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Thermal Stability of Trypsin Inhibitor Activity in Winged Bean (*Psophocarpus tetragonolobus*)

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The thermal stability of trypsin inhibitor activity (TIA) in six varieties of winged beans was investigated. The TIA in winged bean meals was extremely resistant to dry heat treatment. Prolonged cooking (45–60 min) of the whole bean was required to reduce the TIA substantially. Autoclave treatment at 120 °C, 1.05 kg/cm^2, is, however, very effective in destroying TIA in the beans or bean meals. There are significant varietal variations in the effectiveness of autoclave inactivation of winged bean TIA. The TIA in the winged bean meal extracts is, however, heat labile.

The winged bean (*Psophocarpus tetragonolobus*) is indigenous to the humid tropics. Its exceptional agronomic and nutritional peculiarities have been well documented (National Academy of Sciences, 1975; Masfield, 1973; Claydon, 1975). All parts of the plant can be consumed. The bean protein content ranges from 28.8% to 42.5%, with 13.6% to 21.4% oil (Masfield, 1973; Khor et al., 1980). Analysis of the bean protein indicated a more desirable amino acid composition than for most legumes. Raw winged beans are toxic to rats and have a low digestibility (Jaffe and Korte, 1976). Cerny et al. (1971) reported that the nutritive quality of winged bean protein, as assessed by the PER and NPU values, compared well with that of soybean. Other trial experiments have also confirmed the promising properties of the winged bean as livestock feeds (Wong, 1975).

Early studies on the winged bean have indicated the presence of protease inhibitor activity (Sohonie and Bhandarkar, 1954). Several trypsin inhibitors and a chymotrypsin inhibitor have been isolated and purified from the winged bean (Kortt, 1979, 1980; Tan el al., 1979; Chan and de Lumen, 1982a). Trypsin inhibitor activity of the winged beans ranges from 18 500 to 41 000 IU/g of bean (Khor et al., 1980).

The nutritional significance of plant trypsin inhibitors has been extensively investigated (Liener and Kakade, 1980). High levels of trypsin inhibitor activity (TIA) stimulate pancreatic juice secretion and cause pancreatic hypertrophy and growth inhibition. The evidence of the antinutritional role of winged bean TIA came from the studies of Chan and de Lumen (1982b). They demonstrated that feeding rats with casein plus isolated winged bean trypsin inhibitors caused pancreatic hypertrophy and growth inhibition.

Cerny et al. (1971) reported that winged bean TIA could be destroyed easily by moist heat treatment. Jaffe and Korte (1976), however, demonstrated that autoclaved winged beans could still induce pancreatic hypertrophy. Presumably, the TIA in the winged bean variety used by the latter authors is particularly heat resistant and that autoclave treatment could not abolish its deleterious effects.

A cosmopolitant collection of winged bean varieties at the Agricultural University of Malaysia afforded the opportunity for evaluation of the varietal differences of winged bean TIA. This paper reports the results of our investigation on the thermal stability of TIA in six varieties of winged beans grown on the experimental farm at the Agricultural University of Malaysia.

MATERIALS AND METHODS

Materials. Winged beans of varieties 207, 046, 185, 100, 141, and 095 were grown locally at the experimental farm of Agricultural University of Malaysia. Soybeans were obtained from commercial outlets in Kuala Lumpur. Winged bean meals were prepared by grounding the mature beans manually with a pestle and mortar, and the fine powder was stored below 0 °C in a glass container before use. Bovine trypsin and *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) were purchased from Sigma Chemicals. *p*-Nitrophenyl *p*-guanidinobenzoate hydrochloride was from Merck. All other chemicals are of analytical reagent grade and were purchased from Sigma Chemicls and Merck.

Determination of the Purity of Trypsin. The purity of the commercial trypsin was determined by the active-site titration method of Chase and Shaw (1967), using *p*-nitrophenyl *p*-guanidinobenzoate as the titrant. Gen-

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erally, the commercial trypsin is 50–70% pure.

