The formation of 3-ethylpyridine from the heated lysine alone was discussed earlier. This compound, however, was produced in larger quantities from the interaction samples. This may be due to the formation of butanal and acetaldehyde from the triglyceride. The ethylpyridine could be formed by condensation of acetaldehyde, formaldehyde, and ammonia (Millar and Springall, 1966) and/or butanal, formaldehyde, acetaldehyde, and ammonia. The reaction of butanal, formaldehyde, acetaldehyde, and ammonia could yield 3-(*n*-propyl)pyridine, while 4-(*n*-butyl)pyridine may result from the reaction of butanal, formaldehyde, acetaldehyde, propanal, and ammonia. Similar reactions can explain the formation of 2-(*n*-hexyl)pyridine.

The absence or presence of the compounds isolated in this study in actual food systems has yet to be determined. However, the knowledge that these interactions may occur in food is important in (1) preserving the nutritional quality of foods, (2) generation of flavor compounds, and (3) identifying potentially toxic compounds in the food supply.

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Effect of Cysteine on Heat Inactivation of Soybean Trypsin Inhibitors

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Cysteine facilitated the heat inactivation of trypsin inhibitor activity with both purified soybean Kunitz inhibitor and soybean extracts. Effects of cysteine concentration, pH, temperature, and length of treatment on inactivating trypsin inhibitor were studied. Extent of trypsin inhibitor inactivation was determined spectrophotometrically by measuring reductions in rates of production of *p*-nitroaniline from a synthetic trypsin substrate, *N*-benzoyl-DL-arginine-*p*-nitroanilide. Attractive conditions for inactivating soybean trypsin inhibitor were 2.5 mM cysteine, pH 9.0, and 80 °C for 10 min. Under those conditions, inhibitor activity of soybean extract was reduced by 80–90%. In the absence of cysteine, activity was reduced by 29%. Inhibitors inactivated by heat in the presence of cysteine showed no significant reactivation after 60 h at 4 °C or 6 h at room temperature. The treatment results in retention of protein solubility; its nitrogen solubility index after neutralization and lyophilization was 99.7%.

There are several obstacles to realizing the full nutritional potential of soybean protein (Rackis, 1974). Among those are heat-inactivatable antinutritional factors, including trypsin inhibitors. Soybean trypsin inhibitors fall into two classes (Laskowki and Kato, 1980) represented by the well-studied Kunitz and Bowman-Birk inhibitors. The most direct evidence that one or both classes of soybean trypsin inhibitors are antinutritional was provided by Kakade et al. (1973), who found that removing them from water extracts by affinity chromatography on trypsin–agarose columns substantially increased the protein efficiency ratio of the soybean protein. More conventionally, soybean trypsin inhibitors are inactivated by heat (Rackis, 1974).

The Kunitz and Bowman-Birk inhibitors contain two and seven disulfide bonds, respectively (Wolf, 1977). Since disulfide bonds stabilize the native conformations of proteins (Liu, 1977), their cleavage would be expected to make the inhibitor proteins more susceptible to heat denaturation. At least some disulfide bonds in soybean trypsin inhibitors are accessible to reducing agents at room temperature in the absence of denaturants (DiBella and Liener, 1969). Disulfide bonds not readily accessible under such conditions would become accessible during heat denaturation. Their reduction would be expected to facilitate the further denaturation of the protein molecules.

We explored the use of cysteine as a reducing agent to enhance the heat inactivation of soybean trypsin inhibitors. The influence of several variables (cysteine concentration, pH, temperature, and length of treatment) was investigated. We found conditions for inactivating trypsin inhibitors that are considerably milder with added cysteine than those without cysteine. The milder treatment gave excellent retention of protein solubility, as assessed by the nitrogen solubility index.

MATERIALS AND METHODS

Preparation of Soybean Water Extract. Fifty grams of soybeans (Williams variety, grown in Riley County, KS, 1978) was washed with water and then soaked in 150 mL of distilled water for 4 h at 18–21 °C to increase the efficiency of extraction of proteins (Lo et al., 1968). The soaked beans were drained, rinsed, drained again, and

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ground into a slurry with 200 mL of water in a Waring Blendor for 3 min at the highest speed. The slurry then was diluted with water to a total of 450 g and filtered through a muslin cloth, and the filtrate (about 370 mL) was collected as soybean water extract (pH 6.5). The soybean water extract contained 3.78% protein and 7.89% total solids and therefore represented 70% and 65% of the protein and total solids, respectively, of the beans.

