Properties of Trypsin Inhibitor from Winged Bean (*Psophocarpus tetragonolobus*) Seed Isolated by Affinity Chromatography

Jones Chan[1] and Benito O. de Lumen*

The winged bean trypsin inhibitor (WBTI) fraction, isolated from whole winged beans (*Psophocarpus tetragonolobus*) via affinity chromatography on trypsin–Sepharose 4B, was heterogeneous as shown by disc gel electrophoresis. Eight protein bands, each exhibiting trypsin inhibitory activity (TIA), were separated with two of the major protein bands also exhibiting chymotrypsin inhibitory activity (CIA). When the WBTI fraction was subjected to $NaDodSO_4$ electrophoresis, regardless of whether mercaptoethanol was added, the heterogeneity of the fraction was reduced to two homogeneous bands with molecular weights of 20 900 and 16 600. With electrofocusing, the WBTI fraction was separated into five protein bands. The WBTI fraction was stable at acidic conditions but labile in pH greater than 8.0. At 60 °C, the TIA of the WBTI fraction was not affected, but at 100 °C, the thermal-stability curve was triphasic. A high level of the acidic and basic amino acids, proline, serine, and lysine and a low level of methionine characterized the inhibitor fraction, typical of most trypsin inhibitors.

In 1975, after examining 400 neglected tropical plants, the National Academy of Sciences selected the winged bean 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. The winged bean's nutritional advantages lie in the seed, which possesses a protein and oil content equivalent to that of soybeans. Consequently, the nutritonal and functional benefits offered by soybeans can be expected to be offered by winged beans as well. Furthermore, like the soybean, the presence of trypsin inhibitory activity has also been found in the winged bean (Korte, 1974).

The exact nutritional significance of trypsin inhibitors to man and experimental animals have been difficult to evaluate due to the presence of other antinutritional factors in legume seeds (Liener, 1974). Isolation of inhibitors and determination of its biological activity by animal feeding will ultimately have to be done to establish its nutritional importance. Abbey et al. (1979a,b) incorporated partially purified inhibitors from field beans (*Vicia faba*) into rat diets and found that the inhibitors caused depressed growth and pancreatic hypertrophy only when fed in amounts up to 5 times the normal endogenous levels found in field beans. One study where highly purified trypsin inhibitor from soybean was fed showed that 100% of the pancreatic hypertrophy and 30–60% of the growth inhibition effects of raw soybean could be attributed to the purified trypsin inhibitor (Rackis, 1965).

Affinity chromatography provides a means of isolating sufficient quantities of trypsin inhibitors needed for animal feeding experiments. Supplementary to nutritional studies of this kind, the physical and biochemical properties of the isolated inhibitors would have to be determined to establish the identity of the fraction. Kortt (1979) isolated and characterized the trypsin inhibitors from winged bean starting from a protein fraction (Psophocarpin B) recovered by a series of isoelectric precipitations of winged bean extract as performed by Gillespie and Blagrove (1978). As opposed to direct isolation of trypsin inhibitors from the whole bean, it is possible that certain inhibitors could have been lost or excluded in the purification process.

We have isolated trypsin inhibitors from winged bean by affinity chromatography on whole bean extract. Some properties of the isolated inhibitors are reported here. The procedure used in the present study was scaled up to obtain sufficient amounts for rat feeding experiments, and the results are reported in the following paper (Chan and de Lumen, 1982).

MATERIALS AND METHODS

Extraction of Trypsin Inhibitors. One hundred 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 500 mL 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 inhibitors were 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 a 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. Procedures to activate agarose and to immobilize trypsin onto activated agarose were done as described by March et al. (1974) and Loeffler and Pierce (1973), respectively.

Isolation of Trypsin Inhibitors. A 50 × 2.5 cm plastic column (Pharmacia Fine Chemicals, Piscataway, NJ) was packed with 200 mL of trypsin-bound agarose. The gel was first washed with 1 column volume of 0.2 M KCl, pH 2.0 (adjusted with concentrated HCl), followed by 2 column volumes of Tris buffer (0.05 M Tris, 0.1 M KCl, and 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 the Tris buffer, pH 8.0, used in

Department of Nutritional Sciences, University of California, Berkeley, California 94720.

