Trypsin Inhibitor Activity in Winged Bean (*Psophocarpus tetragonolobus*) and the Possible Role of Tannin

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Winged bean (*Psophocarpus tetragonolobus*, variety *Chimbu*) had the highest trypsin inhibitor acitivty (TIA) among several beans examined. Most of the TIA could be heat inactivated. Residual tannin in the cooked bean could account for the heat-resistant TIA, which was approximately 1% of the original. The effects of soaking in different salt solutions prior to cooking on TIA and tannin were studied. Without soaking, the loss of TIA after boiling for 60 min was only 4%. Soaking in water, 10% ash solution, 0.1 N $CaCO_3$, and 0.01 N $NaHCO_3$ resulted in at least 95% total loss (soaking and cooking), indicating the importance of soaking in reducing the TIA. NaOH (1 N) was most effective in descreasing the tannin in the cooked bean. These studies suggest that tannins may play a more important role than the heat-labile, proteinaceous trypsin inhibitor in heat-processed winged bean.

Researchers in recent years have recognized the value of plant protein in supplying the protein needs in developing countries. Leguminous seeds form an important staple in the diet of people in many areas of the world. A survey of underexploited tropical plants by a panel of the National Academy of Sciences (USA) brought attention to winged bean (*Psophocarpus tetragonolobus*) as a promising legume due to its high protein and oil content (National Academy of Sciences, 1975). With research it can become a significant food crop in the tropics.

Limited information from research done so far on winged bean indicates that the chemical composition and nutritive values are similar to that of soybean. Like other legumes, winged bean contains trypsin inhibitor activity (TIA) (Sohonie and Bhandarkar, 1954) which may reduce the digestibility of its protein. Although the trypsin inhibitors make up only 6% of the total protein in soybean, it is estimated that it is responsible for 30–50% of the growth inhibitory effect when monogastric animals ingest unheated soybeans (KaKade et al., 1973).

Our preliminary studies on heat inactivation showed a persistent though small residual amount of TIA after 2 h of boiling. Elias et al. (1979) demonstrated the effects of seed coat color on the protein quality of beans and suggested the possible role of tannins and other polyphenols as trypsin inhibitors which are heat resistant. We have undertaken the present work to determine different conditions of inactivation of TIA by heat and soaking and investigate the role of tannin in TIA.

MATERIALS AND METHODS

Bean Sources. Winged beans (*Chimbu* and *Tpt-1* varieties) were supplied to us by Mr. Louis Lazaroff of the Asia Foundation in San Francisco. These two varieties were considered high yielding in pods and tubers. Other dry beans were obtained from commercial outlets in the Bay Area.

Heat Inactivation of Trypsin Inhibitor. Whole beans or separated hulls and cotyledons were cooked in water at boiling temperature, 80 °C, and 60 °C. Due to the difficulty of removing the hull in the dry bean, the whole bean was soaked at 4 °C until swollen and the hull was peeled off. The hull and cotyledon were air-dried, ground, and extracted.

Extraction and Determination of Trypsin Inhibitor. One gram of ground sample was extracted with 20 mL of ice-cold distilled water by homogenizing for 2 min with an Elvehjem homogenizer run with an electric drill. The extract was centrifuged at 4 °C for 30 min at 13600*g* and the supernatant was used for the assay after appropriate dilution. Water was as effective or better than NaCl solution in extracting TIA in both raw and cooked beans as shown by our preliminary experiments and that of Elias et al. (1979).

Trypsin activity was measured by its hydrolysis of (*p*-toluenesulfonyl)arginine methyl ester (TAME) at 25 °C and measuring the absorbance of (*p*-toluenesulfonyl)arginine at 247 nm with a Cary 14 recording spectrophotometer (Worthington Biochemical Corp., 1977). One unit of trypsin activity is defined as 1 μmol of (*p*-toluenesulfonyl)arginine released per min. One unit of trypsin activity inhibited is defined as one trypsin inhibitory unit (TIU).

