.

Removal of AA plus Cu^{2+} Residues from Samples for Assay. Residues of AA and copper ion interfere with the protein and TI assays. Larson and co-workers (1986) demonstrated that reductants such as ascorbate (1–200 mM) significantly enhance color yield of proteins assayed with the Folin–Ciocalteau reagent (Lowry et al., 1951). We found that addition of $CuSO_4$ to 0.8 mM in our reaction mixture will diminish the color yield of our KTI standard by 10%. In the TI assay, trypsin hydrolyzes the substrate N^{α}-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA) to give *p*-nitroanilide, which absorbs at 410 nm. Added AA (1–10 mM) caused no change in color yield, but additional $CuSO_4$ to 0.8 mM significantly decreased color yields up to 30%. However, serial dilutions (from 1/10 to 1/250) of the reaction mixtures were always made for TI assays. With these dilutions the color yield of the reaction mixture decreased by a maximum of 10%. Because of the effects of AA plus Cu^{2+} on the color yields for both protein and TI assays, AA plus Cu^{2+} residues were removed chromatographically as described in the previous section.

To test for residual AA in our reacted samples, AA was assayed enzymatically with ascorbate oxidase and a tetrazolium salt (Beutler, 1984) purchased as a test kit for enzymatic determination of AA in food and other materials (Boehringer Mannheim Biochemicals, Indianapolis, IN). Metaphosphoric acid, 1.5% (w/v) was used to adjust pH and dilute solutions to a suitable ascorbic acid concentration. We assumed the copper–EDTA complex was removed on our desalting column.

Because of the complexity of components in soy flour treated with AA plus Cu^{2+}, Sephadex G-25 columns were not used for desalting. Instead, AA plus Cu^{2+} plus EDTA residues were removed by protein isolation as described under AA plus Cu^{2+} Treatment for soy flour. Resulting freeze-dried soy protein isolates tested negatively for AA.

Assays for Protein and TI Activity. The protein content of each sample was estimated in triplicate by the Lowry method (Lowry et al., 1951) with KTI as standard.

Table I. Experimental Design Matrix—Ascorbic Acid Concentrations at Each Time and Temperature

	time			
temp, °C	1 h	2 h	5 h	20 h
27	5, 10, 20[a]	5, 10, 20	5, 10, 20	5, 10
50	5, 10, 20	5, 10, 20	–[b]	–[b]
65	5, 10, 20	–[b]	–[b]	–[b]

[a] All ascorbic acid concentrations, in millimolar, for each time and temperature variable contained 0.8 mM $CuSO_4$. [b] Denotes not determined.

TI activity was assayed and quantitated as described by Hamerstrand et al. (1981) with BAPNA as a substrate for trypsin.

Amino Acid Composition. Soy KTI, 10 mg/mL of 0.1 M sodium phosphate buffer, pH 6.5, containing 10 mM AA with 0.8 mM $CuSO_4$ was reacted for 1 h at 65 °C. The reaction was quenched with 1 mM EDTA. Reaction products were dialyzed for 3 days against distilled water; retentates were freeze-dried. Native and oxidized soy KTI were each hydrolyzed at 110 °C for 24 h with 6 N HCl in the vapor phase. The hydrolysates were each concentrated under vacuum, stripped of HCl residues with hot water rinses, filtered, and then derivatized with phenyl isothiocyanate according to the method of Bidlingmeyer et al. (1984). Amino acids analysis was performed on a Waters Pico-Tag amino acid analyzer.

Analytical Techniques for Soy Flour Systems. Aqueous extracts of soy flour with and without AA plus Cu^{2+} treatments were analyzed by high-performance liquid chromatography (HPLC) on a Vydac 218 TP54 (C_{18}) reversed-phase analytical column eluted with an aqueous 20–45% acetonitrile gradient (Peterson and Wolf, 1988).

