approximately 10 min (Richardson, 1981).

Amino Acid Composition. Since the main purpose of the TI purification is to determine its biological activity in rats, the amino acid composition was determined, taking into consideration that the fraction contains eight protein components. Consistent with the amino acid profile of other trypsin inhibitors, WBTI has high levels of acidic and basic amino acids, proline and serine, with a low level of methionine (Table I). When the amino acid profiles of the TI fraction and the raw winged bean are compared, it is interesting to note that the level of lysine and methionine is higher than that of the raw winged bean. With proper heat treatment, the TI can be a nutritional advantage by supplying additional methionine. However, this nutritional advantage can only have significance if the TIs are present in sufficient amounts to compensate for low methionine values in other protein fractions.

ACKNOWLEDGMENT

We thank Professor R. David Cole of the Biochemistry Department and staff, University of California, Berkeley, for doing the amino acid analysis for us.

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Received for review March 16, 1981. Revised manuscript recevied September 16, 1981. Accepted October 20, 1981.

Biological Effects of Isolated Trypsin Inhibitor from Winged Bean (*Psophocarpus tetragonolobus*) on Rats

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By use of affinity column chromatography, sufficient quantities of trypsin inhibitor were isolated from winged bean for rat feeding. Electrophoresis of the isolated trypsin inhibitor and chromatic staining for proteinase inhibition showed that all the eight protein bands possess trypsin inhibitor activity while two of the bands also possess chymotrypsin inhibitor activity. A 28-day feeding study was conducted to examine and compare the effects of feeding raw winged bean (RWB), autoclaved winged bean (AWB), and casein plus isolated winged bean trypsin (WBTI) inhibitor on the protein efficiency ratio (PER), growth rate, and pancreatic, liver, and spleen weights of rats. The RWB diet, along with causing spleen and liver atrophy, was toxic to rats, causing deaths after 12 days of feeding. The AWB diet was not toxic but growth was inhibited. The WBTI diet caused pancreatic and spleen hypertrophy and slight growth inhibition. It is concluded that TI in winged bean was not primarily responsible for the toxicity of raw winged bean.

In 1974, after examining 400 neglected tropical plants, the National Academy of Sciences selected the winged bean (*Psophorcarpus tetragonolobus*) along with 35 other plants as a potential food source which warrants further research (National Academy of Sciences, 1975). The winged bean is indigenous to the tropics, chiefly in Southern Asia and the Western Pacific. All parts of the plant are edible with the pods as the main item of consumption. As in the soybean, the main nutritional advantages of the winged bean lie in the mature seeds, which contain protein and oil level equivalent to that of soybeans. Consequently, the nutritional and functional benefits offered by soybeans can be expected to be offered by winged beans as well.

Currently the seeds are rarely consumed, and their high protein and oil contents are underutilized. But, before full implementation of the seeds can occur, more must be learned about their antinutritional effects.

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0021-8561/82/1430-0046$01.25/0

Under comparable experimental conditions, the antinutritional effects of raw winged beans such as growth inhibition, pancreatic hypertrophy, and ultimately death are more deleterious than those of raw soybean (Jaffé and Vega, 1968). In rats, raw soybeans cause low PER while raw winged beans cause death within a few days. Like that of soybeans, the majority of antinutritional factors present in raw winged beans are heat labile. When raw winged beans are autoclaved, the lethal effects are eliminated, but the capacity to inhibit growth remains. The growth inhibition and pancreatic hypertrophy which developed with the consumption of raw winged beans are symptomatic of trypsin inhibitor intoxication (Jaffé and Korte, 1976).

