THE EFFECT OF CYSTEINE ON HEAT INACTIVATION OF SOYBEAN TRYPSIN INHIBITOR

by

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Dedicated to My Parents

Mr. and Mrs. Kuo-Ching Lei

Golden Wedding Anniversary

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INTRODUCTION

Soybeans are a good source of proteins at an extremely favorable cost. They contribute more protein per unit of land than all of the other food plants or animal sources (Liener, 1972; Rackis, 1977). In addition, the amino acid composition of soybean protein is close to that recommended by FAO (Food and Agriculture Organization). These attributes make the soybean potentially an excellent food crop in countries where protein deficiency in diet is a major nutritional problem. Unfortunately the full benefits of soybean cannot be achieved because the antinutritional factor, trypsin inhibitors, which cause problems in its utilization. These trypsin inhibitors are proteins which inhibit the proteolytic activity of trypsin (Kunitz, 1947). They cause growth depression and pancreatic hypertrophy in animals (Khayambashi and Lyman, 1969). The minimum inactivation of trypsin inhibitor activity to provide maximum nutritive value of soybean products was achieved when 79% of its activity was reduced (Rackis, 1974).

Although high temperature heat treatment can inactivate trypsin inhibitors and improve nutritive value of soybeans to near that of meat and milk (Rackis, 1974), heat denatures other soybean proteins, decreases soybean protein solubility and alters functional properties of soybean protein (Wolf, 1972a). This in turn, limits the usefulness of soybean proteins. Unfortunately, a procedure for simultaneously obtaining high protein solubility,

organoleptic acceptability and good nutrition of soybean product is not yet available. Therefore, the purpose of this study was to search for a method to maintain protein solubility and enhance the applicability of soybeans in food formulation by inactivating trypsin inhibitors of soybean water extract with less heat treatment, i.e., at lower temperature for a shorter heating time.

I attempted to alter the trypsin inhibitor molecules by treating it with the reducing agent, cysteine. I hypothesized that by using cysteine to cleave the disulfide linkage within the molecule of the inhibitors, the inhibitors would be more valnerable to heat denaturation. This hypothesis was tested first with purified and lyophilized soybean trypsin inhibitor (Kunitz), and then with soybean water extract. Other parameters such as pH, cysteine concentration, heating temperature and time were investigated with respect to inactivation of the inhibitors.

LITERATURE REVIEW

A. Composition of Soybean Seeds

Proximate composition

Soybean seeds consist of proteins, lipids, carbohydrates and minerals. On moisture free basis, the average composition of whole soybean seeds is 40.4% protein, 22.3% fat, 31.9% N-free extract plus fiber and 4.9% ash (Horan, 1974). The composition, however, is affected by both environment and variety. Proteins and lipids are the components of soybeans that command most commercial interest. Soybeans have become a major crop in United States agriculture. Annual production was 48 million metric tons for 1978/79; that was 64.9% of the 74 million metric tons of world production (Delury, 1980).

Soybean protein composition

Soybean proteins are quite heterogeneous, most of them are globulins. According to sedimentation coefficients, water extractable soybean proteins of defatted meal may be classified into four fractions: 2, 7, 11 and 15 S. Relative amounts and molecular weights for the components that make up these four fractions are given in Table 1.

The 2 S fraction contains trypsin inhibitors, cytochrome c, allantoinase, and 2 globulins with no known biological activity. The 7 S fraction represents slightly over one-third of the total soluble proteins and contains at least four different proteins. The 7 S globulin comprises about one-half of the 7 S

Table 1. Ultracentrifuge fractions of soybean proteins (Wolf, 1972a).

Protein fraction	Percent of total	Components	Molecular weight
		Trypsin inhibitors	8,000(monomer)
			21,500
		Cytochrome c	12,000
2 S	22	2.3 S globulin	18,200
		2.8 S globulin	32,000
		Allantoinase	50,000
		Beta-amylase	61,700
7 S	37	Hemagglutinins	110,000
		Lipoxygenases	108,000
		7 S globulin	186,000-210,000
11 S	31	11 S globulin	350,000
15 S	11	---	600,000

fraction. It is a glycoprotein consisting of 12 glucosamines and 31 mannose residues per mole. The 11 S globulin, the major protein of soybeans, accounts for the bulk of the 11 S fraction which makes up about one-third of total soluble soybean proteins. The 15 S fraction has not yet been isolated and characterized, it may be a polymer of other proteins (Wolf, 1972a; Orthoefer, 1978).

On the basis of essential amino acid composition, the sulfur-containing amino acids limit the nutritional value of soybean protein (Liener, 1972). However, soybean protein is higher in lysine than other plant proteins (Zimmermann et al., 1967). Therefore, it can be used to increase the nutritional value of various plant protein combinations.

B. Lipoxygenase

Characteristics

Lipoxygenase catalyzes the oxidation of lipids containing a cis, cis-1, 4-pentadiene group by molecular oxygen to hydroperoxides. This enzyme is found in many plants and in some microorganisms, but not in animal tissue. Compared with other plants and microorganisms, soybean has the highest lipoxygenase activity. There are four isoenzymes of lipoxygenase present in soybeans. Crystallized or highly purified soybean lipoxygenase contains no prosthetic groups and requires no coenzyme or metal activator (Rackis, 1972). The optimum temperature of lipoxygenase is generally between 20-30C. The enzyme has a very high

turnover number, and under optimum conditions the crystalline enzyme oxidizes 330 moles of linoleic acid per mole of enzyme per second (Reed, 1966).

Lipoxygenase is important in the generation of flavor compounds from lipids of soybeans. Hydroperoxides, the initial products of lipoxygenase action, undergo a large number of enzymatic and nonenzymatic decompositions and produce a variety of secondary products (Gardner, 1975). The mixture of these secondary products result in the development of the characteristic green-beany flavor of soybean protein products (Wolf, 1975).

Wilkens et al. (1967) found that the off-flavors of soy milk were not present in the intact dry beans but were formed when raw soybeans are macerated. Badenhop and Wilkens (1969) found 1-octen-3-ol was formed in soybeans during soaking. Mattick and Hand (1969) isolated and identified ethyl vinyl ketone as a volatile component which develops in soybeans and contributes to raw bean odor and flavor. Wilkens and Lin (1970) isolated the volatile components from whole-fat soybean milk and found they were very complex. About 80 peaks were evidenced by gas chromatographic analysis of the oxidized milk; 41 compounds were identified and 13 tentatively identified. Among the identified compounds were hexanal, hexanol, 2-hexenal, ethyl vinyl ketone, 2-pentyl-furan and others. No single compound in gas chromatographic analysis could approximate the off-flavor of soybean milk. They concluded that the typical green-beany flavor of soy products is caused by a mixture of many compounds formed

by lipid oxidation.

Inactivation

Heating is the method currently proposed for inactivating heat labile lipoxygenase during processing of soybeans. Total inactivation of this enzyme in dry dehulled soy meats is achieved by immersion cooking at 180F for 15 min. Inactivation also has been achieved at lower temperature by heating at 165F for 15 min in 1% NaOH (Baker and Mustakas, 1973). High initial moisture content of soybeans increased the rate of lipoxygenase inactivation (Wapinski, 1977).

Wilkens et al. (1967) reported that grinding unsoaked, dehulled soybeans with hot water at 80-100C and maintaining the temperature for 10 min would inactivate lipoxygenase and produce an acceptable bland soybean milk product. Mustakas et al. (1969) prepared soybean flour without off-flavor by dry heat at 100C or steaming dehulled beans before milling. They showed that moist heat is more effective than dry heat in the lipoxygenase inactivation. Kon et al. (1970) found that low pH inhibited lipoxygenase. They ground soybeans at low pH (3.85 or below) and neutralized the acid slurry after heating to control the flavor problems of soybean products successfully.

C. Soybean Trypsin Inhibitors

Properties

The presence of trypsin inhibitor in an aqueous extract of soybean was first reported in 1938 by Read and Haas. The soybean

trypsin inhibitor is a globular protein, which inhibits the proteolytic activity of trypsin by rapidly combining with the enzyme to form a tight complex (Kunitz, 1947).

Trypsin inhibitor content of raw soybean meal prepared from several varieties varied within a relatively narrow range of 31-42 mg inhibitor per gram of meal (Rackis, 1966). Among the many kinds of trypsin inhibitors in soybeans, only two, Kunitz and Bowman-Birk inhibitors, have been isolated and well studied. Properties of these two are outlined in Table 2. The Bowman-Birk inhibitor is the smallest protein found in soybeans to date. Its molecule is highly symmetrical with two independent inhibitory sites, one against trypsin and the other chymotrypsin. On the other hand the Kunitz inhibitor has only one active site. It is not effective against chymotrypsin but inhibits trypsin very well. Seven disulfide linkages in Bowman-Birk inhibitor make the molecule compact and explain its resistance to heat, acid, and proteolytic digestion. These agents are more effective in inactivating Kunitz inhibitor, which has only two cystine residues (disulfide linkages) per molecule. The Bowman-Birk inhibitor might be responsible for the residual trypsin inhibitor activity in heated soybean protein products. Reduction of disulfide linkages in the inhibitor causes conformational changes and complete loss of inhibitory activity (Wolf, 1977).

The role of trypsin inhibitors in soybean seeds is not known. They cause growth depression and pancreatic hypertrophy in animals. Growth depression in chicks and rats fed raw soybean

Table 2. Properties of Kunitz and Bowman-Birk trypsin inhibitors (Wolf, 1977).

Properties	Kunitz	Bowman-Birk
Amino acid residues	181	71
Monomer molecular wt	21,500[a]	8,000
Cystine residue/molecule	2	7
Trypsin inhibiting site	Arg^{63}-Ile^{64}	Lys^{16}-Ser^{17}
Chymotrypsin inhibiting site	---	Leu^{43}-Ser^{44}
Stability to heat, acid and proteolytic digestion	Somewhat unstable	Remarkable stable

[a]Wu and Scheraga (1962).

meal was due to the action of the inhibitors as a pancreatic secretagogue, causing a flow of secretion (Khayambashi and Lyman, 1969). The inhibitors induce the formation or release of a hormonal substance capable of stimulating pancreatic enzyme secretion. This in turn causes endogenous loss of essential amino acids (Hill et al. 1953; Lyman and Lepkovsky, 1957). Madar et al. (1974) found when quail ingested native and modified Bowman-Birk inhibitor from soybeans, trypsin inhibitor site rather than chymotrypsin site was responsible for pancreatic hypertrophy and the increased proteolytic activity of the pancreas. The mechanism of pancreatic enlargement caused by ingestion of trypsin inhibitor is still not clear. Adult animals can maintain body weight when fed a raw soybean diet, although pancreatic hypertrophy still occurs (Rackis, 1974). The relationship between trypsin inhibitor activity and PER (Protein Efficiency Ratio) is apparent when 79% of trypsin inhibitor activity was reduced, the maximum PER was reached. Pancreatic hypertrophy no longer occurs in rats when 50-60% of trypsin inhibitor activity was reduced (Rackis, 1974). The nutritional value of soybean is inversely proportional to the trypsin inhibitor content. The level of residual trypsin inhibitor in soybean product can be an index of the adequency of heat treatment (Van Buren et al., 1964).