Aqueous extracts of soy flour were also analyzed calorimetrically on an MC-2 differential scanning calorimeter (Micro Cal, Northhampton, MA) interfaced with an IBM XT microcomputer. The sample and reference solvent volumes were each 0.6 mL; data were collected between 30 and 100 °C at a heating rate of 60 °C/h. The peak temperature, T_{max}, corresponding to the temperature of the maximum heat capacity for the transition, was taken as the denaturation temperature.

Statistical Analysis. All protein and TI activity data from the experimental design matrix of Table I were analyzed with programs within the Statistical Analysis Systems software (SAS Institute Inc., 1987). The basis of all calculations was a control sample at 0 time and 27 °C, with no AA plus Cu^{2+}, performed for each set of experiments. Percentages of original TI activity were calculated with this control as the denominator. Analysis of variance for protein assays and TI activities were run with models from the Table I matrix explained under Results and Discussion.

RESULTS AND DISCUSSION

TI Inactivation with AA plus Cu^{2+} in Model Systems. The KTI activity of the commercial sample used in this study was 0.859 mg of TI/mg of protein on the basis of TI and protein assays and 19 replicates. The amount of $CuSO_4$ selected for our reaction conditions (0.8 mM) was based on changes in KTI inactivation when KTI was treated at 27 °C with 10 mM AA and variable amounts of $CuSO_4$ from 0.008 to 8.0 mM. Only a 4.5% difference in KTI inactivation was observed when the $CuSO_4$ concentration was increased from 0.08 to 8.0 mM. Our KTI reaction system shows $CuSO_4$ is essential to TI inactivation with AA, and a concentration optimum with respect to $CuSO_4$ is between 0.08 and 0.8 mM. Samples containing KTI with 10 mM AA, 0.8 mM $CuSO_4$, and 1 mM EDTA, when reacted for 1 h at 65 °C, gave TI inactivation identical with that for samples with no AA, $CuSO_4$, or EDTA. On the basis of these results, EDTA was used to quench the oxidation reaction with AA plus $CuSO_4$.

Figure 1 shows a typical elution pattern for desalting the KTI model system; tubes 5–9 were pooled as the

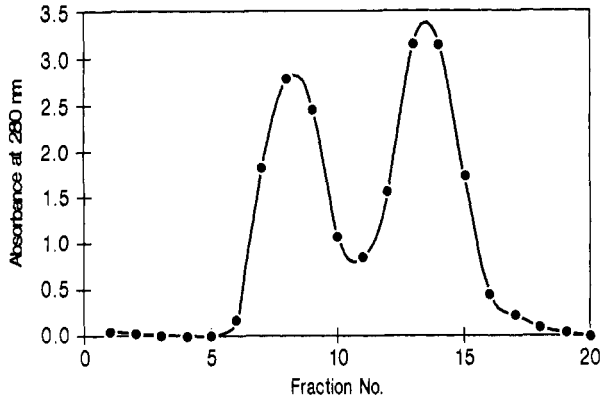

Figure 1. Elution profile of the reaction mixture of soy Kunitz trypsin inhibitor (KTI) treated with ascorbic acid plus cupric sulfate on PD-10 Sephadex G-25M. The 9-mL (1.6 × 50 cm) column resin bed was eluted with 0.1 M sodium phosphate buffer, pH 6.5, at 45 mL/h, collecting fractions of 0.8 mL.

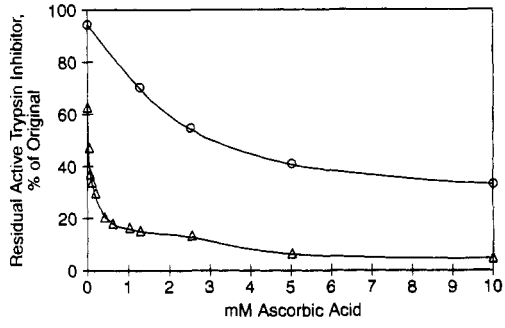

Figure 2. Effect of ascorbic acid (AA) concentration on (△) soy Kunitz trypsin inhibitor (KTI) and (○) Bowman-Birk (BBI) inactivation. Reaction conditions: 3 mg of KTI or BBI/mL of 0.1 M sodium phosphate buffer, pH 6.5, processed for 1 h at 65 °C without and with varied amounts of AA; reactions with AA contained 0.8 mM cupric sulfate as oxidant.