It is well established that trypsin inhibitors (TI) cause pancreatic hypertrophy, but whether or not it also causes growth inhibition is still debatable. This controversy probably arises because, for most of the feeding studies, crude or partially purified preparations of TI were used. Rackis et al. (1962) noted that the purity of some crystalline preparations of soybean TI is only 50%. One study which fed highly purified soybean TI to rats did show that TI was responsible for 100% of the pancreatic hypertrophy and 30–60% of the growth inhibition (Rackis, 1965). In another study, soybean TI was specifically removed from the water extract of raw soybeans by affinity chromatography. When this "TI-free" extract was fed to rat, it was estimated that 40% of the pancreatic hypertrophy and growth inhibition observed in raw soybeans could be accounted for by TI (Kakade et al., 1973).

The degree to which trypsin inhibitors are responsible for the deleterious effects of raw winged bean has not been elucidated. This study attempts to investigate the biological role of trypsin inhibitors in the overall toxic effects observed in raw winged beans by (1) isolating winged bean trypsin inhibitor via affinity chromatography, (2) confirming the inhibitor activity of the components of the isolated fraction, and (3) feeding the fraction to rats under controlled conditions.

MATERIALS AND METHODS

The methods described below for the extraction and isolation of winged bean trypsin inhibitor using affinity column chromatography are those used by Chan and de Lumen (1982). They have been scaled up about 10-fold to obtain sufficient quantities of trypsin inhibitor for the rat feeding experiments.

Extraction of Trypsin Inhibitor. One-thousand grams of winged beans (Chimbu variety) was ground via a Pin-Mill grinder and passed through a 30-mesh screen. The bean powder was defatted by adding 5 L of hexane, stirring the mixture for 30 min, and filtering the suspension through a Whatman No. 1 filter paper. Complete defatting of the powder was achieved after the fifth wash. The inhibitor was extracted by homogenizing the defatted bean powder in cold distilled water (5 mL of water/g of bean powder) for 5 min with a Waring blender. The suspension was stirred overnight with a magnetic stirrer at 4 °C and then centrifuged at 16000*g* for 30 min. The supernatant was collected and the residue was washed twice with 3 L of distilled water and recentrifuged at 16000*g* for 30 min. With further washing of the residue, only negligible amount of inhibitor was extracted. The accumulated supernatant was then filtered through a Whatman No. 1 filter paper and lyophilized.

Preparation of the Affinity Column. Activation of the agarose was done as described by March et al. (1974). Two liters of agarose (Sepharose 4B) was washed with 8 L of distilled water (DW). Four-hundred milliliters of cold DW and 4 L of cold 2 M Na_2CO_3 were added to the agarose. The mixture was cooled to 5 °C with occasional stirring. One-hundred milliliters of an acetonitrile solution of cyanogen bromide (2 g of cyanogen bromide/mL of acetonitrile) was added to the mixture, stirred vigorously for 2 min, transferred to a sintered glass funnel, and washed successively with 20 L of cold 0.2 M Na_2CO_3, pH 9.0, 28 L of cold DW, and 25 L of cold borate buffer (0.02 M H_3BO_3, 0.1 M $CaCl_2$, pH 9.0).

The procedure to immobilize trypsin on activated agarose was adapted from Loeffler and Pierce (1973). The activated agarose was suspended in 4 L of borate buffer, pH 9.0. A solution containing 40 g of trypsin in 1.6 L of borate buffer was added to the agarose and stirred gently with an overhead stirrer for 20 h at 40 °C. Free trypsin was removed from the trypsin-bound gel by washing with borate buffer, pH 4.0, until the filtrate's absorption at 280 nm was negligible. Free trypsin in the combined filtrate was quantitatively measured by using 280-nm absorption and the TAME assay. The amount of trypsin bound was calculated by the difference between the amount of free trypsin before and that after immobilization.

After complete removal of free trypsin, 8 L of 1 M glycine was added and stirred for 4 h at room temperature to mask the unreacted sites of the agarose. A final washing was done by using 0.1 M $CaCl_2$, pH 3.8, and the mixture was stored at 4 °C in the same solution with 2% sodium azide as a preservative.