Assay of Trypsin Inhibitor Activity. Trypsin inhibitor activity (TIA) was determined by using a method modified from Kakade et al. (1974). Trypsin inhibitor extract was prepared by extracting 100 mg of finely ground winged bean meal with 5 mL of 0.005 N sodium hydroxide. With raw meal the extraction time was 1 h, whereas 4 h was used for heat-treated samples. The pH of the suspension was usually 9.3–9.5.

The suspension was diluted to the point where 0.5 mL produces 40–60% trypsin inhibition. Portions (0, 0.3, 0.5, 0.7, and 0.9 mL) of appropriately diluted winged bean meal suspension were pipetted into test tubes and adjusted to 2.0 mL with distilled water. Two milliliters of trypsin solution (0.01 mg/mL of 0.05 M Tris buffer, pH 8.2, containing 0.02 M $CaCl_2$) was added to each test tube and incubated at 37 °C. To each tube, 100 μL of BAPNA solution (20 mg of BAPNA/mL of dimethylformide) was added, followed by thorough mixing. Exactly 10 min later the reaction was terminated by adding 1 mL of 30% acetic acid. The mixture was then centrifuged at 10000*g* for 10 min, and the absorbance of the supernatant was measured at 410 nm against a reagent blank. The reagent blank was prepared by adding 1 mL of 30% acetic acid to a test tube containing trypsin and water (2 mL each) before the 100 μL of BAPNA solution was added. A sample blank is prepared by adding 100 μL of BAPNA solution to the diluted sample extract and water (1 mL each), incubating the mixture at 37 °C for 10 min, and then adding 1 mL of 30% acetic acid followed by the addition of 2 mL of trypsin. The blank readings for the intermediate concentrations of the sample can be estimated by simple mathematical calculations.

TIA is estimated from the residual tryptic activity of the mixture of diluted bean extract and trypsin and is expressed in terms of inhibitor unit (IU) per gram of sample. One inhibitor unit of trypsin inhibitor is defined as the amount of inhibitor that inhibits 1 μg of pure trypsin. Under the conditions used, at 410 nm, 16.1 μg of pure trypsin in 5.1 mL of solution gave a net absorbance increase of 1.0 unit/10 min.

Determination of the Thermal Stability of Trypsin Inhibitor Activity in Winged Bean Meals. The thermal stability of the TIA in winged bean meals was determined by assaying the residual TIA in the heat-treated winged bean meals. The heat-treated winged bean meals were prepared as follows.

Preparation of the Dry Heat-Treated Winged Bean Meals. Dry heat-treated winged bean meals were prepared by heating 100 mg of the finely ground winged bean meals in an oven maintained at 100 ± 1 °C for 2 h.

Preparation of the Autoclaved Winged Bean Meals. The autoclaved winged bean meals were prepared by autoclaving 100 mg of the finely ground bean meals in a test tube at a thickness not exceeding 2 mm at 120 °C, 1.05 kg/cm^2, for 5, 10, 15, or 20 min, after the desired temperature had been reached. The autoclave oven was preheated before use to minimize the time taken to reach the desired temperature (approximately 7 min).

Determination of the Thermal Stability of Trypsin Inhibitor Activity in Whole Winged Beans. Whole winged beans were cooked or autoclaved and ground manually to yield the winged bean meals. Thermal stability of the TIA in the whole winged beans was determined by analyzing the residual TIA in the bean meals prepared.

Preparation of the Cooked Winged Beans. Whole winged beans were cooked in distilled water at boiling temperature for 5, 10, 15, 20, 45, or 60 min, drained off their cooking broths, and dried in an oven at a temperature of 50 °C for 2 h. Appropriate weight corrections were made in the calculation of TIA inactivation to take into account the increase in moisture content of the cooked beans.

Preparation of the Autoclaved Whole Winged Beans. Autoclaved whole winged beans were prepared by autoclaving approximately 2 g of whole winged beans in a shallow pan at 120 °C, 1.05 kg/cm^2, for 5, 10, 15, or 20 min, after the desired temperature had been reached.