Travis and Roberts (1969) reported that human trypsin is not inhibited by trypsin inhibitor (Kunitz). However, Mallory and Travis (1973) isolated two forms of human pancreatic trypsin;

an anionic form and a cationic form. They showed the anionic form was inhibited by soybean trypsin inhibitor (Kunitz) stoichiometrically where the cationic form was inhibited poorly.

Inactivation

Higher initial moisture content and thinner flakes or smaller particles of soybeans accelerate the heat inactivation of trypsin inhibitors (Albrecht et al., 1966; Rackis, 1966). Effects of these factors on destruction of trypsin inhibitors are shown in Table 3.

More heat is required to inactivate trypsin inhibitors in a soybean water-extract than in the seeds. It took 76 min at 98C to completely inactivate trypsin inhibitor activity in soybean milk (Wallace et al, 1971). Horii and Miyazaki (1973) also reported that heat treatment of soybean water extract at 100C for 60 min reduced trypsin inhibitor activity to 16% of the initial value.

pH is another important factor which influences the inactivation of trypsin inhibitors. Soybean trypsin inhibitors were less stable when heated in alkaline solutions than in acid (Obara and Watanabe, 1971; Baker and Mustakas, 1973). Wallace et al. (1971) found that 98C for 40 min was sufficient to inactivate the inhibitor when soybean milk was prepared from carbonate presoaked soybeans; while 76 min at 98C was needed for soybean milk prepared from water presoaked soybeans. They also found the time taken for complete inactivation of the inhibitor

Table 3. Effects of moisture, particle size of soybean product on inactivation of trypsin inhibitor activity (Rackis, 1966).

Soybean product	Moisture content (%)	Steaming 100C (min)	Trypsin inhibitor inactivated (%)
Full-fat flakes	6	0	0
Full-fat flakes	6	15	95
Cotyledons	6	20	60
Cotyledons	6	30	91
Chips	6	20	74
Chips	6	30	96
Whole soybeans	6	20	20
Whole soybeans	25	20	97

was reduced from 76 min to 11 min by increasing pH from 6.8 to 9.9 under the same conditions of heat treatment.

Analytical methods

Gelatin and casein have been widely used as substrates for measuring trypsin inhibitor activity. The methods were originally described by Kunitz (1947) and modified by other investigators. In the gelatin-digestion method, the degree of hydrolysis of gelatin by trypsin with and without trypsin inhibitor is determined by titrating the free carboxyl groups produced in the tryptic hydrolysis of gelatin (Mustakas et al., 1970). In a casein-digestion method, the degree of tryptic hydrolysis of casein is measured spectrophotometrically at 280 nm (Kakade et al., 1969).

Benzoyl-DL-arginine-p-nitroanilide (BAPA), a synthetic substrate of trypsin, was first employed by Erlanger et al. (1961). One of the products of BAPA hydrolysis is p-nitroanilide, a yellow material that can be determined conveniently colorimetrically at 410 nm. Kakade et al. (1969) evaluated BAPA as a substrate for measuring the antitryptic activity of soybean samples. After some modifications, Kakade et al. (1974) reported that an accurate and reproducible procedure for measuring trypsin inhibitor activity of soybean products with BAPA as a substrate was achieved. Reeck (1979) estimated the trypsin inhibitor activity directly by monitoring the rate of p-nitroanilide liberation from tryptic hydrolysis of BAPA spectrophotometrically at 410 nm. This method gives a rapid and accurate measurement of

antitryptic activity.

Wu and Scheraga (1962) used benzoyl-arginine ethyl ether (BAEE) as a tryptic substrate to measure crystalline soybean trypsin inhibitor activity. The method measured the rate of acid production by titrating with KOH as the reaction proceeds. Rackis (1966) reported that this method was not suitable for determining trypsin inhibitor activity of water extracts of soybean meal and of whey solutions. It was only accurate and reproducible with purified trypsin inhibitor preparations. Pringle (1974) stated this method was used successfully to determine the inhibitor activity in the soybean products by the British Arkady Co. Ltd. in England. Wapinski (1977) modified the method and found the modified procedure for the trypsin inhibitor activity is simple and fast.

D. Protein Solubility

Functional properties of soybean proteins

Functional properties can be defined as the set of properties of a protein or protein ingredient that contributes to the desired color, flavor, texture and nutritional value of a food (Martinez, 1979). The importance of these properties with respect to soybean protein varies with different uses, e.g., solubility in beverages, water absorption and binding in sausages, breads and cakes, and foaming in desert toppings. A summary of functional properties of soybean proteins in food applications was shown in Table 4.

Table 4. Functional properties of soybean proteins in food applications (Kinsella, 1979).

Properties	Functional criteria
Organoleptic/kinesthetic	Color, flavor, odor, texture, mouthfeel smoothness, grittiness, turbidity.
Hydration	Solubility, wettability, water adsorption, swelling, thickening.
Surface	Emulsification, foaming (whipping), protein-lipid, film formation, lipid-binding, flavor binding.
Structure	Elasticity, grittiness, cohesiveness.
Rheological	Chewiness, viscosity, adhesion, net-work-crossbinding, aggregation, stickness, gelation, dough formation, texturizability, fiber formation, extrudability.
Other	Compatibility with additives, enzymatic antioxidant.

Protein solubility

Solubility is an important property governing food applications of soybean proteins. Proteins with low solubility indexes have limited functional properties and more limited uses (Wolf, 1972a; Kinsella, 1979). Soluble ingredients are always easier to formulate into foods, therefore, solubility is often used as a quality control consideration and as a good indicator of the potential use of soybean proteins.

Soybean proteins are sensitive to moist heat and rapidly insolubilized by steaming. Disulfide bond reducing agents such as mercaptoethanol and cysteine cleave intermolecular bonds and cause disaggregation of protein, which in turn, enhance solubility, solubility of soybean protein is also influenced by pH and salt concentration. At low or zero ionic strength and pH values around neutrality, protein and water interactions are greatest and maximum solubility occurs (Wolf, 1972b; Kinsella, 1979). Soybean products which have been heated to improved their flavor and nutritional value may lack functionality because of poor protein solubility.

Analytical methods

Colloidal stability is often used to measure protein solubility (Hermansson, 1979), where the amount of dispersible nitrogen in the supernatant is measured after centrifugation. Protein dispersibility index (PDI) and nitrogen solubility index (NSI) are commonly employed as the official methods of the American Oil Chemists' Society (1978). The slower stirring technique

used in NSI method generally yields lower results than those obtained by fast stirring method for PDI.

E. Uses of Soybeans in Food Products

General uses of soybean proteins

Soybean meal, soy flour, soy protein concentrate, soy protein isolate, textured and modified soy proteins are marketed and used in food products (Orthoefer, 1978). The utilization of soy protein in foods is for functional properties as well as nutritional fortification. Bakery products, comminuted processed meats and breakfast cereal are by far the most common items that are fortified with soy proteins (Kinsella, 1979). Soy proteins used in food systems along with functionality and mode of action are outlined in Table 5.

Soybean milk

Soybean milk is simply a milky-looking liquid of a water extract of dry mature soybeans. It is an important food in China, commonly used as a hot breakfast drink. According to Lo et al. (1968a), soybean milk was developed by a Chinese philosopher, Whai Nain Tse, and used in China before the Christian era. Yellow-seeded varieties of soybeans are recommended for making soybean milk (Smith and Beckel, 1946).

Soybean milk has approximately the same protein content of cow's milk (3.5 - 4.0%) (Liener, 1977) and is a good substitute for cow or human milk in feeding infants and children. When the Japanese invaded China (1937), soybean milk was used

Table 5. Functional properties performed by soybean protein preparations in food systems[a](Kinsella, 1979).

Functional property	Mode of action	Food system	Preparation used
Solubility	Protein solvation, pH dependent.	Beverages	F,C,I,H.
Water absorption and binding	Hydrogen-binding of HOH, entrapment of HOH, no drip.	Meats, breads, sausages, cakes.	F,C.
Viscosity	Thickening, HOH binding.	Soups, gravies.	F,C,I.
Gelation	Protein matrix formation and setting.	Meats, curds, cheese.	C,I.
Cohesion-adhesion	Protein acts as adhesive material.	Meats, baked goods, pasta products, sausages.	F,C,I.
Elasticity	Disulfide links in gels deformable.	Meats, bakery.	I.
Emulsification	Formation and stabilization of fat emulsions.	Sausages, bologna, soup, cakes.	F,C,I.
Fat adsorption	Binding of free fat.	Meats, donuts, sausages.	F,C,I.
Flavor-binding	Adsorption, release, entrapment.	Simulated meats, bakery.	C,I,H.
Foaming	Forms stable films to entrap gas	Whipped toppings, chiffon desserts, angel cakes.	I,W,H.
Color control	Bleaching of lipoxygenase.	Breads	F.

[a]F, C, I, H, W denote soybean flour, concentrate, isolate, hydrolyzate and soybean whey, respectively.

extensively for feeding babies and children in Chinese refugee camps. This afforded a unique opportunity for observation, on a relatively large scale, the nutritional effects of soybean milk as a supplementary food. A group of the children who did not drink the milk served as a control in comparing its value in building body height and weight. The results showed that children who received soybean milk put on more weight than those not receiving the soybean milk. Among children in the control groups, those over one year old who received soybean milk also showed a somewhat greater monthly increase in body height, although not so marked as the monthly increase in body weight (Smith and Beckel, 1946). Soybean milk can also be used to nourish babies allergic to cow's milk (Rackis, 1972). Clinical sensitivity to cow's milk has estimated to occur in 0.3% to 7% of all children (Heiner et al., 1964).

The main deficiency of soybean protein as compared with the protein of cow or human milk is sulfur-containing amino acids. But, theoretically, at least essential amino acids provided by the soybean milk protein should satisfy the requirements of infants to the same extent as cow or human milk when administered at the same level of protein intake (Liener, 1977).