Isolation of Trypsin Inhibitor. A 126 × 5 cm glass column was packed with 2 L of trypsin-bound agarose. The gel was first washed with 1 column volume of 0.2 M KCl, pH 2.0, followed by 2 column volumes of Tris buffer (0.05 M Tris, 0.1 M KCl, 0.02 M $CaCl_2$, pH 8.0) before the application of the bean extract.

A 9% bean extract was prepared by dissolving the lyophilized bean sample in Tris buffer, pH 8.0. After centrifugation at 16000*g* for 15 min, the supernatant containing total TIU, less than the trypsin units in the column, was applied to the column. Tris buffer, pH 8.0, was the eluting solvent used to wash out non-trypsin-inhibitor compounds. The flow rate of the column was 2 mL/min. Low 280-nm absorption of the fractions was the indicator when complete removal of non-trypsin-inhibitor compounds was achieved. At this point, in order to dissociate the trypsin–trypsin inhibitor complex, the eluting solvent was changed to 0.2 M KCl, pH 2.0, adjusted with concentrated HCl. When the 280-nm absorption of the fractions was negligible, the chromatographic run was terminated. The column was then washed with 0.1 M $CaCl_2$, pH 3.8, and stored at 4 °C. The inhibitor fraction was dialyzed for 4 days in distilled water at 4 °C and then lyophilized.

Trypsin Inhibitor Assay. Trypsin inhibitory activity was measured, according to "Worthington Enzyme Manual" (1972), by the decrease in the rate in which trypsin hydrolyzed *p*-toluenesulfonyl-L-arginine methyl ester (TAME), as monitored at 247 nm with a Cary 14 recording spectrophotometer. One unit of trypsin activity represents the hydrolysis of 1 μmol of TAME/min at 25 °C and pH 8.1 in the presence of 0.01 M calcium ion. One unit of trypsin activity inhibited is defined as one trypsin inhibitory unit (TIU).

Disc Gel Electrophoresis. The Ornstein (1964) and Davis (1964) procedure for disc gel electrophoresis was used. The acrylamide concentration of the gels was 7.5%. The lyophilized trypsin inhibitor was dissolved in 1:10 dilution of the stock Tris–glycine buffer, pH 8.3, to yield a final concentration of 1 mg/mL. The electrophoretic system was run at 4 °C for 4.5 h with a constant current

Table I. Composition of Diets

ingredients	g/100 g of diet			
	casein	RWB[f]	AWB[f]	casein + TI (WBTI)[g]
protein[a]	12.0	28.0	30.0	12.0
corn oil[b]	5.0	1.0	1.0	5.0
vitamin premixture[c] (2 × NRC)	1.0	1.0	1.0	1.0
mineral mixture[d]	4.0	4.0	4.0	4.0
choline bitartrate mixture[e]	1.0	1.0	1.0	1.0
DL-methionine	0.3	0.3	0.3	0.3
powdered sucrose	75.7	65.3	63.3	75.7

[a] Vitamin-free casein or winged bean, Chimbu variety. [b] Mazola corn oil. [c] The mixture provides the following (mg/kg of diet): thiamin hydrochloride, 2.5; riboflavin, 5.0; pyridoxine hydrochloride, 14; niacin, 30; calcium pantothenate, 16; vitamin B-12 (0.1% triturate in mannitol), 10; folacin, 4; biotin, 0.6; menadione, 1; vitamin A palmitate (250 000 IU/g), 16; vitamin D_2 (500 000 IU/g), 4; *dl*-α-tocopherol (250 IU/g), 308. [d] UCB-1Rb (Cohen et al., 1967) provides the following (in mg/100 g of diet): $CaCO_3$, 827; $CaHPO_4$, 1288; Na_2HPO_4, 742; KCl, 815; $MgSO_4$, 262; $MnSO_4 \cdot H_2O$, 18; $CuSO_4$, 1.48; ferric citrate, 34; $ZnCO_3$, 2.6; KIO_3, 0.11. This supplied, at 4.0% of the diet (in mg of each element per 100 g of diet), the following: Ca, 711; P, 456; Na, 240; K, 427; Cl, 381; Mg, 52; Mn, 5.7; Cu, 0.59; Fe, 5.7; Zn, 1.3; I, 0.068; Se, 0.125. [e] The choline bitartrate mixture is composed of 37.1 g of choline bitartrate and 237.9 g of sucrose. [f] Protein and oil contents of the RWB and AWB diets were adjusted according to the following data: RWB, 36.3% protein, 17% oil, and 8% H_2O; AWB, 33.3% protein, 14% oil, and 13% H_2O. [g] Freeze-dried isolated winged bean trypsin inhibitor was added in an amount (0.67 g/100 g of diet) equivalent to the total inhibitor activities of RWB without replacing other diet components.