Soaking Treatments. About 100 g of winged bean was soaked at room temperature in sufficient salt solutions to cover the beans for 6 and 24 h. After soaking, the beans were drained and cooked in known amounts of water for 60 min, drained of their cooking broths, and air-dried. The soaking solutions, raw soaked beans, cooking broths, and cooked beans were assayed for TIA and tannin content. The soaking solutions were as follows: (a) water, (b) 1 N NaOH, (c) 0.01 N $NaHCO_3$, (d) 4% HAc, (e) 1% $CaCO_3$, and (f) 10% ash solution. We selected the aqueous alkaline solutions due to their ability to decrease tannin (Price et al., 1979). HAc was included to determine the effect of an acid solution. Ash is an inexpensive available source of alkali used in some developing countries to treat beans. The 10% ash solution was obtained by soaking 100 g of hardwood ash in 900 mL of water overnight with stirring at room temperature and filtering.

Assay of Tannin. Tannin was assayed with the Folin–Denis reagent according to Sherman et al. (1953) and expressed as tannic acid. Tannin extract was prepared by the same procedure except that the color reagent was not added. The extract was freeze-dried and the residue dissolved in water.

RESULTS AND DISCUSSION

The TIA of the two varieties of raw winged bean was examined and compared with that of other dry beans (Table I). Variations on TIA were observed within a variety but the *Chimbu* variety of winged bean consistently had more TIA than the other beans. *Tpt-1* variety had slightly less than large lima, which had the highest TIA

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Table I. Trypsin Inhibitor Activity in Winged Bean as Compared to Other Beans

bean	TIU/g of flour[a]
winged bean (*Psophocarpus tetragonolobus*)	
variety *Chimbu*	3691
variety *Tpt-1*	1716
large lima (*Phaseolus lunatus*)	1952
pink (*Phaseolus vulgaris*)	419
blackeye (*Vigna unguiculata*)	619
garbanzo (*Cicer arietinum*)	419
dark red kidney (*Phaseolus vulgaris*)	1040

[a] Average of three determinations and two levels of trypsin. One TIU (trypsin inhibitory unit) is defined as one unit of trypsin activity inhibited. One unit of trypsin activity is equal to 1 μmol of TAME hydrolyzed per minute.

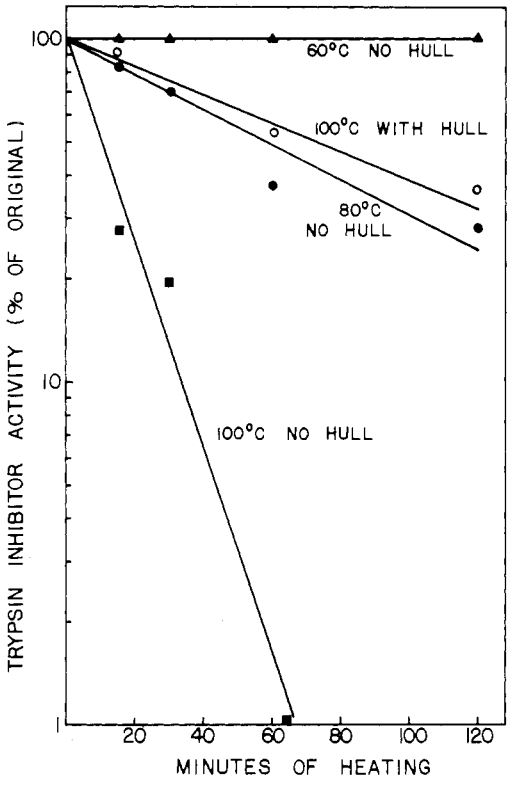

Figure 1. Effects of temperature, time, and hull on heat inactivation of trypsin inhibitor in winged bean.

among the other beans tested. It is possible that these differences in TIA may not hold upon examination of a larger pool of samples, but the trend shown here is consistent in this particular batch of beans. The relatively high amount of TIA in the *Chimbu* variety should be considered in the processing of winged bean for food use. A low level of TIA and other antinutritional factors should be used as one of the criteria for selecting suitable varieties now being tested in The Philippines (Batugal, 1979) and Indonesia (Thompson, 1979).