Preparation of soybean milk

Traditional Chinese soybean milk is processed from the whole beans. Beans are soaked in cold water until hydrated; ground finely with added water in a stone mill to form a slurry; added additional water to the slurry to the desired concentration

(usually bean to water in the ratio of 1 to 8 by weight); filtered to remove the insoluble residue; and the milk is boiled for 20-30 min to improve flavor. Unfortunately, the characteristic flavor of soybean milk is considered as undesirable flavor to non-Oriental populations. Numerous modifications of the traditional process have been reported to improve the flavor. These modifications include hot water extraction (Wilkens et al., 1967), acid grinding (Kon et al., 1970) and alkaline soaking (Badenhop and Hackler, 1970; Khaleque et al., 1970). Nelson et al. (1976) blanched beans before grinding and homogenized whole soybean slurry without filteration to reduce the beany flavor and increase total solids.

As the soaking time for soybeans is increased, larger quantities of water-soluble solids are leached to the soakwater. For a minimum loss the soaking time should be only long enough to permit the soybeans to nearly double their initial dry weight. This facilitates grinding of the beans (Lo et al., 1968a). Soaking at higher temperature drastically reduces the yield of soybean milk. For example soaking temperature higher than 80C almost completely inhibited filtration of the milk (Wilkens and Hackler, 1969). When the temperature of extraction is above 85C there are substantial reductions in the solids extracted in soybean milk and difficulties are encountered in filtrating the soybean milk (Lo et al., 1968b). Johnson and Snyder (1978) found that heating reduced the solids yield of soybean milk and heating before grinding of the beans decreased

the yield more than heating during grinding the beans.

Hackler et al. (1965) reported that the protein efficiency ratio of heat-processed soybean milk is dependent upon both time and temperature of treatment. Maximum nutritional value of the protein of the soybean milk is attained within 5-10 min when the milk is heated at 121C or in 60 min at 93C, conditions required to inactivate about 90% of the trypsin inhibitor activity.

MATERIALS AND METHODS

MATERIALS

A. Soybeans

The soybean variety used for this study was Williams, a recognized variety and registered with Crop Science Society of America, grown in Riley County, Kansas in 1978.

B. Soybean Water Extract (Soybean Milk)

Soybean water extract was prepared from 50 g of whole soybean that were washed with water and then soaked in 150 ml water for 4 hr at 18-21C. The soaked beans were rinsed with water, drained, and ground into a slurry with 200 ml water in a waring blender at the highest speed for 3 min, diluted with water to a total weight of 450 g. The mixture was filtered through a muslin cloth and the filtrate collected as soybean water extract. The residue (pulp) was discarded. pH of resulting extract was 6.5.

C. DL-Cysteine HCl (Cysteine Solution)

Sixty milligrams of cysteine (DL-cysteine HCl, Sigma, #C-9768) was dissolved in 2 ml of water. This solution was prepared just before use to minimize oxidation and formation of cystine.

D. Others

Test tubes (16 x 125 mm) and a thermostatical-controlled

shaker water-bath (New Brunswick Scientific, Model G76) were used in the trypsin inactivation study. A 1-N KOH solution was used to adjust the pH of the soybean water extracts. Distilled water was used throughout this study and expressed as water.

METHODS

A. Trypsin Inhibitor Activity Assay Method

The existing methods for measuring trypsin inhibitor activity in food samples, such as the method developed by Kakade et al. (1974), involve time consuming sample preparation, in which, samples need to be pre-treated by drying, defatting, re-extracting with alkaline solution and diluting. Therefore, I have used a simple and rapid trypsin inhibitor assay (Swartz et al., 1977) based on the BAPA method of Erlanger et al. (1961) for assaying trypsin. N-Benzoyl-DL-arginine-p-nitroanilide (BAPA) is cleaved by trypsin into N-benzoyl-arginine and p-nitroanilide.

$$\text{BAPA} \xrightarrow{\text{trypsin}} \text{N-benzoyl-arginine} + \text{p-nitroanilide}$$

The concentration of the yellow colored p-nitroanilide can be determined spectrophotometrically at 410 nm. By continuously monitoring the changing absorbance at 410 nm, the trypsin activity is determined. In a separate assay, the inhibition of trypsin by sample with suspected trypsin inhibitor is determined.

Trypsin stock solution: Ten milligrams of trypsin (from bovine pancreas, 2x crystallized, dialyzed and lyophilized,

Sigma) was dissolved in 25 ml, 0.001 M HCl solution.

Tris-buffer: A 0.5 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$ was prepared by dissolving 60.57 g Tris (Tris hydroxymethyl-aminomethane) and 2.94 g $CaCl_2$ in 900 ml water, adjusted to pH 8.2 with HCl, and diluted to one liter.

Substrate stock solution: One hundred milligrams of BAPA (Sigma) was dissolved in 1.00 ml dimethyl sulfoxide.

Sample preparation: Samples were diluted 20x with water to decrease turbidity and tryptic inhibitory 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 incubated at 25C 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, with a teflon cuvette cap in place, the cuvette was inverted quickly several times to mix the contents. The changes in absorbance at 410 nm were recorded and plotted (absorbance at 410 nm vs. time) with a Perkin-Elmer 552 recording spectrophotometer. For each set of assay a reference assay was performed, in which water was substituted for sample. From the initial, straight portion of curve, the slope or initial reaction rate, $\Delta A/min$, was calculated. Samples were assayed in triplicate and the initial rates were averaged. Therefore, the slopes of residual trypsin activity of sample and trypsin activity of reference were determined. Inhibitory activity (I) expressed as percent inhibition was calculated by the following equation: $I(\%) = (1 - S'/S) \times 100$, where S' and S are the slopes of sample

containing and reference assay, respectively. One trypsin inhibitor unit (TIU) was defined as the amount of trypsin inhibitor which causes 50% inhibition of trypsin activity in each assay.

Between each assay, the cuvette was washed with dimethyl sufoxide and rinsed thoroughly with water.

Feasibility test of the method: Attempts were made to relate sample volume with trypsin inhibitor activity. Different sample sizes, 5, 10, 15, 20 and 25 µl, of diluted soybean water extract (20x dilution) were used for trypsin inhibitory assay. Each sample was assayed five times. In order to establish the reproducibility of the assay method three different concentrations of trypsin inhibitor in soybean water extract were prepared with alkali, cysteine and heat treatment. These samples were assayed for inhibitory activity ten times independently. The reference trypsin activity also was assayed ten times. Mean and standard deviation of the ten assay were calculated. The method employed to adjust trypsin inhibitor activity is described in the following section.

B. Inactivation of Trypsin Inhibitors

Pure soybean trypsin inhibitor (Kunitz)

Trypsin inhibitor solution preparation: Nine milligrams of trypsin inhibitor (chromatographically prepared, lyophilized from soybeans, Sigma, #T-9003) was dissolved in 60 ml of pH 9.0 Tris- buffer (0.05 M) to make the inhibitor concentration of

0.15 mg/ml.

Procedure: Thirty aliquots of 2 ml-inhibitor solutions in test tubes were divided into three sets, 10 of each. Ten microliters of cysteine solution was added to one set of tubes and 20 µl to another, the third set had no cysteine added and was used as control. The mixture then was heated at 80C in a shaker water-bath. Ten heating times were employed for each set (0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min). After heating, tubes were placed in an ice-water bath immediately to cool the content quickly. The residual inhibitor activity of each tube was assayed with no delay.

Trypsin inhibitors in soybean water extract

a. Alkali and heat treatment

Soybean water extract of one preparation was divided into two equal parts, one was adjusted to pH 9.0 with a KOH solution, the other part was not adjusted (pH 6.5). Ten aliquots of 5 ml of each part were prepared in test tubes, and heated at 70C in a shaker water-bath. Heating time was controlled from 0 min to 90 min at 10 min intervals. After heating, test tubes were placed in an ice-water bath immediately to cool the contents quickly. Residual trypsin inhibitor activity of each tube was assayed with no delay.

The same procedure was followed for the 80C-heating test.

b. Alkali, DL-cysteine HCl and heat treatment

A soybean water extract was adjusted to pH 9.0 with a KOH solution. Four sets of 10 aliquots each of 5 ml extracts in

test tubes were prepared. A 25 µl aliquot of the cysteine solutions (0.75 mg cysteine) was added to samples in one set; 50 µl (1.5 mg cysteine) to those in a second set; and 100 µl (3.0 mg cysteine) to samples in the third set. The fourth set had no cysteine added was used as control. These tubes were then placed in an 80C shaker water-bath. Ten heating times (0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min) were employed for each set. After heating, tubes were placed in an ice-water bath immediately, and the residual inhibitor activity was assayed with no delay. A schematic showing compositions and treatments of samples in each of the sets is shown in Fig. 1.

c. Alkali, L-cysteine HCl·H_2O and heat treatment

L-cysteine HCl·H_2O solution: Since L-cysteine was considerably less expensive than the DL form, attempts were made to utilize it for the assay. Therefore 66.8 mg L-cysteine HCl·H_2O (United State Biochemical Corporation, #14035, assay: > 98.0%), equivalent to 60 mg of DL-cysteine HCl, was dissolved in 2 ml water.

Only 50 µl and 100 µl of L-cysteine·HCl·H_2O solution were used to enhance inactivation of trypsin inhibitor in the 5-ml samples of soybean water extract. The procedure was the same as described for DL-cysteine HCl above. Heating times were 10 and 30 min. Residual trypsin inhibitor activity was assayed in triplicate.