of 2.7 mA/tube. The gels were stained for protein with 0.25% Coomassie blue R-250 in methanol, acetic acid, and water (5:1:5) for 30 min and then destained with 7% acetic acid and 7% methanol in water.

Chromatic Protease Inhibitor Staining. Staining for trypsin and chymotrypsin inhibitory activity in electrophoretic gels was performed with the method of Uriel and Berges (1968) as modified by Rosenberg et al. (1976). The hydrolysis of acetyl-DL-phenylalanine β-naphthyl ester was done with either a trypsin concentration of 0.01 mg/mL or a chymotrypsin concentration of 0.02 mg/mL. The β-naphthol formed was reacted with *o*-dianisidine, producing a colored product. Destaining was done for 30 min with 2% acetic acid.

Feeding Study. The rats were fed for 28 days on either casein, raw winged bean (RWB), autoclaved winged bean (AWB), or casein plus winged bean trypsin inhibitor (WBTI) as the sole protein source. The casein diet served as the control and contained no TIA. The RWB diet contained 3080 trypsin inhibitory units/g (TIU/g) of diet. The WBTI diet consisted of adding isolated WBTI to casein at a TIU/g of diet level equivalent to that of the RWB diet. Since the freeze-dried WBTI was added at a low level (0.67 g/100 g of diet), there was no need to replace other ingredients in the diet. The AWB diet contained a negligible amount of TI. For each diet, a 10% protein level was chosen. Ground winged beans (Chimbu variety) were used. AWB was prepared by autoclaving RWB at 121 °C for 25 min. Table I shows the composition of the different diets fed to the rats. The protein and fat were adjusted to the desired percentage at the expense of sucrose. Six 21-day-old, male Spraque–Dawley rats were randomly assigned to each treatment group. They were

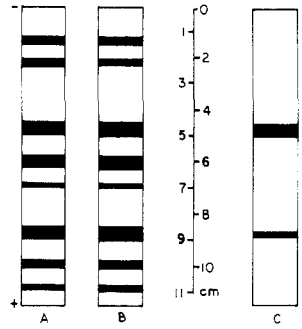

Figure 1. Electrophoresis of WBTI isolated by affinity column chromatography. The acrylamide concentration of the gels was 7.5%. By use of a Tris–glycine buffer, the gel was run at a pH of 8.3 for 4.5 h. A constant current of 2.7 mA was applied per tube. (A) Trypsin inhibitory activity; (B) protein bands; (C) chymotrypsin inhibitory activity. The dark bands in gels A and C actually represent absence of staining.

fed ad libitum and were weighed twice weekly except during the first week when they were weighed every other day. At the end of 28 days, the rats were sacrificed with chloroform. Immediately their pancreas, liver and spleen were excised and weighed. The data were first analyzed with a one-way analysis of variance. If the null hypothesis was rejected, Tukey's Studentized Range Test was used to determine where differences existed.