Inactivation of Trypsin Inhibitor. *(A) Cysteine Solution.* A solution containing 30 mg of cysteine (Sigma Chemical Co., C-9768) per mL of water was prepared just before use to minimize cysteine oxidation.

(B) Purified Soybean Trypsin Inhibitor (Kunitz). The Kunitz inhibitor solution prepared contained 0.15 mg of Type I-S lyophilized soybean inhibitor (Sigma Chemical Co., T-9003) per mL of 0.05 M Tris buffer, pH 9.0. Aliquots of 2 mL, with 0, 10, or 20 μL of cysteine stock solution, were added then heated at 80 °C in a shaker–water bath for 10 heating times for each set (0, 2, 4, 6, 8, 10, 15, 20, 25, and 30 min). Immediately after heating, each tube was cooled quickly in ice water. The residual inhibitor activity of each tube was assayed immediately.

(C) Soybean Water Extract. (1) Alkali and Heat Treatment. Soybean water extract (pH 6.5) was divided into two equal parts. One was adjusted to pH 9.0 with KOH; the other part was not adjusted. Ten test tubes each with 5-mL aliquots of the pH 6.5 and 10 of the pH 9.0 extracts were heated at 70 °C in a shaker–water bath. At 10-min intervals, from 0 to 90 min, tubes were removed and immediately placed in ice water. Residual trypsin inhibitor activity of each tube was then assayed. The same procedure was repeated except samples were heated at 80 °C instead of 70 °C.

(2) Alkali, Cysteine, and Heat Treatment. A soybean water extract was adjusted to pH 9.0 with a KOH solution. Four sets of test tubes were prepared; each contained 10 tubes and each tube contained 5-mL aliquots of the extract. A 25-μL aliquot of stock cysteine solution (previously described) was added to tubes in one set, 50 μL to those of another, and 100 μL to those of a third set. The fourth set with no cysteine added was the control. All tubes then were placed in an 80 °C shaker–water bath for 10 heating times (0, 2, 4, 6, 8, 10, 15, 20, 25, and 30 min). After heating, the tubes were placed in ice water immediately, and the residual inhibitor activity was assayed.

For determination of whether the inactivation of trypsin inhibitor with cysteine is permanent, samples that had been heat inactivated in the presence of added cysteine were held for 12, 36, and 60 h at 4 °C and 1, 2, 3, and 6 h at 18–21 °C and assayed for inhibitor activity.

Assay Method for Trypsin Inhibitor Activity in Soybean Water Extracts. A simple and rapid trypsin inhibitor assay (Swartz et al., 1977) based on the BAPA method of Erlanger et al. (1961) was used.

Trypsin Stock Solution. Ten milligrams of Type I, pancreatic bovine trypsin (2× crystallized, dialyzed, and lyophilized; Sigma Chemical Co., T-8253) was dissolved in 25 mL of 0.001 M HCl; Tris buffer was 0.5 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$.

Substrate Stock Solution. One hundred milligrams of BAPA (*N*-benzoyl-DL-arginine-*p*-nitroanilide; Sigma Chemical Co., B-4875) was dissolved in 1.00 mL of dimethyl sulfoxide.

Sample Preparation. Samples of soybean water extracts were diluted 20× with water to decrease turbidity and trypsin inhibitor activity.

Assay Procedure. A 20-μL sample and 30 μL of trypsin stock solution were added to 1 mL of Tris buffer, and the mixture was incubated at 25 °C in a water bath for 10 min. The incubated mixture was transferred to a quartz cuvette, 10 μL of BAPA stock solution then was added, a Teflon cuvette cap was put in place, and the cuvette was inverted quickly several times to mix the contents. Changes in absorbance at 410 nm were recorded continuously with a Perkin-Elmer 552 recording spectrophotometer. For each set of samples, a reference assay was performed with water substituted for the sample. From the initial, linear portion of time course, the slope or initial reaction rate, ΔA/min, was calculated. The slopes of residual trypsin activity of sample assay and trypsin activity of reference assay were determined and expressed in trypsin inhibitor units (TIU's). One TIU is defined as the amount of trypsin inhibitor that causes 50% inhibition in the standard reference assay. All samples were assayed in triplicate, and the initial reaction (slopes) rates were averaged.

Before each assay, the cuvettes were rinsed with dimethyl sulfoxide and then thoroughly with water.