protein fraction. All reaction mixtures including controls with no AA plus Cu^{2+} were chromatographed prior to the protein and TI assays. A material balance study for residual AA with KTI plus 10 mM AA before and after chromatography showed almost 100% recovery of AA in tubes 11–15 of Figure 1. No study was made to determine the completeness for removal of the copper–EDTA complex.

Analysis of variance with variables of time, temperature, and AA concentration, on the 20 observations designated in Table I, yielded an overall mean protein concentration of 1.13 ± 0.23 mg/mL. This does not differ significantly ($P < 0.10$) from the protein content of KTI control samples (1.19 ± 0.22 mg/mL). Therefore, rather than calculate TI activity on the basis of milligrams of TI per milligram of protein, we calculated TI of treated samples as percent of original activity by using the TI assay data in milligrams of TI per milliliter recorded from the control sample for each day's run.

To determine the effect of AA concentration on KTI and BBI inactivation, an experiment was run with 3 mg of either KTI or BBI/mL of 0.1 M sodium phosphate buffer at pH 6.5 and levels of AA of 0.01, 0.05, 0.10, 0.20, 0.40, 0.60, 1.0, 1.25, 2.5, 5.0, and 10, all with 0.8 mM $CuSO_4$ reacted for 1 h at 65 °C. In Figure 2, our results indicate that the AA/Cu^{2+} system inactivates KTI more effectively than it does BBI. Because of the thermal stability of BBI, heat alone at 65 °C for 1 h will inactivate less than 6% BBI, while 10 mM AA plus $CuSO_4$, when combined with heat, inactivated almost 70% BBI.

The effect of heat on KTI was evaluated in model

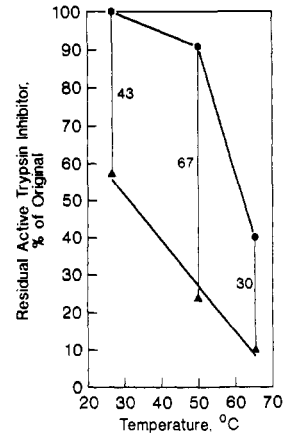

Figure 3. Effect of temperature on soy Kunitz trypsin inhibitor (KTI) inactivation. (●) 3 mg of KTI/mL of 0.1 M sodium phosphate buffer, pH 6.5, processed for 1 h. (▲) 3 mg of KTI/mL of 0.1 M sodium phosphate buffer, pH 6.5, with 5, 10, or 20 mM AA plus 0.8 mM $CuSO_4$, processed for 1 h. The regression equation for TI, as percent of original activity, is $89.61 - 1.26x$, where x is the temperature in °C; $R^2 = 0.98$.

systems with and without AA and $CuSO_4$ (Figure 3). On the basis of 11 observations at 27 °C, 6 observations at 50 °C, and 3 observations at 65 °C, we observed only a slight effect of heat over the entire temperature range in percent protein ($P = 0.13$). However, a change in temperature from 27 to 50 °C reduced ($P < 0.01$) the percent of original TI activity by 9 percentage points, while a change from 50 to 65 °C reduced the residual active TI by an additional 51 percentage points.