Although removing the hull is difficult and not generally practiced in cooking beans, the effects of dehulling on the rate of trypsin inhibitor inactivation were studied. Heating the whole bean for 2 h at 100 °C inactivated only 65% of the TIA, while heating at 60 °C did not have any effect after 2 h (Figure 1). However, when the hull was removed, 2 h of heating at 80 °C inactivated about 68%, and almost 99% inactivation was achieved at 100 °C after 2 h. The persistence of a residual amount of TIA, approximately 1% of the original, was observed in the whole bean even after 3 h of boiling, indicating a heat-resistant trypsin inhibitor. The slow inactivation of TIA in the whole bean can be due to the slow heat penetration through the seed coat and the relatively high concentration of a heat-resistant trypsin inhibitor in the seed coat. Evidence in the literature points to tannins as the probable heat-resistant trypsin inhibitors (Elias et al., 1979; Tamir and Alumot, 1969; Goldstein and Swain, 1965; Sosulski, 1979).

Table II. Trypsin Inhibitor Activities and Tannin Contents of Raw and Cooked[a] Whole Winged Bean, Cotyledon, and Hull

	trypsin inhibitor,[b] TIU/g	tannin content,[b] mg/g
whole bean, raw[c]	9750	1.58
whole bean, cooked	6420	0.62
cotyledon, raw	6400	1.08
cotyledon, cooked	3600	0.42
hull, raw	2938	2.88
hull, cooked	2400	2.70

[a] Boiled for 60 min. [b] All values are averages of three determinations. [c] *Chimbu* variety. This batch is different from that of Table I.

Table III. Trypsin Inhibitor Activity of Tannin Extract from Winged Bean, Commercial Tannin, and Soybean Trypsin Inhibitor as Affected by Boiling[a]

sample	TIU/mg of sample[b]
tannin (commercial), unboiled	204
tannin (commercial), boiled	218
tannin extract from winged bean, unboiled	188
tannin extract from winged bean, boiled	185
soybean trypsin inhibitor, unboiled	338
soybean trypsin inhibitor, boiled	0

[a] Boiled for 60 min at 100 °C. [b] All values are averages of three determinations.

The TIA and tannin contents of whole bean, hull, and cotyledon and the effects of cooking on them are shown in Table II. Tannin was highest in the raw hull, almost three times that of raw cotyledon and twice that of raw whole bean. These values are lower than those reported by Elias et al. (1979) in colored seed coats of *Phaseolus vulgaris*. Cooking had the least effect on tannin content and TIA of the hull, suggesting that the heat-resistant trypsin inhibitor in the hull was due to tannin. Tannin content of the whole bean and cotyledon were reduced by approximately 60%, while that in the hull was reduced by only 6%, suggesting that most of the tannin found in the cooking broth came from the cotyledon. Similarly, cooking reduced TIA in the whole bean by 34%, in the cotyledon by 44%, and approximately 16% in the hull.

Considering that the bean is approximately 12% hull and 88% cotyledon, the hull contributed about 50% of the total tannin in the cooked bean. Since the TIA of the hull was not significantly decreased and the tannin was not removed by the cooking conditions employed here, the hull contributed significantly to the total TIA activity in the cooked whole bean.

To evaluate further the role of tannin in TIA, tannin extract from winged bean, commercially available tannin (Nutritional Biochemical Corp., Cleveland, OH) and soybean trypsin inhibitor (Sigma Corp., St. Louis, MO) were assayed for TIA. The specific TIA of commercial tannin and the tannin extract from winged bean were almost the same and were about 30% lower than the soybean trypsin inhibitor (Table III). The TIA in the two tannin samples were not affected at all by boiling for 60 min while that

Table IV. Effects of Salt and Acid Soak Treatments before Cooking on the Distribution of Tannin in Cooked Winged Bean and Broth

	treatment						
tannin content[a]	none	water	NaOH (1 N)	$NaHCO_3$ (0.01 N)	$CaCO_3$ (0.1 N)	HAc (0.6 N)	ash solution (10%)
% of the original in cooked bean	70	56	33	46	50	53	50
% of the original in broth	29	34	65	49	49	52	63

[a] Values are averages of two experiments.