RESULTS

Extraction, Isolation, and Electrophoresis of WBTI. The water extraction for TI used in these experiments yielded an average of 8800 TIU/g of winged bean from three extractions. These results are similar to what we obtained earlier in a smaller scale extraction (Chan and de Lumen, 1982). The elution pattern of the TI extract when applied to the agarose–trypsin column is identical with that we obtained using a much smaller column, although the elution volumes of the two protein peaks in the present work is about 60-fold that of the smaller scale fractionation. The major protein peak eluted by the first solvent (pH 8.0, 0.5 M Tris, 0.1 M KCl, 0.02 M $CaCl_2$) did not have any TIA. The minor protein peak eluted by the second solvent (0.2 M KCl, pH 2.0) contained the trypsin inhibitor, and the 280-nm absorption and the TIA coincided with one another. Measured by its specific activity, the isolated WBTI was purified 8-fold when compared to the extract before chromatography. Approximately, a total of 8.5×10^6 TIU in 18.5 g of freeze-dried samples were isolated for the feeding study. This represented an average yield of 19.2 g of freeze-dried WBTI/kg of winged bean.

The isolated WBTI exhibited eight protein bands in disc gel electrophoresis (Figure 1). Each of these protein bands showed TIA while two exhibited both TIA and chymotrypsin inhibitory activity (CIA). These results are identical with our previous studies (Chan and de Lumen, 1982) and indicate that all the protein components of the isolated fraction prepared on a large scale possess protease inhibitor activities. Assuming that tryptic cleavage of the active site in the inhibitor would destroy activity, the fact that all of the proteins possess activity after passage through the column suggests that there was no enzymatic modification. Furthermore, if cleavage occurred in a peptide bond enclosed by disulfide linkage, reduction would increase the number of protein bands in electrophoresis. It was shown in our previous studies (Chan and de Lumen, 1982) that reduction with mercaptoethanol did not affect the number of protein bands. Therefore, it is unlikely that the inhibitor bands represent enzymatically modified forms.

Table II. Relative Organ Weights, PER, and First Week Feed Consumption of Rats After Consuming the Four Treatment Diets for a Period of 28 Days[a]

	g/100 g of body wt					1st week feed
	liver	spleen	pancreas	wt gained, g	PER	consumed, g
casein control	5.35 ± 0.28[a]	0.277 ± 0.0083[a]	0.493 ± 0.49[a]	133.0 ± 5.84[a]	3.97 ± 0.072[a]	50.2
raw bean (RWB)	2.73 ± 0.06[b]	0.100 ± 0.0081[b]	0.518 ± 0.05[a]	*b*	*b*	22.2
heated bean (AWB)	4.65 ± 0.18[a]	0.230 ± 0.0093[a]	0.533 ± 0.04[a]	92.75 ± 8.15[b]	3.05 ± 0.050[b]	43.0
casein + TI (WBTI)	4.78 ± 0.26[a]	0.338 ± 0.0270[c]	0.740 ± 0.02[b]	115.2 ± 2.40[a]	3.72 ± 0.048[c]	45.7

[a] All the values are expressed as mean ± SEM. All groups not designated with the same letter within the same column are statistically different at $p < 0.05$. [b] Rats died between day 7 and day 12.

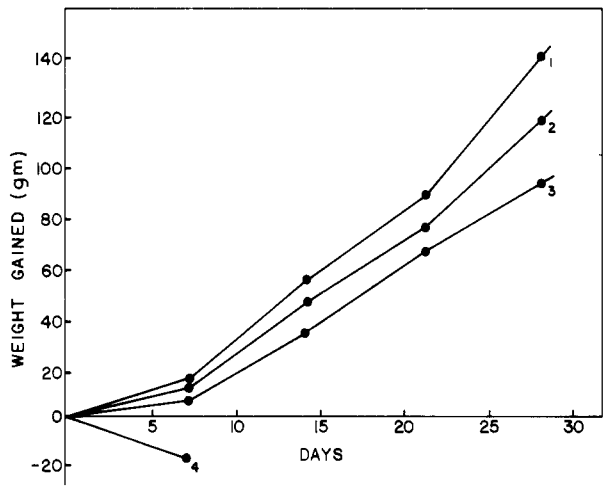

Figure 2. Weight gains of rats on the four treatment groups during the 28-day feeding period. (1) Casein control; (2) casein plus isolated winged bean trypsin inhibitor (WBTI); (3) autoclaved winged bean (AWB); (4) raw winged bean (RWB).