[1]Present address: Shaklee Corporation, Research Center, Hayward, CA 94545.

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equilibrating the column. After centrifugation at 16000g for 15 min, the supernatant was applied to the column with a total trypsin inhibitory unit less than the total trypsin unit of 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, for dissociation of the trypsin–trypsin inhibitor complex, the eluting solvent was changed to 0.2 M KCl, pH 2.0. When the 280-nm absorption of the fractions was negligible, the chromatographic run was terminated. The column was then washed with 0.1 M $CalCl_2$, 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 by the decrease in the rate by which trypsin hydrolyzed *p*-toluenesulfonyl-L-arginine methyl ester (TAME), as monitored at 247 nm with a Cary 14 recording spectrophotometer (Worthington, 1972). 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 1 trypsin inhibitor unit (TIU).

Disc Gel Electrophoresis. Ornstein's (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 a 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 of 2.7 mA/tube. The gels were stained in 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.

$NaDodSO_4$ Gel Electrophoresis. $NaDodSO_4$ gel electrophoresis was conducted according to the procedure described by LKB-Produkter AB (1977). A phosphate buffer system was used for the preparation and electrophoretic running of the slab gel. The acrylamide concentration of the gel was 10%. It was run at 4 °C with a constant current of 80 mA instead of the recommended current of 190 mA because running the gel at a current higher than 80 mA generated too much heat. The standards for the molecular weight determination consisted of phosphorylase *b* (M_r 94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400). Mercaptoethanol provided the reducing condition to the electrophoretic system. The gel was fixed, stained, and destained according to the method of LKB-ProdukterAB (1977).

Electrofocusing. Electrofocusing on polyacrylamide gels was performed according to the method of LKB-ProdukterAB (1977). Ready-made LKB ampholine polyacrylamide gel plates, with a pH range of 3.5–9.5, were run at 10 °C, under a constant current of 20 mA. Lyophilized trypsin inhibitor was dissolved in Tris–glycine buffer to a concentration of 1 mg/mL, 15 μL of which was applied to the gel. The pH gradient of the gel was established via a Corning pH microelectrode. The fixing, staining, and destaining of the gel were performed as described in the above-mentioned method.

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 either with a trypsin concentration of 0.01 mg/mL or a with 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.

Amino Acid Analysis. Four hundred milligrams of lyophilized WBTI was hydrolyzed, in vacuo, in 6 N HCl for 22 h at 110 °C. The amino acid compositions of the WBTI were analyzed by running the hydrolyzates through a single-column Beckman amino acid analyzer, Model 121, according to the method of Moore and Stein (1963).

pH Stability of WBTI. The effects of pH on trypsin inhibitory activity (TIA) were determined by incubating winged bean trypsin inhibitors (WBTI) in various pH solutions and then assaying for TIA with the TAME assay. A 20 mg/mL WBTI solution was prepared by dissolving lyophilized WBTI in Tris–glycine, pH 8.3. The pH solutions were prepared according to the method of Gomori (1955). Three different buffer systems were employed to establish a pH range from 1 to 12: potassium chloride buffer (0.2 M KCl; 0.2 M HCl) for pH 1–2; citrate buffer (0.1 M citric acid; 0.1 M sodium citrate) for pH 3–6; Tris–acid maleate buffer (0.2 M Tris–maleic acid; 0.2 M NaOH) for pH 7–12. One-tenth milliliter of trypsin inhibitor solution was added to 9.9 mL of a buffer system at a certain pH, incubated for 20 min at 25 °C, and diluted 100 × with a Tris buffer (0.05 M Tris; 0.01 M $CaCl_2$; pH 8.1). This dilution was performed in order to eliminate the effect of pH on the TAME assay.

Determination of Thermal Stability of WBTI. In 12 × 1.5 cm test tubes, 2-mL aliquots of 0.002 mg/mL WBTI were immersed in a 100 °C water bath for 0–60 min. Aliquots of the inhibitor solution were removed at different time intervals, cooled at ambient temperature, and assayed for TIA. The same procedure was performed when the thermal stability of WBTI was tested at 60 °C.