Thermal Stability of the Trypsin Inhibitor Activity in Winged Bean Meal Extracts. Thermal stability of the TIA in winged bean meal extracts was determined by assaying the TIA in the boiled winged bean meal extracts. For preparation of boiled winged bean meal extract, 3 mL of boiling distilled water was added to 100 mg of winged bean meal in a test tube. The suspension was mixed, incubated in a boiling water bath for 5 min, and cooled rapidly to room temperature by an ice bath. Two milliliters of 0.015 N sodium hydroxide was added to the suspension and stirred for 4 h. TIA in the appropriately diluted suspension was then determined as described above.

Thermal Stability of the Trypsin Inhibitor Activity in Soybean. For comparison, the effects of various types of heat treatments on TIA in soybean were examined by using the methods described above.

Table I. Effect of Dry Heat Treatment on Trypsin Inhibitor Activity in Winged Bean Meals

winged bean variety	trypsin inhibitor act.,[a] IU (g of sample)$^{-1}$	
	untreated (raw sample)	120 °C, 2-h treatment
207	15 200 ± 900	15 000 ± 1100
046	27 000 ± 1800	27 100 ± 1900
185	32 100 ± 2900	31 700 ± 3000
100	24 300 ± 2100	24 500 ± 1800
141	16 500 ± 1300	16 600 ± 1400
095	32 400 ± 3500	32 300 ± 3100
soybean	18 900 ± 800	18 100 ± 1200

[a] Mean ± standard deviation; $n = 4$.

RESULTS

Thermal Stability of Trypsin Inhibitor Activity in Winged Bean Meals. Dry heat at 100 °C for 2 h inactivated less than 5% of the TIA in winged bean meals (Table I). Autoclave treatment, on the other hand, is much more effective in destroying the TIA in the meals. Data in Table II show that the effects of autoclave treatments on TIA in the winged bean meals of variety 207 are very similar to that on the TIA in soybean meals. Twenty minutes of autoclave treatment at 120 °C, 1.05 kg/cm^2, of winged bean meals generally destroyed more than 80% of the TIA. The data also show significant variability in the effectiveness of autoclave treatment in destroying the TIA in different varieties of winged bean meals. Thus, whereas 5-min autoclave treatment of winged bean meals of varieties 046, 185, 100, and 095 destroyed only approximately 20% of the TIA, the same treatment on winged bean meals of varieties 141 and 207 destroyed 46.1% and 58.6% of the TIA, respectively. Table II also shows that the residual TIA in the winged bean meals of the six varieties of beans that have been subjected to the same autoclave treatment differs significantly.

The effects of dry heat treatment and autoclaving on the TIA in soybean meals are also shown in Tables I and II for comparison. There was no significant loss of TIA in soybean meal after 2 h of 100 °C heating. On the other

Table II. Effects of Autoclave Treatments on Trypsin Inhibitor Activity in Winged Bean Meals

	inactivation of TIA by autoclave treatments[a]							
	5-min autoclave		10-min autoclave		15-min autoclave		20-min autoclave	
winged bean variety	% destroyed	residual TIA[b]	% destroyed	residual TIA	% destroyed	residual TIA	% destroyed	residual TIA
207	58.6 ± 1.9	6300	71.7 ± 2.6	4300	86.2 ± 4.7	2100	91.5 ± 3.5	1300
046	20.0 ± 9.5	21600	63.3 ± 9.1	9900	71.5 ± 9.8	7700	85.2 ± 7.9	4000
185	17.5 ± 8.0	26500	62.3 ± 9.8	12100	71.3 ± 9.6	9200	84.4 ± 2.1	5000
100	18.1 ± 8.3	19900	52.7 ± 4.4	11500	55.6 ± 5.5	10800	82.3 ± 7.6	4300
141	46.1 ± 7.4	8900	63.6 ± 8.7	6000	81.8 ± 1.4	3000	87.9 ± 4.2	2000
095	24.4 ± 7.3	24500	75.3 ± 3.5	8000	76.9 ± 5.3	7500	95.1 ± 2.1	1600
soybean	68.3 ± 5.3	6000	80.4 ± 6.3	3700	90.1 ± 1.0	1900	94.7 ± 1.1	1000

[a] Mean ± standard deviation; $n = 5$. [b] In IU per gram of meal.