The weekly weight gains of the rats in the different diet groups are shown in Figure 2. At any time point of the experiment, the weight gains were highest in the casein control, followed by WBTI and then AWB. The rats fed RWB died between days 7 and 12.

DISCUSSION

In designing these experiments, we had the choice of adding the isolated WBTI to heat-treated winged bean or to casein. We decided to add it to casein to separate out the effects of the isolated WBTI, apart from any residual antinutritional factor that may be present in the heat-treated bean. Also, this would allow comparison of the WBTI diet with the casein control without the added factor of differences in protein quality and availability.

This study shows that isolated winged bean trypsin inhibitor not only caused pancreatic hypertrophy but also caused growth depression. This agrees with the observations of Rackis (1965) that TI was responsible for 100% of pancreatic hypertrophy and 30–60% of the growth inhibition observed in raw soybean and that of Kakade et al. (1973), who concluded that 40% of the pancreatic hypertrophy and 40% of the growth inhibition was due to TI. As far as we know, this is the only study of its kind where sufficient quantities of trypsin inhibitor were isolated from winged bean by affinity column chromatography, its in vitro activity was confirmed, and it was fed to rats under controlled conditions. From Table II, the WBTI group had significantly higher pancreatic weight and significantly lower PER than the control group. It is interesting to note that the PER of the heated bean group (AWB) was lower than the WBTI group, yet no pancreatic hypertrophy was observed in AWB. One possible explanation is the presence of a heat-stable factor which inhibited growth but did not cause pancreatic hypertrophy. A similar factor had also been observed in the soybean when it was shown that although 95% of the soybean extract's TIA was destroyed with 0.1 N HCl at 55 °C for 18 h, its growth inhibiting capacity was unaffected (Liener, 1951). Another explanation for the lower PER of AWB compared to that of WBTI is a difference in amino acid composition and/or availability. Casein provided the protein in WBTI while that of AWB came from the heated winged bean. In general, plant proteins have lower digestibility and amino acid availability resulting from factors inherent in the seed and the decrease resulting from processing (Bressani and Elias, 1968).

Besides causing pancreatic hypertrophy and growth depression, winged bean trypsin inhibitor also appeared to cause spleen hypertrophy. No other study has investigated or shown this effect of TI, and before any conclusions can be made, further research is required.

Since the lethal effects observed in the raw bean group was not found in the WBTI group, it can be concluded that isolated winged bean trypsin inhibitor was not responsible for the lethal effects of raw winged beans. Increasing evidences appear to indicate that hemagglutinins may cause growth inhibition and, at higher levels, mortality in certain legumes. Hemagglutinins are heat-labile proteins (Liener and Hill, 1953). Pure preparations of hemagglutinins from kidney and black beans caused substantial growth depression, which increased with increasing dosage. At levels greater than 1% of the diet, mortality ensued (Honavar et al., 1962). Furthermore, removal of hemagglutinating activity by the affinity column from kidney bean protein improved the nutritional quality of the bean protein (Pusztai and Palmer, 1977). Strong hemagglutinating activity, but lower than that of kidney beans, had been found in winged bean (Claydon, 1978). The possibility that winged bean hemagglutinins also exert similar deleterious effects as does kidney bean is not remote and merits further investigation.

Along with the liver and spleen astrophy observed in the raw winged bean diet, pancreatic hypertrophy was not apparent, contradicting the findings of Jaffé and Korte (1976). One plausible explanation may be that the excision of organs was not immediate. Because the rats in this treatment died unexpectedly, often in the middle of the night, immediate excision of the organs was not possible. Consequently, dehydration of the organs could taken place, changing the organs' actual weight and masking the hypertrophy of the pancreas. In addition, since the majority of rats died within 7 days of the study, the time needed for significant hypertrophy of the pancreas to occur may be insufficient.