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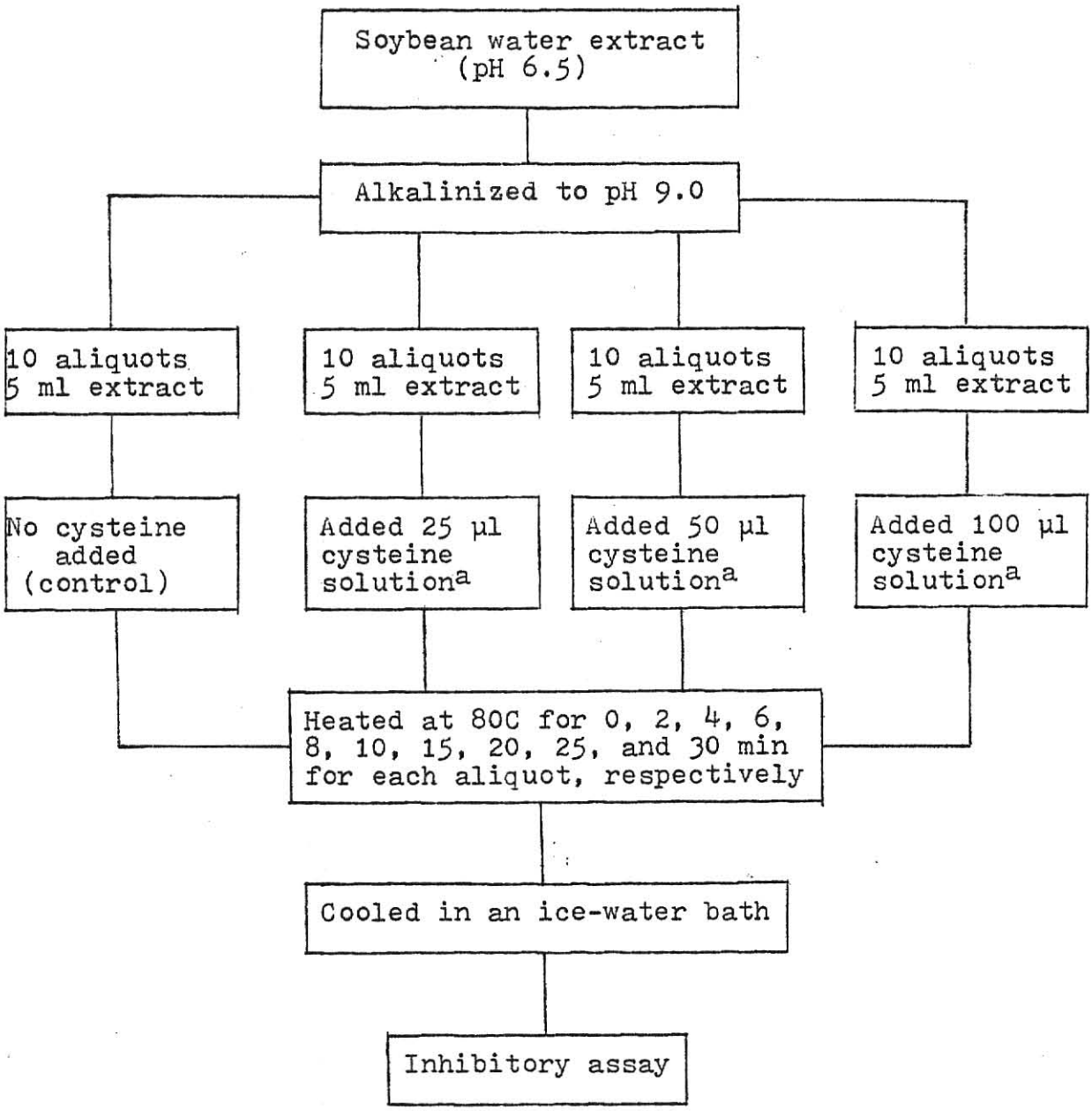
Fig. 1. Flow chart of alkali, cysteine and heat treatment on inactivation of trypsin inhibitor in soybean water extract.



[a]The concentration of the cysteine solution was 30 µg/µl.

Analysis of soybean water extract with pure soybean trypsin inhibitor (Kunitz) added

Trypsin inhibitor solution: Soybean trypsin inhibitor (19.2 mg) was dissolved in 0.64 ml water. This relatively high concentration (1.2 mg/0.04 ml) was employed to prevent diluting the soybean water extract.

Procedure: A 0.56 ml aliquot of the above inhibitor solution (16.8 mg of the inhibitor) was added to 14 ml soybean water extract with a micropipette (1.2 mg inhibitor per ml of extract). The mixture then was adjusted to pH 9.0 with KOH solution. Ten microliters of the cysteine solution was added into 1 ml aliquots of the sample mixture in test tubes, mixed thoroughly, and then heated at 80C in a shaker water-bath. Heating times were 0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min. After heating, tubes were cooled in an ice-water bath immediately. Residual trypsin inhibitor activity was assayed with no delay.

Reactivation test

In order to determine whether inactivation of trypsin inhibitor with cysteine is relatively permanent, samples with added cysteine were held for up to 60 hrs at 4C and 6 hrs at room temperature.

a. Refrigeration temperature

Four sets of samples were prepared as in "alkali, DL-cysteine HCl and heat treatment" section, except heating times were 10 and 30 min for each set. After the ice-water bath treatment and adjusting pH to 7.0, the residual trypsin inhibitor activity was assayed after storing 12, 36, and 60 hr at 4C. The

0-hr time was the control.

This reactivation test was repeated three times.

b. Room temperature

A separate set of samples was prepared as described for the refrigeration temperature test (with 50 µl cysteine solution per 5 ml of soybean water extract). The procedure was the same except the tubes were allowed to stand at room temperature for 1, 2, 3 and 6 hr. Again the 0-hr time was the control. Residual trypsin inhibitor activity were assayed.

This analysis also was repeated three times.

C. Solubility Test: Nitrogen solubility Index (NSI)

Sample preparation: Five different treatments of soybean water extract were prepared for NSI test:

(1) Alkali, cysteine and heat treated soybean water extract: A 1.5 ml aliquot of a cysteine solution was added to 150 ml of an alkalinized soybean water extract (pH 9.0) in a 250 ml-Erlenmeyer flask (10 µl cysteine solution per ml of the extract), and mixed. This material was heated to 80C, held for 10 min and then cooled in an ice-water bath.

(2) Alkali and heat treated soybean water extract: Prepared as in (1), but no cysteine was added.

(3) Blanching, alkali, cysteine and heat treated soybean water extract: The soybean water extract was prepared as in the MATERIALS section, except the soaked beans were blanched in boiling water for 30 sec before grinding. The extract was treated as in (1).

(4) Blanching, alkali and heat treated soybean water extract: Prepared as in (3), except no cysteine was added.

(5) Boiled soybean water extract: A 150 ml sample of soybean water extract in a 250 ml-Erlenmyer flask was heated in a boiling-water bath for 30 min, and cooled in an ice-water bath.

These five samples were adjusted to pH 7.0 with a 0.1 N HCl solution, lyophilized and ground to fine powder for NSI determination.

Nitrogen solubility index determination: Nitrogen solubility index was determined by modifying a slow stirring method described in the official methods of the American Oil Chemists' Society (1978). About 5 g of the sample was weighted accurately into a 400 ml-beaker; 200 ml of 30C water was added in several small portions and dispersed thoroughly with a glass rod. The glass rod was washed with a final portion of water. The mixture was stirred at 120 rpm with a magnetic stirrer for 120 min while keeping the beaker immersed in a 30C-water bath. The mixture was transferred to a 250 ml-volumetric flask by carefully washing the contents of the beaker into the flask with water. Two drops of antifoam agent (GE 66) were added and the volume brought to the mark with water. After content of the flask was mixed thoroughly, it was allowed to stand for a few minutes. About 40 ml of the mixture was decanted into a 50 ml-centrifuge tube, and centrifuged for 10 min at 1500 rpm. The supernatant was filtered through a funnel containing a glass wool plug (Pyrex brand wool #3950). The

clean filtrate was collected and 25 ml used for nitrogen determination by the Kjeldahl analysis (A.O.A.C. 1970). The total nitrogen of the sample also was determined by the Kjeldahl method. The nitrogen solubility index was calculated as:

% NSI = (% water soluble nitrogen/% total nitrogen) x 100.

D. Proximate Analysis of Soybean Water Extract

Total solids determination: Total solids of soybean water extract was determined as weight remaining after 4 hr in a forced draft oven at 100C.

Crude protein determination: Macro-Kjeldahl method (A.O.A.C. 1970) was used for the protein determination. A protein factor of 6.25 was used.

Fat determination: Fat content of soybean water extract was determined by the Mojonnier method (Mojonnier Milk Tester Bulletin 101), with a Model D electrically operated Mojonnier Milk Test.

E. Organoleptic Analysis

Two cysteine treated soybean milk samples were prepared as described in (1) and (3) in the sample preparation steps of the NSI test (one was alkali-cysteine-and heat treated, the other one was blanched, alkali-cysteine-and heat treated). The soybean milks were adjusted to pH 7.0 with a 0.1 N HCl solution. Each of them was blended with equal parts of cow's milk (Kansas State University grade A pasteurized - homogenized milk) and sweetened

with 2% sugar. Two taste panels, one composed of 10 Chinese and the other of 10 Americans were employed to test these milks. The panelists were asked to rate the samples on a seven point hedonic scale in which a sample "liked very much" was assigned a score of seven and one "disliked very much" assigned a score of one. The samples were presented in random order, and coded with random three-digit numbers.

RESULTS AND DISCUSSION

A. Trypsin Inhibitor Activity Assay Method

The trypsin inhibitor assay method was applied directly to soybean extract samples without pre-treatment, to determine its accuracy and the feasibility. Fig. 2 shows the linear increase in inhibition of trypsin with increasing amount of a 20 fold diluted raw soybean water extract. It is apparent that the trypsin inhibitor activity is directly proportional to the volume of samples used in the assay system. The soybean aqueous sample itself had significant absorbance and/or light scattering at 410 nm. When more than 25 µl of the 20 fold diluted sample was used in the assay, the absorbance at 410 nm was too great to yield accurate results.

Reproducibility of the assay method was found to be quite satisfactory. Among ten independent assays, the standard deviation of the reference (with no sample added) was $\pm 2.1\%$ of the mean, the standard deviations of samples with 2.7%, 21.5% and 56.5% inhibitory activity were $\pm 3.7\%$, $\pm 3.2\%$ and $\pm 4.2\%$ of the mean, respectively. The small standard deviations indicated the high reproducibility of the assay.