Because the experiment was not completely balanced in Table I, the analysis was split into parts to best examine the effects of time, temperature, and AA concentration, individually and in combination. In analysis 1, under the 1-h reaction time in Table I, an analysis of variance with temperature, AA level, and temperature times AA level interaction was performed with response variables for both protein and residual active TI, as percent of original. The only significant ($P < 0.01$) effect found was a temperature effect on residual TI activity. The residual active TI, as percent of original, was 56.9 ± 6.5, 23.7 ± 10.1, and 9.8 ± 4.0 at 27, 50, and 65 °C, respectively (shown in Figure 3) for AA plus Cu^{2+} treated KTI. With heat alone, KTI activity was 100% at 27 °C, 91% at 50 °C, and 40% at 65 °C. AA (5, 10, or 20 mM) was responsible for inactivation of 43.1% TI at 27 °C, 67.3% at 50 °C, and 30.2% at 65 °C. Our results indicate that the AA plus Cu^{2+} redox system with AA at 5–20 mM will indeed inactivate KTI at 27 and 50 °C. With heating above 50 °C, the contribution of heat toward the inactivation of KTI is greater than that caused by the redox system.

In analysis 2, under the 2-h reaction time of Table I, temperatures of 27 and 50 °C were compared over 5, 10, and 20 mM levels of AA. In the analysis of variance neither time, AA level, nor time times AA level interaction affected percent protein ($P > 0.10$), and only temperature differences affected percent residual active TI ($P < 0.01$). At 27 °C the percent residual active TI was 64.0 ± 17.0 and at 50 °C was 16.8 ± 1.1. Data from analyses 1 and 2 were combined (temperature 65 °C was not run at 2 h and therefore was deleted from this analysis) and a temperature times time interaction was tested to see if temperature effects between 27 and 50 °C were different at 2 h versus 1 h. The effect was not significant ($P > 0.20$).

A third analysis was run at a constant 27 °C with times at 1, 2, and 5 h and AA levels of 5, 10, and 20 mM, and a fourth analysis was performed at 20 h at AA levels 5 and

Table II. Comparison of the Amino Acid Compositions of Native and Oxidized[a] Soy Kunitz Trypsin Inhibitor (KTI)

amino acid	molar ratio,[b] % native	oxidized	difference
Asx	15.18	19.40	+4.22
Glx	10.21	10.73	+0.52
Ser	4.69	4.62	−0.07
Gly	8.44	8.79	+0.35
His	1.49	1.18	−0.31
Thr	3.75	3.76	+0.01
Ala	5.45	5.45	0
Arg	5.66	5.51	−0.15
Pro	6.87	6.34	−0.53
Tyr	2.11	1.20	−0.91
Val	7.13	7.16	+0.03
Met	1.55	0.86	−0.69
cystine	1.38	0.17	−1.21
Ile	7.57	7.75	+0.18
Leu	8.01	7.87	−0.14
Phe	4.68	4.46	−0.22
Lys	5.82	4.75	−1.07

[a] Oxidation of 10 mg of KTI/mL of 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM ascorbic acid with 0.8 mM $CuSO_4$ reacted 1 h at 65 °C. [b] Molar ratio (percent) is expressed as the molar concentration of each amino acid per total amino acids.

10 mM. There were no significant effects of time ($P > 0.15$), AA level, or interaction of time times AA level on percent protein or percent residual active TI.

From the AA concentrations, reaction times, and temperatures evaluated, effective KTI inactivation with the AA plus Cu^{2+} redox system can be achieved with 5 mM AA, treated for 1 h at 50 °C. With a reaction temperature of 65 °C further KTI inactivation can be achieved. Our analyses indicate that this further KTI inactivation is caused by action of heat alone.