Nitrogen Solubility Index (NSI) and Proximate Composition Determination. Before NSI was determined, samples were adjusted to pH 7.0 with a 0.1 N HCl solution, lyophilized, and ground to a fine powder. NSI was determined by a slow stirring method described by American Oil Chemists' Society (1978), except that we used a magnetic stirrer.

Total solids of soybean water extract were determined gravimetrically (4 h in a forced draft oven at 100 °C). Protein contents were determined by the Kjeldahl method (AOAC, 1975).

RESULTS

Effect of Cysteine on Kunitz Soybean Trypsin Inhibitor. When a solution of purified and lyophilized Kunitz trypsin inhibitor (0.15 mg/mL Tris buffer, pH 9.0) was heated at 80 °C, only 13% was inactivated after 2 min and 29% after 30 min. But when heated at 80 °C for 2 min after adding 0.15 and 0.30 mg of cysteine/mL of inhibitor, 47% and 78% were inactivated, respectively. Figure 1 illustrates the cysteine-enhanced inactivation of the inhibitor at pH 9.0, with the rate and degree increasing as cysteine concentration increased. Even before heat treatment, cysteine itself inactivated the inhibitor somewhat.

Assay of Trypsin Inhibitor Activity in Water Extracts of Soybeans. Existing methods for measuring trypsin inhibitor activity in food samples, such as that of Kakade et al. (1974), involve time-consuming sample preparation that includes drying and defatting. To explore systematically the effect of cysteine on heat inactivation of trypsin inhibitors in water extracts of soybeans, we wanted a simpler, faster assay procedure. In the method we used, trypsin inhibitor activity is determined directly in diluted soybean extracts. With a recording spectrophotometer, we measured the ability of a diluted sample to decrease the rate of hydrolysis of a synthetic trypsin substrate, BAPA, by a standard amount of bovine trypsin. That approach is possible becauses of the high trypsin inhibitor content of soybean extracts. To obtain trypsin inhibitor levels that were conveniently measured, we diluted extracts 20-fold. Such dilution reduced sample turbidity to levels that can be tolerated electronically to allow continuous display of increasing absorbance at 410 nm on a strip-chart recorder. The assay was accurate and precise (Lei, 1980).

It should be noted that our assay is not suitable for use with solid samples and that it determines only the activity of inhibitors that are extracted. The method of Kakade et al. (1974), on the other hand, was specifically designed

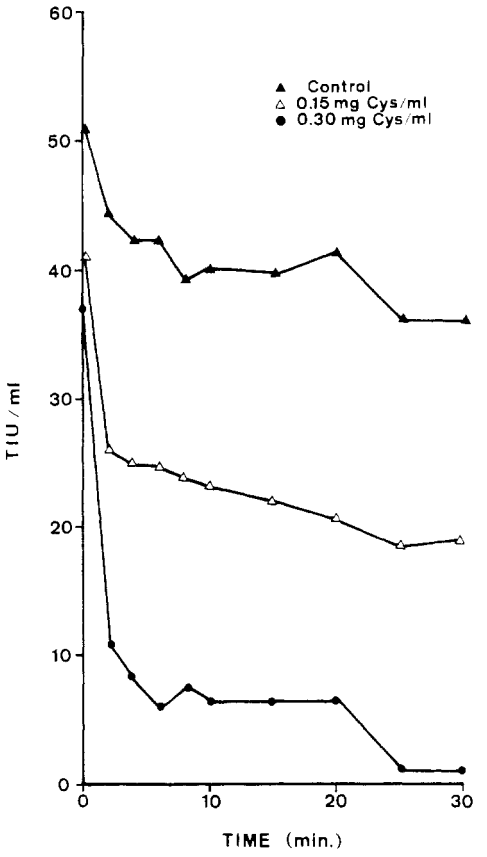


Figure 1. Effect of cysteine on heat inactivation of Kunitz soybean trypsin inhibitor at pH 9.0 and 80 °C. Concentration of inhibitor = 0.15 mg/mL.