B. Inactivation of Trypsin Inhibitors

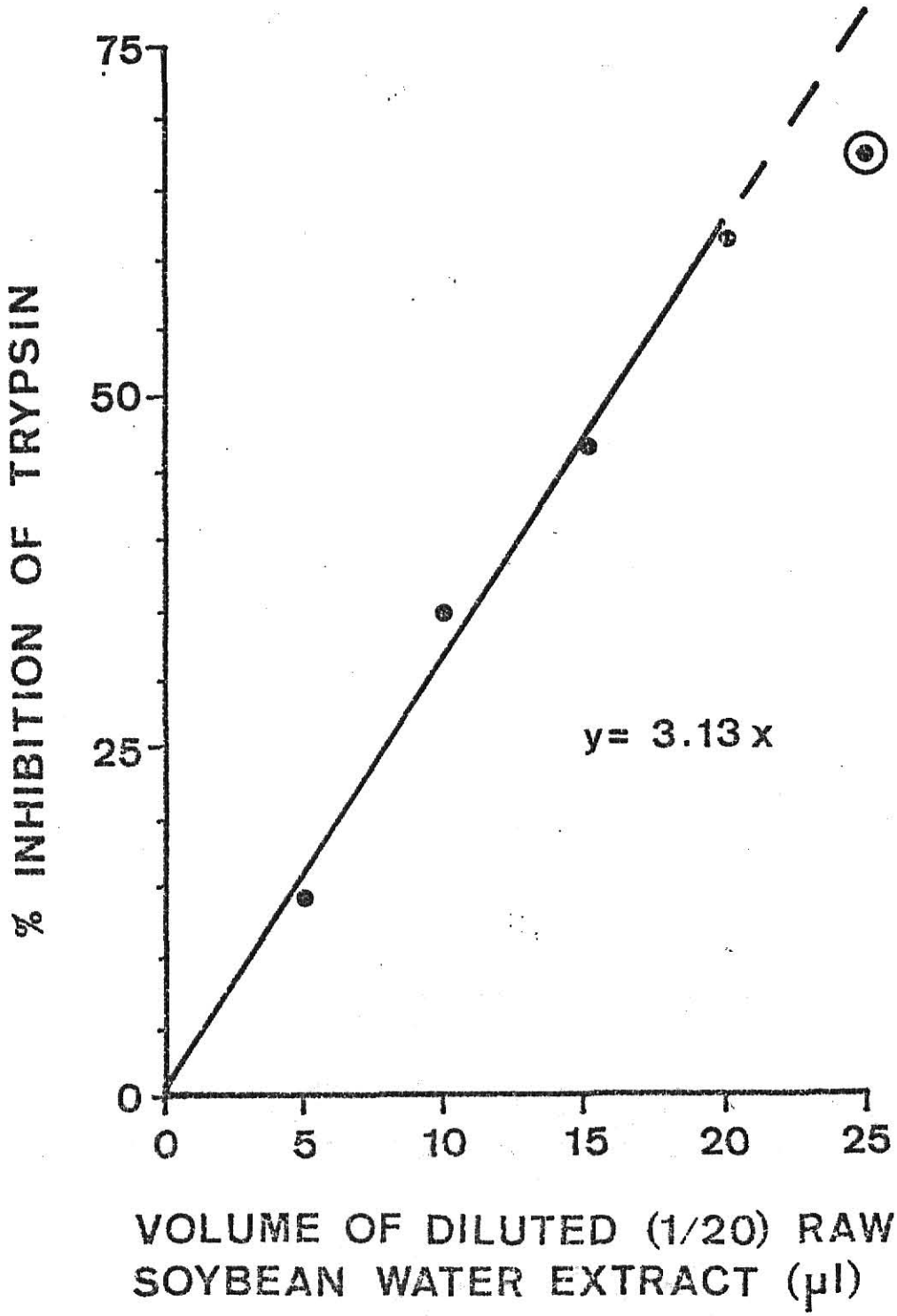
Effect of cysteine on soybean trypsin inhibitor (Kunitz)

When a solution of purified and lyophilized trypsin inhibitor (0.15 mg/ml Tris-buffer, pH 9.0) was heated at 80C for 2 and 30 min, only 13% and 29% was inactivated, respectively.

THIS BOOK CONTAINS NUMEROUS PAGES THAT WERE BOUND WITHOUT PAGE NUMBERS.

THIS IS AS RECEIVED FROM CUSTOMER.

Fig. 2. Analysis of trypsin inhibitors in diluted raw soybean water extract. Dashed line is an extrapolation of the linear portion of the curve.


75
50
25
0
% INHIBITION OF TRYPSIN
y= 3.13 x
0
5
10
15
20
25
VOLUME OF DILUTED (1/20) RAW SOYBEAN WATER EXTRACT (µl)

However when it was heated with the addition of 0.15 mg and 0.30 mg cysteine per ml of inhibitor solution at 80C for 2 min, 47% and 78% was inactivated. Fig. 3 shows that the cysteine enhanced the inactivation of the inhibitor at pH 9.0. The rate and degree of inactivation of the inhibitor increased as the concentration of cysteine increased. Even before heat treatment, cysteine itself inactivated the inhibitor to some extent. The steep decline of the curve during 2 min heating time indicated that the reaction of cysteine on the inhibitor was very fast. Though the mechanism of inactivating trypsin inhibitor by cysteine was not investigated, it seems that, at pH 9.0 with some heat treatment, cysteine worked as disulfide linkage reducer. In reducing the disulfide linkage of the inhibitor molecule, the function of the inhibitor was altered.

Effects of pH and temperature on trypsin inhibitors in soybean water extract

A combination of pH adjustments and heat has more effect on the inactivation of trypsin inhibitors than heat alone. Fig. 4 shows that pH 9.0 is more effective than pH 6.5 in inactivating the trypsin inhibitors. pH is more effective at 80C than at 70C. This can be observed by comparing the increase in inactivation at 80C (e.g. 20% for 90 min) and at 70C (e.g. 10% for 90 min) when pH was increased from 6.5 to 9.0. Because the combination of pH 9.0 and 80C was more effective than other combinations, it was chosen for further experiments.

Fig. 3. Effect of cysteine on inactivation of pure soybean trypsin inhibitor (Kunitz) at pH 9.0 and 80C heating.

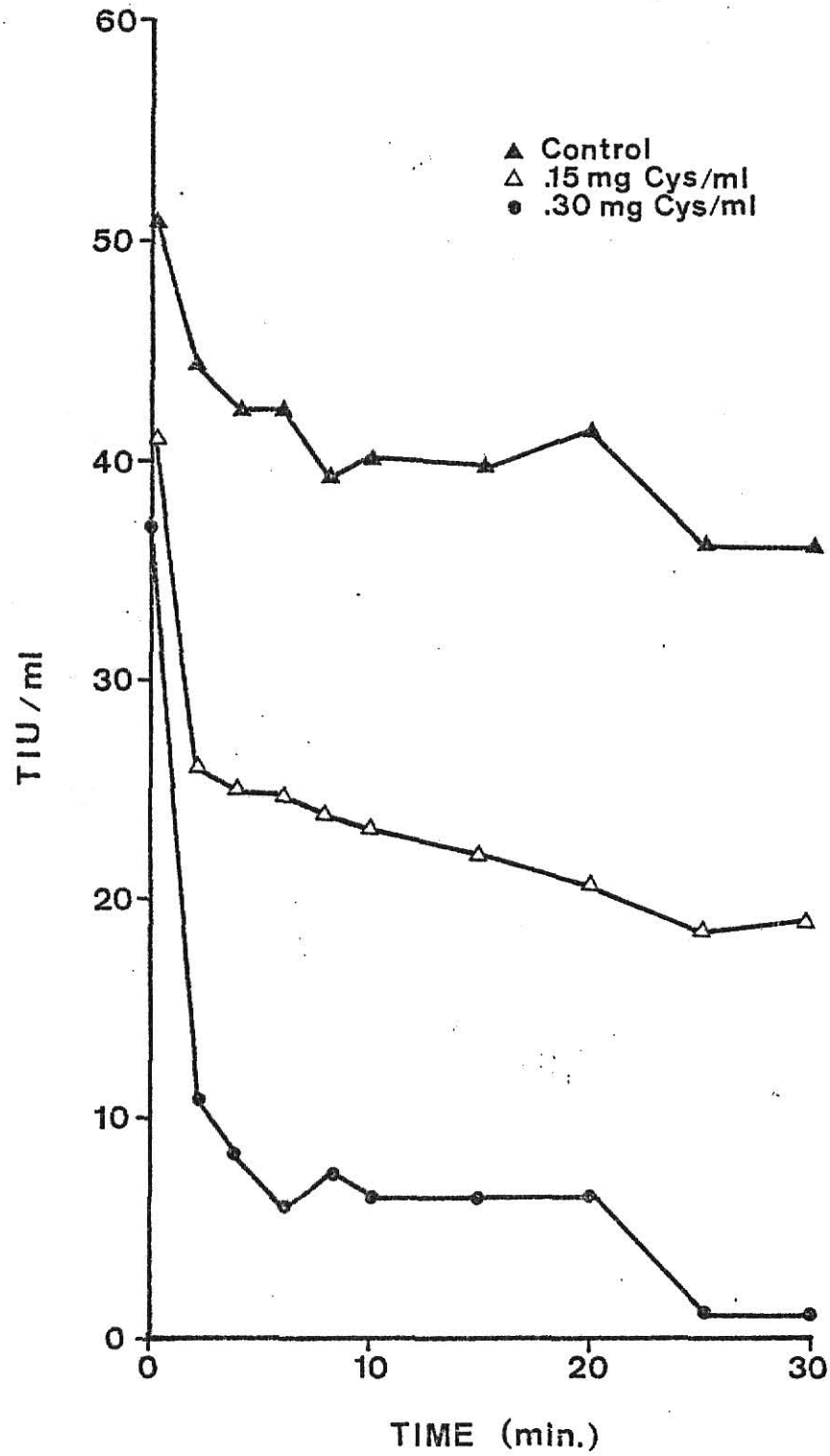
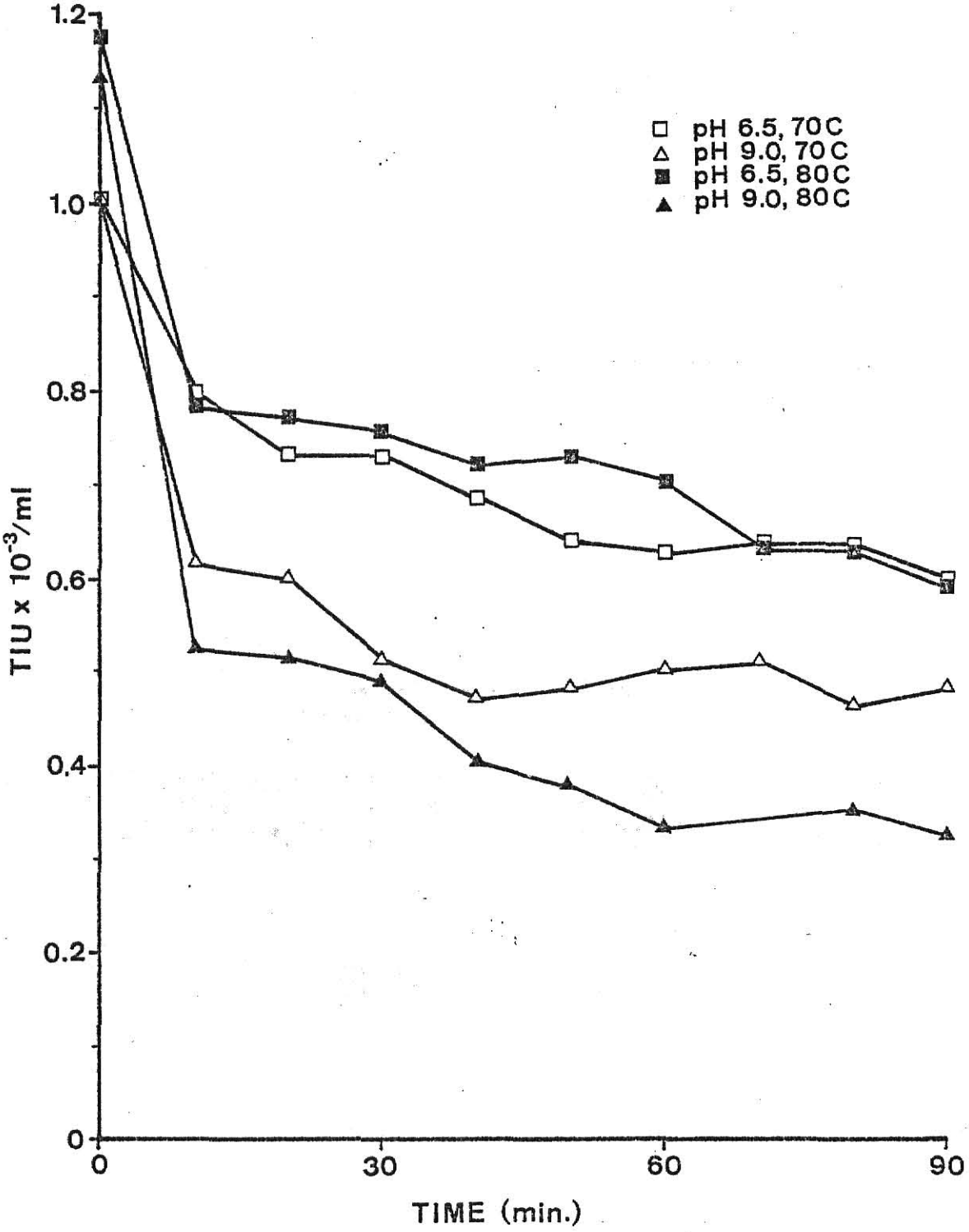