Changes in Amino Acid Composition of KTI with AA plus Cu^{2+} Treatment. In Table II, our results of AA plus Cu^{2+} reaction on KTI showed marked increase of aspartic acid along with major decreases in cystine and methionine; also observed were significant decreases in histidine, tyrosine, and lysine. Uchida and Kawakishi (1988) likewise observed significant losses of histidine, lysine, and tyrosine when they reacted bovine serum albumin with an ascorbic acid plus copper ion system. Ortwerth and Olesen (1988) noted a major decrease in lysine along with increased aspartic and glutamic acids in ascorbic acid induced cross-linking of lens protein. Fleming (1983) showed that disulfides can complex directly with ascorbate, which would account for decreased cystine content. The AA plus Cu^{2+} redox system is known to oxidize histidine and methionine residues of many proteins (J. R. Whitaker, University of California, Davis, personal communication). Since L-ascorbate with dioxygen in the presence of transition-metal ions can generate hydrogen peroxide (Liao and Seib, 1987), we evaluated hydrogen peroxide with $CuSO_4$ as an inactivator of KTI. We found that KTI inactivation with 10 mM hydrogen peroxide with 0.8 mM $CuSO_4$ is just as effective as 10 mM AA plus 0.8 mM $CuSO_4$ reacted with KTI for 1 h at 65 °C. Hydrogen peroxide (0.3–4.0% v/v) is known to oxidize methionine in soy protein isolate to methionine sulfoxide (Chang et al., 1982). The mode of action of the AA plus $CuSO_4$ as an inactivator of KTI may involve hydrogen peroxide generation and consequent free radicals from action of copper ion on hydrogen peroxide acting on the protein.

Action of AA plus Cu^{2+} in Defatted Soy Flour. Soy flour slurries, treated with AA plus Cu^{2+}, either buffered or unbuffered, when heated at 65 °C for 1 h retained most TI activity despite the fact that heat treatment alone inactivated 34–46% of the original activity (see Table III).

Table III. Trypsin Inhibitor (TI) Activity of Processed Soy Flour Slurries[a]

medium	TI, % original activity: heat alone[b]	treated and heated[c]
0.2 M Tris, pH 8.5	65.8	92.8
0.1 M sodium phosphate, pH 6.5	54.4	97.5
water, unbuffered	59.6	135.4[d]

[a] 10 mg of defatted soy flour/mL. [b] Slurry heated 1 h at 65 °C. [c] Slurry treated with 10 mM ascorbic acid plus 0.8 mM $CuSO_4$ prior to heating. [d] Supernatant of extract was enriched with TI due to precipitation of 11S protein.

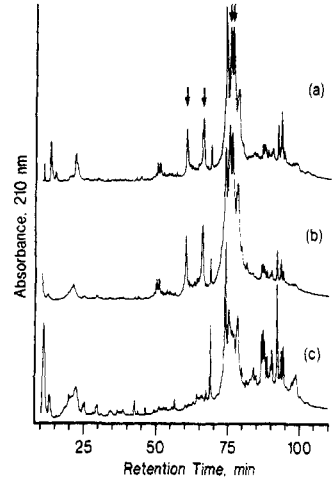

Figure 4. HPLC elution profile of (a) water extract of defatted soy flour, (b) (a) heated for 1 h at 65 °C, and (c) (a) heated with 10 mM ascorbic acid plus 0.8 mM cupric sulfate for 1 h at 65 °C. A Vydac 218 TP54 reversed-phase column was eluted at 1 mL/min with a linear 20–45% acetonitrile gradient in H_2O (with 0.1% trifluoroacetic acid) for 90 min and then held for 20 min. Total time was 110 min at 60 °C. Arrows designate peaks associated with soy 11S proteins (Peterson and Wolf, 1988).

The >100% TI activity in unbuffered aqueous extracts treated with 10 mM AA plus 0.8 mM $CuSO_4$ and heated for 1 h at 65 °C resulted from precipitation of soy glycinin (11S) from the slurry during protein isolation, with consequent enrichment of TI. Supernatants from centrifuged aqueous extracts of defatted soy flour treated with 10 mM AA plus 0.8 mM $CuSO_4$, which had been heated at 65 °C for 1 h, differed visibly from supernatants of aqueous extracts with no AA plus $CuSO_4$ that had been treated at either 27 or 65 °C for the same time. Supernatants from the unbuffered centrifuged extracts with AA plus $CuSO_4$ were clear and had a pH of 5.9, whereas the 27 and 65 °C supernatants were milky dispersions with pH of 6.7.