Table III. Effects of Heat Treatments on Trypsin Inhibitor Activity in Whole Winged Beans (Variety 207)

duration of treatment, min	% inactivation of TIA by cooking[a]	% inactivation of TIA by autoclaving[a]
5	17 ± 3	60 ± 6
10	17 ± 3	73 ± 6
15	22 ± 2	90 ± 8
20	35 ± 3	92 ± 9
45	70 ± 5	n.d.[b]
60	86 ± 4	n.d.

[a] Mean ± standard deviation; $n = 3$. [b] n.d. = not determined.

Table IV. Thermal Stability of Trypsin Inhibitor Activity in Winged Bean Meal Extracts

winged bean variety	% inactivation of TIA in extract by 5-min boiling[a]
207	83 ± 5
046	76 ± 6
185	83 ± 5
100	85 ± 7
141	77 ± 6
095	83 ± 5
soybean	10 ± 2

[a] Mean ± standard deviation; $n = 3$.

hand, 5–10 min of autoclaving was sufficient to reduce the TIA substantially.

Thermal Stability of the Trypsin Inhibitor Activity in Whole Winged Beans (Variety 207). The effects of cooking on the TIA in whole winged beans (variety 207) are shown in Table III. Cooking the beans for 5 to 15 min reduced the TIA in the whole beans by only 17–22%. Prolonged cooking (45–60 min) was required to reduce the TIA substantially.

The effects of autoclaving (120 °C, 1.05 kg/cm^2) on the TIA in the whole winged beans are also shown in Table III. There is no significant difference in the effects of autoclaving on TIA in the whole winged beans and the winged bean meals.

Thermal Stabilty of the Trypsin Inhibitor Activity in Winged Bean Meal Extracts. Table IV shows that boiling the winged bean meal extracts for 5 min reduced the TIA substantially (76–85%). On the other hand, boiling the soybean meal extract destroyed less than 10% of the TIA.

DISCUSSION

Determination of Trypsin Inhibitor Activity in Winged Bean Meals. Kakade et al. (1974) reported a procedure for measurement of TIA in soybean products, using an uncentrifuged extract, the trypsin substrate BAPNA, and an extraction pH of 8.4–10.0. The method was reported to be particularly useful in the evaluation of heat destruction of TIA in soybean samples.

Various methods of assaying winged bean TIA have been reported (Kortt, 1979; Tan et al., 1979; Chan and de Lumen, 1982a). The method described here is a slight modification of the procedure reported by Kakade et al. (1974). The modification include the use of 0.005 N sodium hydroxide as the extraction medium. With this extraction medium, the pH of the suspension was usually 9.5–9.8. At this pH region, maximum amount of TIA was extracted. Dimethylformide, rather than dimethyl sulfoxide, was used as the solvent for BAPNA. The BAPNA stock solution prepared this way is stable for 1 week. Uncentrifuged extract was used for TIA assay. There was, however, no significant difference in the TIA in uncentrifuged and centrifuged extracts. There is a 2-fold variation of TIA in the six varieties of winged beans examined. TIA in the soybean meal sample determined by the same method is comparable to that in winged bean meals.

Thermal Stability of Trypsin Inhibitor Activity in Winged Bean Meals and Whole Winged Beans. TIA in the winged bean meals is extremely resistant to dry heat treatment. Prolonged cooking is required to reduce the TIA in whole winged beans substantially. Autoclave treatment, on the other hand, is very effective in destroying the TIA in winged bean meals or whole winged beans.

In contrast, the TIA in the winged bean meal extracts is relatively heat labile. Boiling the winged bean meal extracts for 5 min generally destroyed more than 75% of the TIA in the extracts. This observation is in agreement with the finding of Chan and de Lumen (1982a), who reported that heating the isolated winged bean trypsin inhibitors for 7 min at 100 °C destroyed 60% of the TIA. Thus, the extracted winged bean trypsin inhibitors are less resistant to heat as compared to other plant proteinase inhibitors that remain essentially unaltered after heating at 80–100 °C for 10 min (Richardson, 1981). The difficulties in destroying the TIA in winged bean meals or whole winged beans by dry heat treatment or cooking may be due to the inefficiency of heat penetration through the bean meals or beans.