60
50
40
30
20
10
0
TIU/ml
0
10
20
30
TIME (min.)
▲ Control
△ .15 mg Cys/ml
● .30 mg Cys/ml

Fig. 4. Effects of pH and temperature on inactivation of trypsin inhibitors in soybean water extract.


1.2
1.0
0.8
0.6
0.4
0.2
0
TIU x 10^{-3}/ml
0
30
60
90
TIME (min.)
pH 6.5, 70C
pH 9.0, 70C
pH 6.5, 80C
pH 9.0, 80C

Since the initial trypsin inhibitor concentrations (TIU) of samples that were heated at 80C were higher than those at 70C, part of the 80C-curve in Fig. 4 (pH 6.5) was above that of the 70C-curve (pH 6.5). In fact, the heat treatment at 80C was more effective on inactivation of the inhibitors than that of 70C. This effect can be seen more clearly in Fig. 5 in which % TI (trypsin inhibitor) remaining vs. heating time was plotted. During heating, TIU decreased rapidly during the first 10 min and then slowly. It is possible that there are two kinds of inhibitors, one that is inactivated easily and the other is more heat resistant.

Effect of cysteine on trypsin inhibitors in soybean water extract

Cysteine was shown to be an effective agent for inactivating Kunitz trypsin inhibitor (Fig.3), it also functioned effectively with the trypsin inhibitors in soybean water extract (Fig. 6), even though the soybean water extract contained a complicated mixture of soybean protein (Table 1). Fig. 6 also shows that the extent of inactivating the inhibitors is a function of cysteine concentration.

The drastic decrease of TIU (Fig. 6) probably occurs because the 10 min-heat treatment and cysteine quickly break the disulfide linkages of trypsin inhibitor. This undoubtedly alters the conformation of inhibitor molecules. After free cysteine is depleted or its concentration is lower than an effective threshold, any subsequent inactivation is dependent solely on heat. This accounts for the flat segments of the curve in the figures.

Fig. 5. Percent trypsin inhibitor remaining in soybean water extract after alkali and heat treatment.

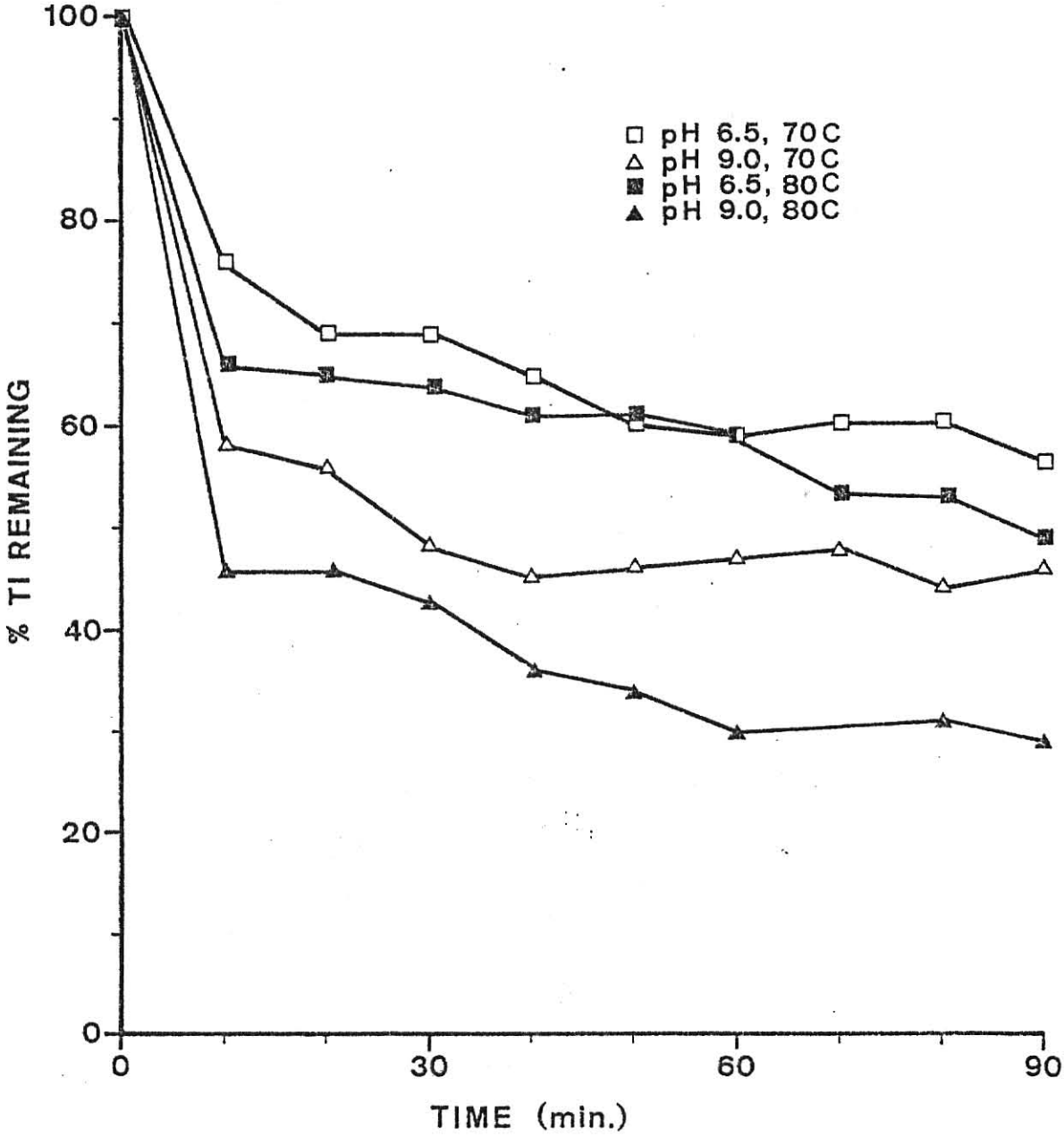
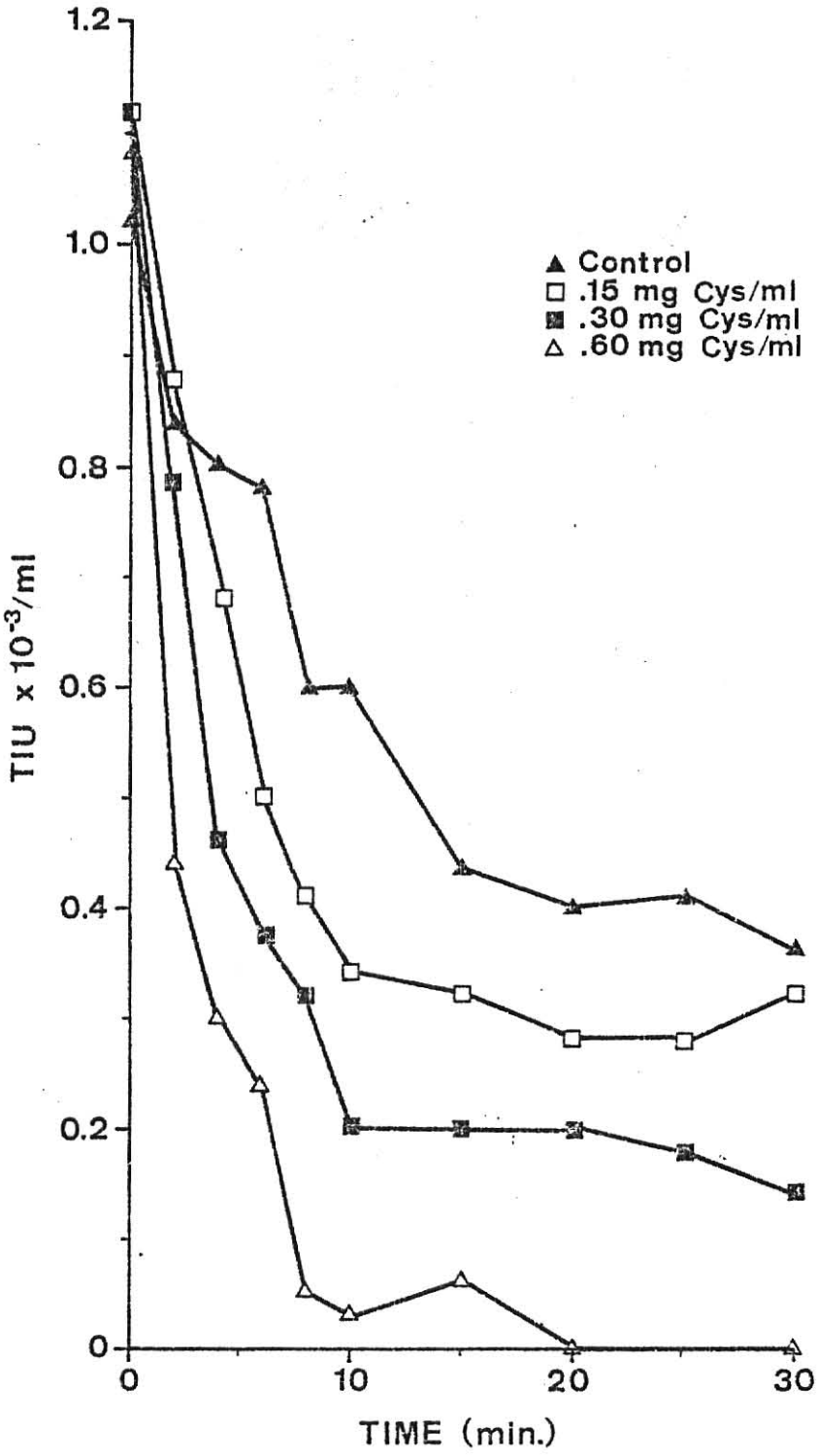