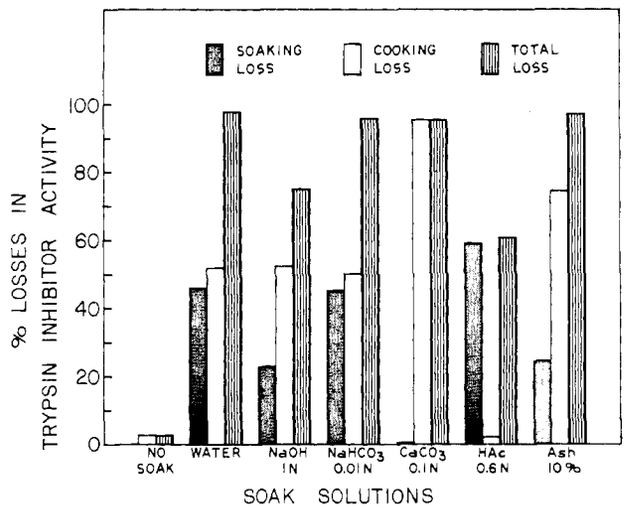

Figure 2. Effects of soaking and cooking on trypsin inhibitor of winged bean.

of the soybean trypsin inhibitor was completely inactivated. This was further evidence that the TIA of tannin was heat resistant in contrast to that of the soybean tryspin inhibitor which is proteinaceous in nature (Jirgensons, 1966).

Soaking of beans before cooking is common practice in developing countries to soften the texture and hasten the cooking process. Soaking beans in a salt solution formulated by Rockland et al. (1967) resulted in a considerable reduction in cooking time. Aqueous alkalies had been used to reduce the amount of tannin in sorghum grain (Price et al., 1979). We therefore examined the effects of soaking winged bean in various salt solutins on TIA and tannin.

The data shown in Figure 2 were obtained from samples soaked for 6 h. The same trend was observed from samples soaked for 24 h. TIA was assayed in the cooked bean and the broth. Total losses in TIA (loss due to soaking in salt solution and cooking in water for 60 min) were at least 95% in water, 0.01 N $NaHCO_3$, 0.1 N $CaCO_3$, and 10% ash solution. Total losses with 1 N NaOH and 0.6 N HAc were 75 and 61%, respectively, with all of the remaining TIA in the cooked bean and none in the cooking broth. Losses due to soaking alone were calculated from the TIA in the raw soaked bean and the soaking solution. Soaking losses varied among the treatments with the highest being 95% with 0.1 N $CaCO_3$ and the lowest 23% with 1 N NaOH and 10% ash solution. It is interesting to note that 0.6 N HAc prevented any significant inactivation during cooking which accounted for about 2% of the total loss. Cooking unsoaked beans resulted only in a total loss of 4%. The remaining TIA was distributed 66% in the cooked bean and 30% in the cooking broth. In contrast, the TIA in the cooking broths were 1% or less of the original in all the soak treatments.

The heat resistance of TIA in the HAc-soaked beans is unusual. The low pH (2.8) of the soaking solution might have denatured proteins in the periphery of the seed and formed a heat barrier in addition to the seed coat. This might have prevented further denaturation of proteins in the interior of the cotyledon. It is worth noting also that water was just as effective as the other salts in reducing TIA. However, salts are known to cause favorable texture changes which were not evaluated in these experiments. Since ash is an inexpensive source of alkali and other salts in developing countries, its effects on texture and flavor, mineral content, and vitamins should be evaluated.

It should also be noted that without soaking, more than half of the original TIA remained in the cotyledon and the TIA in the broth was still active after cooking for 60 min. The importance of soaking in reducing TIA was clearly demonstrated in these experiments.

Since the TIA of tannin was demonstrated to be heat resistant, it was important to determine the effects of the treatments on the distribution of the tannin between the cooked bean and the broth. The percentages given in Table IV were based on the original tannin content of the raw bean. Recoveries of tannin varied from 90 to 116%. Without soaking, 70% of the original tannin remained in the bean after soaking for 6 h and cooking for 60 min. In general, similar trends were observed after 24 h of soaking. NaOH (1 N) was most effective, reducing the tannin content by 70%. The rest of the treatments decreased the tannin by approximately 50%. The remaining tannin in the cooked bean with an average total value of 115 TIU per gram of bean (0.62 mg/g × 185 TIU/mg; Tables II and III) could account for the residual 1% of the original TIA which was heat resistant.