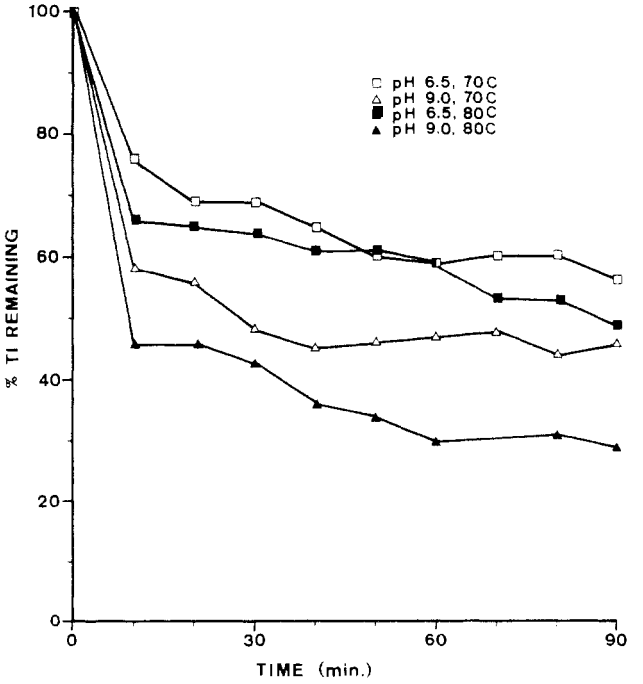


Figure 2. Percent trypsin inhibitor remaining in soybean water extract after alkali and heat treatment.

to determine the total inhibitor activity, including that in insoluble forms.

Effect of pH and Temperature on Trypsin Inhibitors in Soybean Water Extract. Figure 2 shows that heat treatment at 80 °C more effectively inactivated the inhibitor than that at 70 °C and pH 9.0 was more effective than pH 6.5. During heating, trypsin inhibitor activity first decreased rapidly and then decreased more slowly.

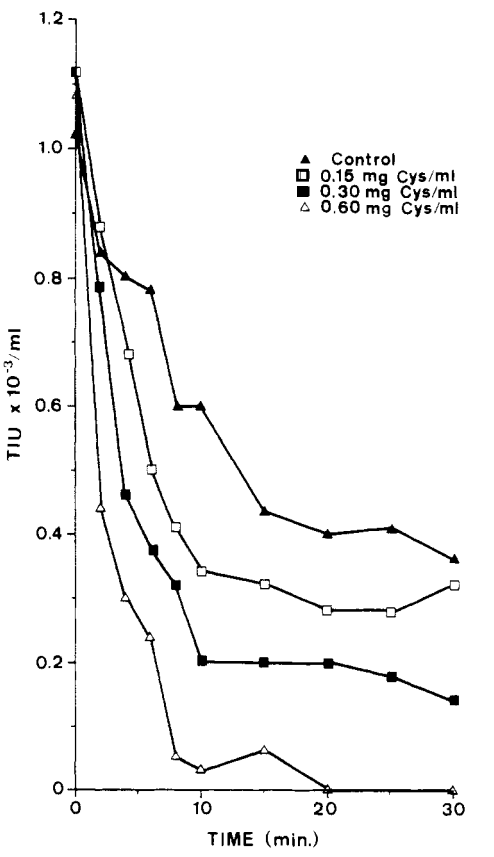


Figure 3. Effect of cysteine on inactivation of trypsin inhibitors in soybean water extract at pH 9.0 and 80 °C heating.

Effect of Cysteine on Trypsin Inhibitors in Soybean Water Extract. Cysteine facilitated the heat inactivation of purified Kunitz trypsin inhibitor (Figure 1); it also functioned effectively on the trypsin inhibitor activity in soybean water extract (Figure 3), even though the soybean water extract contained a complicated mixture of proteins (Wolf, 1972). Figure 3 shows inactivation of the inhibitors as a function of cysteine concentration.

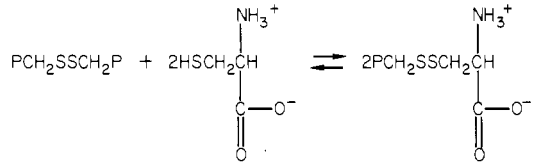
Our results show that adding 0.3 mg of cysteine/mL of alkali soybean water extract at pH 9.0 and heating for 10 min at 80 °C were enough to inactivate more than 80% of the inhibitors. Complete inactivation was attained by adding 0.6 mg of cysteine/mL of soybean water extract at pH 9.0 and heating at 80 °C for 20 min.

Reactivation Test. When we investigated the possible reformation of disulfide linkages of the trypsin inhibitor molecule previously reduced by cysteine and heat treatment, no significant reactivation of inhibitor activity was apparent after 60 h at 4 °C or 6 h at room temperature (Lei, 1980).