Another effect observed in the raw bean group was the low food intake of the rats. The raw bean group consumed approximately half as much feed as the other groups during the first week of the study. Unpalatability of the raw beans or the presence of a factor which depressed food

intake may account for this observation. In either case, both were destroyed or reduced with heat, as indicated by the increase in food intake in the heated bean group. The presence of a food intake depressing factor was probably the principal cause of the low food intake because any unpleasant taste of the raw beans should have been masked by the sugar, which constituted 75% of the raw bean diet. Inevitably, the depression of food intake by this factor will result in growth depression and untimely death. Liener (1953) and Turner and Liener (1975) concluded that soybean hemagglutinin caused growth depression by reducing food intake. A similar food intake depressing effect of raw winged bean is possible since strong hemagglutinating activity has been found in the winged bean seed.

The main objective of this study is to demonstrate the biological activity of isolted WBTI and determine its relative contribution to the toxicity of raw winged beans on rats. The limitation of this approach is that one cannot observe the combined and possibly synergistic effects of all the antinutritional factors in the raw bean. Such effects can put sufficient stress on the rats, leading to death as opposed to the individual and separate effects of the isolated factors. Within this limitation, we conclude that WBTI was not primarily responsible for the toxicity of raw winged bean but it caused pancreatic and spleen hypertrophy and growth depression.

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Received for review April 23, 1981. Accepted September 28, 1981.

Tocopherols of Winged Bean (*Psophocarpus tetragonolobus*) Oil

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Oil samples extracted from 27 varieties of winged bean seeds were analyzed directly for individual tocopherols after dissolution in the mobile phase by high-performance liquid chromatography (HPLC) and ultraviolet detection at 295 nm. γ-Tocopherol was found to be the dominant form of tocopherol with traces of α-, β-, and δ-tocopherols. The samples showed a low of 8 and a high of 130 mg of γ-tocopherol/100 g of oil while most of the samples fell in the range of 23–44 mg/100 g of oil. Less variation was observed in the oil content of the different varieties which averaged 14.7%. On the basis of published data on fatty acid composition of winged bean oil, the tocopherol to polyunsaturated fatty acid ratio was calculated to be 0.2 mg of *d*-α-tocopherol equiv/g of polyunsaturated fatty acids, a value similar to that of soybean and less than that of a number of vegetable oils. The nutritional and functional significance of the predominance of γ-tocopherol in winged bean oil is discussed.

The winged bean (*Psophocarpus tetragonolobus*) is considered to be a very promising source of protein and oil in the humid tropics where it is native, grows well, and tolerates a wide range of altitudes (National Academy of Sciences, 1975). The chemical composition of the mature seeds is very similar to that of soybean and therefore offers the same uses as soybean. The oil may be extracted and used for cooking while the defatted meal can be used as a protein source for humans and livestock. However, in its present limited use as a backyard vegetable crop, it is greatly underutilized as a source of protein and oil.

Effective utilization of winged bean oil for cooking and as a source of lipids and lipid-soluble nutrients requires information on its chemical composition and physical properties. The fatty acid composition of winged bean oil is similar to that of peanut oil (Ekpenyong and Borchers, 1980; Garcia et al., 1979; Cerny et al., 1971). Total unsaturated fatty acids account for 65% of the total, and the ratio of the unsaturates to saturates is about 2. As far as we know, there is only one report on vitamin E analysis of winged bean oil, given by Cerny et al. (1971), who obtained a value of 126 mg of total tocopherol/100 g of oil. Vegetable oils contain tocopherols as natural antioxidants and are rich sources of vitamine E in the human diet

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0021-8561/82/1430-0050$01.25/0