The retention of a small amount of TIA in the cooked bean may not be significant nutritionally. Rackis et al. (1975) determined that maximum body weight and protein efficiency ratios (PER) were obtained with rats fed soybean meals in which 80% of the TIA was inactivated and no pancreatic hypertrophy occurred in rats fed soybean flour in which 50–60% of TIA had been destroyed. However, the inclusion of cooking broth may increase the amount of heat-resistant TIA to a nutritionally significant level. For example, Elias et al. (1979) demonstrated that cooked beans supplemented with methionine and fed to rats without the cooking broth had higher protein quality values than sample fed with the broth. The effect was more pronounced with red and black beans (*Phaseolus vulgaris*) than with white coated beans, suggesting that the color of the seed coat was related to the protein quality of the beans. Therefore, the color of the seed coat should be considered in the selection of suitable varieties of winged bean. It is also recommended that the cooking broth not be consumed.

Tannins are not a homogeneous chemical group and are closely related only in consisting largely of natural polyhydric phenols. Goldstein and Swain (1963) suggested that the term "tannin" be reserved for those phenolic compounds whose degree of hydroxylation and molecular size is sufficient to form complexes with proteins and other polymers under suitable conditions of pH and concentration. Van Buren and Robinson (1969) presented evidence

that the interaction of tannin with proteins is affected by the types of tannin and protein, pH, and concentration. Due to the nonspecific nature of this interaction, it is possible that the tannins referred to in this work can inhibit other enzymes in the gastrointestinal tract besides trypsin. This property and its heat resistance indicate that tannins may play a more important role than the heat-labile proteinaceous trypsin inhibitor in heat-processed beans. This can be demonstrated more definitively by animal feeding experiments.

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Interaction of Iron(II) with Lactose

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A change in specific rotation of a lactose–$FeCl_2$ mixture indicated the formation of a soluble lactose·$FeCl_2$ complex. Maximum complex formation occurred within 1 h. Addition of NaOH to solutions containing lactose and $FeCl_2$ precipitated insoluble $Fe(OH)_2$ and lactose. Maximum lactose precipitation occurred at molar ratios $NaOH/FeCl_2$ = 2.0 and $FeCl_2$/lactose = 6.0 with 60% yield. Reaction reached completion in less than 30 min. Concentration of iron remaining in solution was some 15 times greater than expected, indicating the formation of a soluble lactose·$Fe(OH)_2$ complex. The lactose–iron precipitate is a gel consisting of approximately 70% water. The remainder is composed of an insoluble lactose·$Fe(OH)_2$ adduct and insoluble $Fe(OH)_2$. The amount of complex and lactose precipitated is a function of the relative insolubility of the metal hydroxide and the solubility of the adduct.

Complexing of metal ions with lactose has important biological and environmental implications. In the manufacture of cheese, enormous amounts of whey solutions and effluents are produced as byproducts. Lactose comprises about 5% of these whey solutions. Lactose is an excellent substrate for microorganisms. This presents a disposal problem since direct addition of the whey to sewage systems would raise the biological oxygen demand of the system appreciably. Removal of the lactose eliminates much of this problem. Methods for the removal of lactose include reverse osmosis and ultrafiltration (Sammon, 1974; Fenton-May et al., 1972) and precipitation of lactose using alkaline-earth metals (Olano et al., 1977a,b; McCommins et al., 1980; Kwon, 1979). Most researchers believe these precipitation reactions are a consequence of complex formation between lactose and the metal ion (Cerbulis, 1973; Herrington, 1934a,b; Kwon, 1979; McCommins et al., 1980; Olano, 1977a,b).

Most studies with carbohydrates have utilized alkali or alkaline-earth metals for complexation, but a few have been directed to iron–carbohydrate interactions. Charley et al. (1962) used iron(III)–fructose chelates to study penetration and transport of iron across biological membranes. Davis and Deller (1966) also studied the binding of iron(III) to carbohydrates. Cerbulis (1973) used $FeCl_3$ and NaOH alone, and in combination with CaO, to recover proteins and lactose from acid whey. Imamura and Kawamoto (1974) and Imamura et al. (1975) prepared and purified an iron(III)–lactose complex with a molar ratio of 1:1 and molecular weight of 5–10 000.

Very little is known about the reaction of lactose with divalent nonalkaline earth metals. There is still much to be learned about the precipitation process itself and the chemistry of lactose complexation. This study, utilizing iron(II), is an attempt to fill some of these informational gaps. The specific objectives of this study were to inves-

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