100
80
60
40
20
0
% TI REMAINING
0
30
60
90
TIME (min.)
pH 6.5, 70C
pH 9.0, 70C
pH 6.5, 80C
pH 9.0, 80C

Fig. 6. Effect of cysteine on inactivation of trypsin inhibitors in soybean water extract at pH 9.0 and 80C heating.


1.2
1.0
0.8
0.6
0.4
0.2
0
TIU x 10⁻³/ml
0
10
20
30
TIME (min.)
▲ Control
□ .15 mg Cys/ml
■ .30 mg Cys/ml
△ .60 mg Cys/ml

If the addition of cysteine was below a certain concentration, heat treatment at 80C was unable to inactivate the remaining inhibitor. Therefore, the concentration of cysteine in the reaction was very important.

It is concluded that cysteine enhances the inactivation of not only Kunitz trypsin inhibitor model system but also trypsin inhibitors in an aqueous soybean extract.

Rackis (1974) reported that when 79% of trypsin inhibitor was inactivated, the maximum PER of soybean product was obtained. Our results shows the addition of 1.5 mg cysteine to 5 ml alkali soybean water extract at pH 9.0 and heated for 10 min at 80C were enough to inactivate more than 80% of the inhibitors. Complete inactivation of inhibitors was attained when 3.0 mg of cysteine was added to 5 ml alkali soybean water extract at pH 9.0 and heated at 80C for 20 min.

Effect of L-cysteine HCl·H2O on trypsin inhibitors in soybean water extract

L-Cysteine HCl·H_2O (United State Biochemical Corporation, #14035, assay: > 98.0%) is much cheaper than DL-cysteine HCl (Sigma, #C-9768) which has been used throughout this study. Our results shows that L-cysteine HCl·H_2O is as effective as DL-cysteine HCl for inactivating trypsin inhibitor (Table 6 and 11). From the economical point of view, it is more feasible to use L-cysteine HCl·H_2O ($38/kg) for processing of the soybean water extract (soybean milk).

Eighty-nine percent inactivation of trypsin inhibitor can

be obtained by adding 1.67 mg L-cysteine $HCl \cdot H_2$) to 5 ml alkali soybean water extract at pH 9.0 and heating at 80C for 10 min. The cost of this amount of cysteine was only 1.3¢ per liter soybean milk and would undoubtedly be less in bulk amounts.

Table 6. Effect of L-cysteine $HCl \cdot H_2O$ on inactivation of trypsin inhibitors in soybean water extract[a].

L-Cysteine $HCl \cdot H_2O$ (mg/ml)	Reduced trypsin inhibitor (%)	
	10 min	30 min
0.334	89	96
0.668	95	99

[a]Soybean water extract was alkalinized to pH 9.0, added cysteine (as in the table) and heated at 80C for 10 min and 30min.

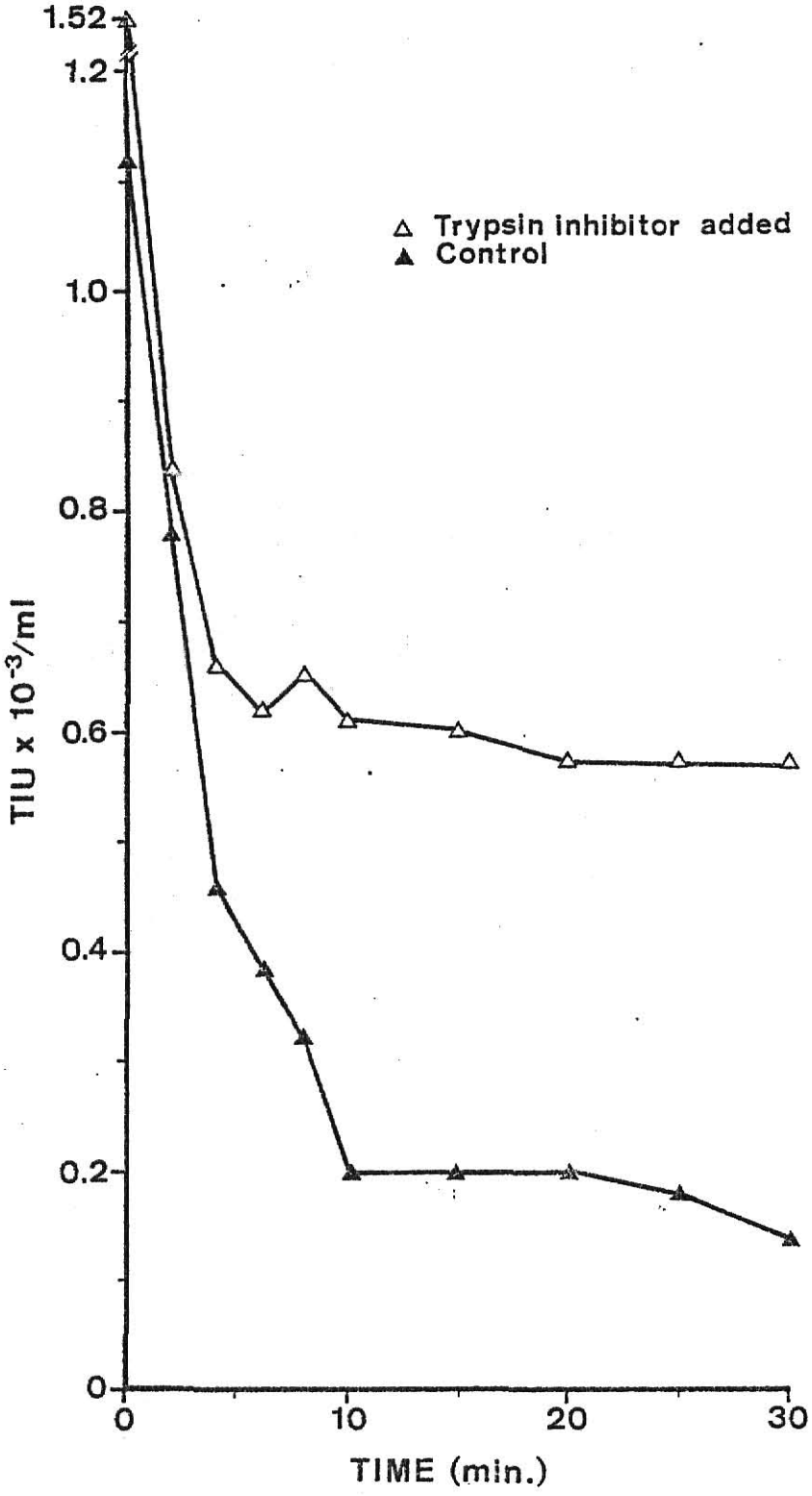
Effect of cysteine on trypsin inhibitors in soybean water extract with pure trypsin inhibitor (Kunitz) added.

Fig. 7 shows the addition of pure trypsin inhibitor (400 TIU) shifted the curve up about the amount of added trypsin inhibitor after 10 min heating (average difference between two curves was 400 TIU after 10 min heating time), which demostrated that the concentration of cysteine was a critical factor on the enhancement of inactivating trypsin inhibitors.

Reactivation test

The possible reformation of the correct disulfide linkages

Fig. 7. Effect of cysteine on inactivation of trypsin inhibitors in soybean water extract with pure trypsin inhibitor (Kunitz) added. At pH 9.0 and 80C heating.


1.52
1.2
1.0
0.8
0.6
0.4
0.2
0
TIU x 10^{-3}/ml
△ Trypsin inhibitor added
▲ Control
0
10
20
30
TIME (min.)

of trypsin inhibitor molecule which has been reduced by cysteine was investigated. There appears to be no significant reactivation of the inhibitor activity after 60 hr at 4C or 6 hr at room temperature (Appendix Table 13 and 14).

C. Nitrogen Solubility Index (NSI)

The NSIs of lyophilized soybean products are shown in Table 7. Products of treatment 1 (0.30 mg cysteine/ml extract added and heated at 80C for 10 min at pH 9.0) had the highest % NSI. Nearly all of the protein solubility was retained (99.70% NSI). The cysteine added products (treatment 1 and 3 in Table 7) had higher % NSI than the control (see no cysteine added, treatment 2 and 4 in Table 7). This probably was due to cleavage of intermolecular disulfide bonds with resulting disaggregation of the soybean proteins (Wolf, 1972b). Although boiling inactivated trypsin inhibitor, it reduced the % NSI (49.80% NSI). Cysteine enhanced the inactivation of trypsin inhibitor with less heat (80C). This in turn, retains solubility properties of lyophilized products and makes them readily dispersible in water with no dispersing agent.

D. Proximate Composition of Soybean Water Extract

Proximate composition of soybean water extract is shown in Table 7. Blanched samples (treatment 3 and 4) showed lower protein and total solids content than samples without blanching (treatment 1 and 2). Since blanching denatured protein with heat (30 sec in boiling water), the protein and total solids

Table 7. Proximate composition and nitrogen solubility index (NSI) of soybean water extract exposed to various treatments.