HPLC patterns for either 27 or 65 °C supernatants without AA plus Cu^{2+} were similar (see Figure 4), whereas the pattern for the supernatant from an aqueous extract treated with AA plus $CuSO_4$ and heated at 65 °C differed in peaks designated with arrows. AA plus Cu^{2+} caused decrease of peaks associated with soy glycinin (i.e., 11S) (Peterson and Wolf, 1988).

Differential scanning calorimetry further established that defatted soy flour extracted with water containing 10 mM AA plus 0.8 mM $CuSO_4$ extracted both the 7S and 11S proteins as shown in Figure 5a. This slurry gave two endotherms with T_{max} = 70 and 92 °C associated with soy β-conglycinin (7S) and soy glycinin (11S), respectively (Hermansson, 1986; Varfolomeyeva et al., 1986). After centrifugation, the resulting supernatant yielded only one endotherm at T_{max} = 70 °C. Apparently, the pH drop from 6.7 to 5.9 caused by AA in the unbuffered extract will

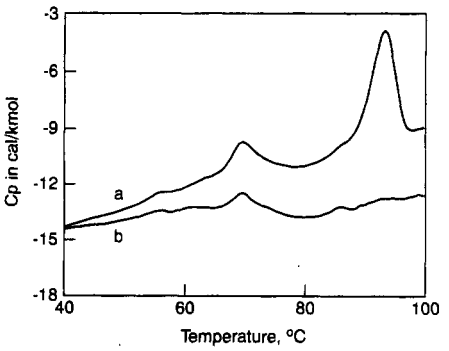

Figure 5. Thermograms comparing water extracts of defatted soy flour treated with ascorbic acid (AA) and cupric sulfate (Cu^{2+}) (a) original slurry, 10 mg of soy flour/mL of water with 10 mM AA plus 0.8 mM Cu^{2+} and (b) supernatent after centrifugation 14900*g* for 20 min at 5 °C. Scan rate was 60 °C/h.

precipitate the 11S protein. The resulting supernatant is therefore enriched in the low molecular weight 2S, which contains the TIs, and the 7S storage proteins.

11S globulin constitutes about 31% of the total protein in soy. Even if all 11S protein were precipitated, some TI inactivation should have occurred. The AA plus $CuSO_4$ system may have generated an artifact with TI activity or may have converted a natural inactivator in the soy flour slurry, which is activated in the presence of heat, to its oxidized or reduced state. This modified natural inactivator may then lose its ability to inactivate TIs. In support of the natural inactivator hypothesis, Ellenrieder and coworkers (Ellenrieder et al., 1980, 1981) isolated an uncharacterized protein from soybeans that enhanced thermal inactivation of purified soybean TIs. The effect was appreciable after a few minutes of treatment at 96 °C. These same researchers (Ellenreider et al., 1981) found that carboxymethylation of free mercapto groups caused loss of the accelerating effect. Additional evidence in support of the accelerating effect of free mercapto groups on TI inactivation is the research reported by Tanahashi et al. (1988). They established that the free SH groups on purified 11S protein actively interact with the disulfides of purified soy KTI and BBI which, when heated, resulted in significant reduction of antitryptic activity. The 7S protein with no sulfhydryl groups had no effect on trypsin inhibitor inactivation. Free mercapto groups can aid in the thermal inactivation of soy TIs (Friedman and Gumbmann, 1987). The research of Ellenreider and coworkers and also Tanahashi and his co-workers implicates TI inactivation via sulfhydryl interchange. Our research may indirectly support their findings if ascorbic acid is acting as an oxidant in a soy flour slurry.

Conclusion. Our results indicate that AA plus $CuSO_4$ effectively inactivates soy KTI and BBI in model systems with no or moderate heat treatment. When the AA plus $CuSO_4$ system is applied to soy flour extracts, however, TI activity is preserved in spite of heat treatment. The reason for TI preservation in heated soy flour slurries has not yet been determined.

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