NSI of Heated Soybean Water Extracts. The protein solubility of soybean water extract that was heated for 10 min at 80 °C at pH 9.0 with 0.3 mg of cysteine/mL was very high: the neutralized and lyophilized protein had an NSI of 99.7%. This high solubility was not due to the inclusion of cysteine but to the comparative mildness of the heat treatment, since the NSI of a control sample heated under the same conditions but without added cysteine was 98.9%. In contrast, we obtained an NSI of 49.8% for the protein of soy aqueous extract that was heated at 100 °C for 30 min. (That length of heating at 100 °C is less than that which other investigators have found necessary for inactivation of trypsin inhibitors in aqueous extracts of soybeans (Horii and Miyazaki, 1975).) In our hands it resulted in lowering the trypsin inhibitor activity by greater than 90%.

DISCUSSION

Our results demonstrate that cysteine facilitates heat inactivation of trypsin inhibitors in water extracts of soybeans. Although our approach was empirical and we did not attempt to determine the mechanism by which cysteine exerts its effect, a likely mechanism is apparent from relevant studies on well-defined systems. Exchange reactions between disulfide bonds and thiol-containing compounds are well documented (Liu, 1977). Since disulfide bonds help stabilize the native conformations of proteins (Liu, 1977), cysteine's effects in the inactivation of trypsin inhibitors likely result from cleavage of disulfide bonds in the inhibitors by thiol (cysteine)/disulfide exchange reactions:



In the above reaction, PCH_2SSCH_2P represents a disulfide bond (cystine residue) in a protein and the product of the reaction is a mixed disulfide. Although one disulfide bond in the Kunitz inhibitor is readily accessible under nondenaturing conditions (DiBella and Liener, 1969), many disulfides in the mixture of soybean trypsin inhibitors are likely to be buried. As they unfold in response to heat treatment, the disulfide bonds would become accessible to cysteine and undergo thiol/disulfide exchange. The cleavage of disulfide bonds would facilitate denaturation of the protein molecule.

Disulfide bonds within any protein in the soybean extract would be potentially available for reaction with cysteine. As we do not know the total concentration of disulfide bonds in the soybean extracts, it is not possible to estimate the extent to which thiol/disulfide exchange reactions would proceed. Stated somewhat differently, we do not know how many of the disulfide bonds in soybean trypsin inhibitors are likely to be mixed disulfides after heat treatment in the presence of cysteine. Neither can we speculate on the extent to which such a mixed disulfide might react with cysteine to yield a cysteine residue within a protein and free cystine.

Despite our ignorance of mechanistic details and our lack, at this point, of nutritional and functional data, we believe there are several possible benefits of the use of cysteine in heat inactivation of soybean trypsin inhibitors that merit mentioning at this stage in our research.

First, because less heat is required to inactivate the extracted inhibitors in the presence of cysteine, less energy would be required for the purpose in a large-scale, industrial setting. At this time, this comment is relevant only to preparation of soy milk, since we have not yet examined the effect of cysteine during heat inactivation of trypsin inhibitors in other forms of soy proteins.

Second, heat denaturation in the presence of cysteine might render renaturation of the inhibitors less likely. Heat denaturation in the absence of a reducing agent should leave the disulfide bonds in a molecule intact. On the other hand, denaturation in the presence of cysteine would break some or all of the disulfide bonds and thus allow the formation of scrambled disulfides upon air oxidation. One would anticipate that, as originally shown for ribonuclease A (Anfinsen, 1973), a trypsin inhibitor with incorrect pairing of cysteine residues in disulfide bonds would be inactive, so including cysteine during heat inactivation might reduce the likelihood of trypsin inhibitor activity reappearing by spontaneous renaturation of the inhibitors.

Finally, it seems likely that the excellent retention of protein solubility after heat treatment in the presence of cysteine would confer functional properties on the soy proteins that would be useful in some food applications [i.e., those where solubility is desirable; see Kinsella (1979)]. It should be noted that heat treatment has been shown to have a beneficial nutritional effect beyond the inactivation of trypsin inhibitors (Kakade et al., 1973; Rackis et al., 1975). Through heating, an increase in digestibility of soy protein is achieved that is independent of trypsin inhibitor inactivation (Kakade et al., 1973). We have not yet studied the nutritional value of soy proteins in extracts that have been supplemented with cysteine and heated to inactivate trypsin inhibitors. It is possible, however, that an increased digestibility of soy protein results from such a treatment, even though the heating is mild, since the major protein components of soy milk, glycinin and conglycinin, contain disulfide bonds (Kitamura et al., 1976; Koshiyama, 1968). Those proteins' denaturation (and, possibly, increased digestibility) might therefore be expected to be facilitated by cysteine, just as is the inactivation of the trypsin inhibitors.

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