Soybean water extract					
Treatment[a]	Description	Protein %	Fat %	Total solid %	NSI[b] %
1	pH 9.0 0.30 mg cysteine per ml extract added 80C, 10 min	3.78	2.20	7.89	99.70
2	pH 9.0 80C, 10 min	3.78	2.26	7.88	98.91
3	Blanching pH 9.0 0.30 mg cysteine per ml extract added 80C, 10 min	3.26	2.24	7.31	94.56
4	Blanching pH 9.0	3.26	2.34	7.29	91.56
5	Boiled, 30 min	3.53	2.14	7.75	49.80

[a]2 and 4 were control tests for 1 and 3, respectively.

[b]Before NSI was determined, samples were neutralized and lyophilized.

yield of the extract are reduced (Johnson and Snyder, 1978). It is apparant that almost all of the reduced solids of the blanched samples are protein. Although blanching reduced the solids yield of products, it is necessary to inactivate lipoxygenase to prevent the beany flavor of soybean products.

E. Organoleptic Analysis of Cysteine Treated Soybean Milks

An organoleptic analysis of two samples of soybean water extract mixed with cow's milk is shown in Table 8. Both samples were acceptable to Chinese members of a panel but not to Americans. Chinese preferred the product prepared with unblanched beans but Americans preferred the product prepared with blanched beans. The acceptability of the products was dependent on the nationality and obviously familiarity with the product.

Table 8. Average organoleptic score of cysteine treated soybean milks mixed with cow's milk[a].

Sample	Chinese panel	American panel
Prepared with blanched beans	5.1 ± 1.4[b]	3.7 ± 1.3
Prepared with unblanched beans	5.8 ± 0.9	2.1 ± 1.6

[a]Score 7 for sample was liked very much, score 1 for sample was disliked very much.

[b]Standard deviation of mean (n = 10).

CONCLUSIONS

Based on the results of this study, the following conclusions are drawn:

1. The trypsin inhibitor assay method used throughout this study is simple, fast, accurate and reproducible.

2. Alkalinization of soybean water extract to pH 9.0 is effective in promoting inactivation of trypsin inhibitors at 70C and 80C.

3. Cysteine enhances the inactivation of trypsin inhibitors not only in a Kunitz inhibitor model-aqueous system but also in soybean water extract.

4. The inhibitors are inactivated completely when 5 ml alkalinized soybean water extract (pH 9.0) is treated with 3.0 mg cysteine and heated at 80C for 20 min.

5. Treatment of 5 ml alkalinized soybean water extract (pH 9.0) with 1.5 mg cysteine at 80C for 10 min results in 80-90% inactivation of inhibitors. This is enough to attain maximum PER value of soybean product according to Rackis (1974).

6. The NSI value of an alkali-cysteine (pH 9.0, with 0.3 mg/ml cysteine)-and 80C heat treated, neutralized and lyophilized soybean product was 99.70%.

7. Cysteine treated soybean milks were acceptable to Chinese members of a panel but not to Americans. The acceptability of the products was dependent on the familiarity with the products.

8. The cost of L-cysteine $HCl \cdot H_2O$ for processing one liter of soybean milk was only 1.3¢ (334 mg/l) and would undoutedly be less in bulk amounts.

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APPENDIX

Table 9. Effect of cysteine on inactivation of pure soybean trypsin inhibitor (Kunitz)[a].

Cysteine conc. (mg/ml)	Heating time (min)									
	0	2	4	6	8	10	15	20	25	30
	TIU/ml sample									
Control (without Cys)	50.8	44.4	42.3	42.3	39.2	40.2	39.7	41.3	36.0	36.0
0.15	41.3	27.0	24.9	24.9	23.8	23.3	22.2	20.6	18.5	19.1
0.30	37.0	11.4	8.2	6.0	7.6	6.5	6.5	6.5	2.2	2.2

[a]Inhibitor solution: 0.15 mg/ml in Tris buffer pH 9.0. Heat treatment at 80C.

Table 10. Effects of pH and temperature on inactivation of trypsin inhibitors in soybean water extract.

T	pH	Heating time (min) 0	10	20	30	40	50	60	70	80	90
		TIU x 10^{-3}/ml sample									
70C	6.5	1.062	0.802	0.734	0.734	0.686	0.640	0.628	0.638	0.638	0.600
	9.0	1.062	0.618	0.600	0.512	0.470	0.484	0.502	0.512	0.646	0.484
80C	6.5	1.190	0.784	0.774	0.756	0.720	0.730	0.702	0.630	0.630	0.586
	9.0	1.126	0.522	0.514	0.484	0.406	0.378	0.334	---	0.352	0.324

Table 11. Effect of cysteine on inactivation of trypsin inhibitors in soybean water extract[a].

Cysteine conc. (mg/ml)	Heating time (min) 0	2	4	6	8	10	15	20	25	30
	TIU x 10^{-3}/ml sample									
Control (with no Cys)	1.02	0.84	0.80	0.78	0.60	0.60	0.44	0.40	0.41	0.36
0.15	1.12	0.88	0.68	0.50	0.42	0.34	0.35	0.28	0.28	0.32
0.30	1.12	0.78	0.46	0.28	0.32	0.20	0.20	0.20	0.18	0.14
0.60	1.08	0.44	0.30	0.24	0.05	0.03	0.06	0.00	----	0.00

[a]Soybean water extract was adjusted to pH 9.0, heated at 80C.

Table 12. Effect of cysteine on inactivation of trypsin inhibitors in soybean water extract with pure soybean trypsin inhibitor (Kunitz) added.

Treatment[a]	Heating time (min) 0	2	4	6	8	10	15	20	25	30
	TIU x 10^{-3}/ml sample									
Inhibitor added	1.52	0.84	0.66	0.62	0.65	0.61	0.60	0.57	0.57	0.57
With no inhibitor added	1.12	0.78	0.46	0.38	0.32	0.20	0.20	0.20	0.18	0.14

[a]Sample were adjusted to pH 9.0 and heated at 80C.

Table 13. Reactivation test--refrigeration temperature. Soybean water extract was adjusted to pH 9.0, added with cysteine and heated at 80C for 10 or 30 min. The trypsin inhibitor activity of the products was assayed after storage at 4C for 12, 36 and 60 hr.

Cysteine conc. (mg/ml)	Test[a,b]	10 min at 80C				30 min at 80C			
		0-hr	12-hr	36-hr	60-hr	0-hr	12-hr	36-hr	60-hr
		TIU x 10^{-3}/ml							
0.60	1	0.062	0.094	0.066	0.066	0.000	0.000	0.000	0.000
	2	0.010	0.062	0.090	0.054	0.000	0.000	0.000	0.046
	3	0.062	0.030	0.134	0.040	0.000	0.000	0.000	0.000
0.30	1	0.158	0.166	0.094	0.058	0.094	0.000	0.000	0.000
	2	0.160	0.124	0.170	0.178	0.040	0.020	0.120	0.036
	3	0.092	0.110	0.048	0.092	0.000	0.030	0.000	0.030
0.15	1	0.472	0.294	0.272	0.354	0.314	0.252	0.206	0.230
	2	0.302	0.332	0.330	0.298	0.242	0.218	0.280	0.262
	3	0.306	0.320	0.324	0.214	0.286	0.190	0.252	0.174
0	1	0.522	0.492	0.420	0.450	0.370	0.314	0.252	0.260
	2	0.492	0.498	0.450	0.388	0.312	0.280	0.330	0.316
	3	0.460	0.420	0.460	0.410	0.336	0.380	0.324	0.266

[a]Test 1, 2 and 3 were conducted at three different times.

[b]Each test-result is an average of three inhibitor assays.

Table 14. Reactivation test--room temperature. Soybean water extract was adjusted to pH 9.0, added with cysteine (0.30 mg/ml) and heated at 80C for 10 or 30 min. The trypsin inhibitor activity of the products was assayed after holding at room temperature for 1, 2 and 6 hr.

Test[a,b]	10 min at 80C					30 min at 80C				
	0-hr	1-hr	2-hr	3-hr	6-hr	0-hr	1-hr	2-hr	3-hr	6-hr
	TIU x 10^{-3}/ml									
1	0.120	0.140	0.184	0.120	0.192	0.050	0.080	0.000	0.026	0.104
2	0.124	0.162	0.108	0.100	0.120	0.038	0.094	0.000	0.070	0.080
3	0.090	0.090	0.090	0.100	0.078	0.010	0.000	0.000	0.010	0.000

[a]Test 1, 2 and 3 were conducted at three different times.

[b]Each test-result is an average of three inhibitor assays.

THE EFFECT OF CYSTEINE ON HEAT INACTIVATION OF SOYBEAN TRYPSIN INHIBITOR

by

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AN ABSTRACT OF A MASTER'S THESIS

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Soybean protein nutritional value is inversely proportional to soybean trypsin inhibitor activity. Conventional heating inactivates the inhibitor but reduces soybean protein solubility. This greatly reduced its applicability in food formulation.

I hypothesized that the trypsin inhibitor of soybean water extract would be more vulnerable to heat denaturation when cysteine, a reducing agent, was added to the extract. By investigating effects of pH, cysteine concentration, heating temperature and heating time, I attempted to obtain the best nutritional value and functional properties of soybean protein.

A rapid analytical method with minimal sample preparation was developed for trypsin inhibitor activity. The assay method is simple, fast, accurate and reproducible. The method is based on the inhibition of trypsin by a soybean sample. N-Benzoyl-DL-arginine-p-nitroanilide is cleaved by trypsin into N-benzoyl-arginine and p-nitroanilide. The concentration of the yellow colored p-nitroanilide can be determined spectrophotometrically at 410 nm. By continuously monitoring the changing absorbance at 410 nm, the trypsin activity is determined. In a separate assay, the inhibition of trypsin by sample with suspected trypsin inhibtor is determined.

The optimum conditions for destroying trypsin inhibitor were obtained when 1.5 mg of cysteine was added to each 5 ml sample of soybean water extract at pH 9.0 and heated at 80C for 10 min, which reduced 80-90% of soybean trypsin inhibitor activity and reached maximum PER of the product. The product showed no

significant reactivation of soybean trypsin inhibitor activity after 60 hr at 4C or 6 hr at room temperature. Soybean protein solubility was expressed by nitrogen solubility index (NSI). The NSI of neutralized and lyophilized product was 99.70%. Nearly all proteins retained their solubility after treatment. The cysteine treated products were acceptable to people who were familiar with soybean milks. The cost of L-cysteine $HCl \cdot H_2O$ for processing one liter of soybean milk was only 1.3¢ and would undoubtedly be less in bulk amount.