Thus, winged beans have an obvious advantage when the bean proteins are used in the preparation of protein isolates and concentrates because of TIA in the winged bean meal extracts can be easily destroyed by simple heat treatment.

Nutritional Significance of the Thermal Stability of Trypsin Inhibitor Activity in Winged Beans and Winged Bean Meals. It is well established that trypsin inhibitors cause pancreatic hypertrophy and are responsible for 30–60% of the growth inhibition in soybean. Rackis et al. (1975) found that 70–80% of the TIA in soybean needed to be destroyed in order to achieve max-

imal gains in weight and in PER's with rats. No pancreatic hypertrophy occurred in rats fed soy flour in which 55–69% of the TIA had been destroyed.

Although Chan and de Lumen (1982b) have demonstrated by rat feeding studies that winged bean trypsin inhibitors caused pancreatic hypertrophy and growth inhibition, there was, however, uncertainty regarding the biological threshold level of TIA at which these biological effects occurred. In this connection, it is important to note that there is signifciant varietal differences in the effectiveness of autoclave inactivation of TIA and the level of TIA in the winged beans. Thus, even though 20-min autoclave treatment generally destroyed 80% of the TIA in the winged bean meals, the residual TIA in the six varieties of winged bean meals after this treatment differs significantly, ranging from 5000 to 1300 IU (g of seed)$^{-1}$.

It is also interesting to note that the residual TIA in the winged bean meals of varieties 046, 185, 100, and 095 after 5 min of autoclave treatment is higher than the TIA of untreated varieties 141 and 207. On the other hand, after 5 min of autoclave treatment, TIA in winged bean meals of varieties 141 and 207 was substantially reduced. Thus whereas 5-min autoclaving is almost certainly inadequate to abolish the deleterious effect of TIA in winged bean meals of varieties 046, 185, 100, and 095, the same treatment may be sufficient for varieties 141 and 207.

Even though prolonged heat treatment could destroy virtually all the winged bean meal TIA, excessive heat treatment could cause functional as well as nutritional damage to protein. The breeding of varieties of winged beans with thermolabile TIA should therefore offer a more satisfactory solution to eliminate the uncertainties entailed in the heat processing of the winged beans.

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Evaluation of the Bleidner Technique for Analysis of Soil-Bound 3,4-Dichloroaniline Residues

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By radiochemical and conventional analysis, the effectiveness of the Bleidner distillation process for recovery of the herbicide residue 3,4-dichloroaniline (DCA) from its humic complexes was evaluated. An increase of alkali concentration to 12.5 N and the extension of the distillation period to 23 h attained the quantitative recovery of DCA from its freshly formed humic complexes. However, during 99 days of incubation in soil, DCA recovery efficiency declined to less than 70%. From a field soil treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) at the rate of 1.76 kg ha^{-1} year^{-1} for the past 10 consecutive years, Bleidner distillation recovered 1 ppm of DCA, virtually all from humic complexes. The mineralization rate of bound DCA and the decline kinetics of DCA recovery both lead to the conclusion that the total bound DCA accumulation in the analyzed soil did not exceed 2.5 ppm and that in soils of the examined type the accumulation of bound DCA residues does not constitute a problem.

3,4-Dichloroaniline (DCA) is the major biodegradation product of several phenylcarbamate (Herrett, 1969), phenylurea (Geissbühler, 1969), and acylanilide (Bartha and Pramer, 1970) herbicides. At recommended treatment levels, 80–90% of the DCA released from herbicides in soil becomes solvent inextractable (Chisaka and Kearney, 1970) primarily by covalent binding to soil organic matter (Bartha, 1971; Hsu and Bartha, 1974). Humus-bound DCA appears to be a source of low-level crop contamination (Still and Mansager, 1969; Still et al., 1980) and gives rise to concern that xenobiotic residues might accumulate in humus (Bartha, 1981). A direct analytical technique for humus-bound DCA residues is clearly needed to resolve the regulatory and environmental concerns asssociated with humus-bound residues. Unfortunately, alkaline or

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