RESULTS AND DISCUSSION

Extraction and Isolation of WBTI. The extraction procedure for trypsin inhibitors from winged bean (Chimbu variety) yielded a trypsin inhibitor concentration of 8857 TIU/g of winged bean (average of three determinations). de Lumen and Salamat's (1980) extraction procedure for WBTI is similar to the procedure used in this study but is less involved. The overnight extraction of TI with water and the two extra washings of the bean are omitted (see Materials and Methods). From two different batches of winged beans (Chimbu variety), they extracted a trypsin inhibitor concentration of 3691 and 9750 TIU/g of winged bean. These data illustrate the wide range of TIA variability between batches of the same variety of winged bean that makes it difficult to make comparisons of the efficiency of different extraction procedures.

The affinity chromatographic elution profile for the isolation of WBTI is illustrated in Figure 1. Two protein peaks and one trypsin inhibitor peak are present. The first protein peak contains no TIA and represents elution of non-trypsin-inhibitor compounds with Tris buffer, pH 8.0. The second protein peak contains TIA and represents elution of the trypsin inhibitor fraction with 0.2 M KCl, pH 2.0. Elution with this buffer caused a faint pink color to appear in the column and in the eluate. Even with complete elution of the trypsin inhibitor fraction, the pink coloration still persisted in the column and changes to a tannish color when the column was washed with a Tris buffer, pH 8.0. This suggests that pH-sensitive pigments may be attached to the column. In the eluate, the pink coloration is not dialyzable in either water or 0.001 M HCl, thus indicating that pigment may be affixed to the trypsin

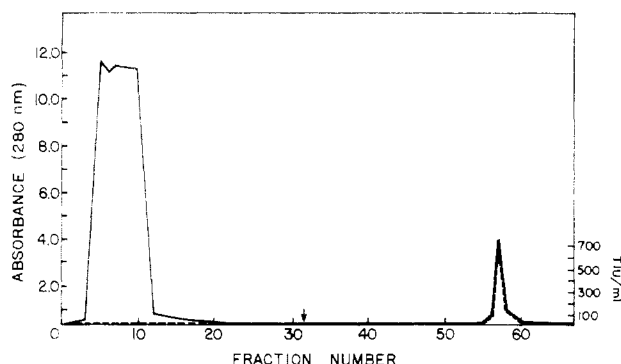

Figure 1. Affinity column separation of winged bean trypsin inhibitor. Two hundred milligrams of the lyophilized winged bean extract in 20 mL of Tris buffer, pH 8.0, was added to a column (50 × 2.5 cm) of agarose-bound trypsin. The arrow indicates a change of eluent from Tris buffer, pH 8.0, to 0.2 M KCl, pH 2.0. Four-milliliter fractions were collected. (—) indicates 280-nm absorption and (- - -) indicates TIA.

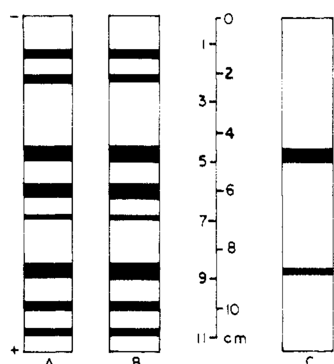

Figure 2. Electrophoresis of WBTI isolated by affinity column. The acrylamide concentration of the gels was 7.5%. By use of a Tris–glycine buffer, the gel was run at a pH of 8.3 for 4.5 h. A constant current of 2.7 mA was applied per tube. (A) Trypsin inhibitory activity. (B) Protein bands. (C) Chymotrypsin inhibitory activity. The bands in gels A and C actually represent the absence of staining.

inhibitor. Whether this will affect the physical character of the TI is not known, and further research is necessary before any inferences can be made.

Electrophoretic Properties. The WBTI fraction is heterogeneous as indicated by disc gel electrophoresis (Figure 2). Eight protein bands, each exhibiting TIA, were separated. Two of the major protein bands exhibited both TIA and chymotrypsin inhibitory activity (CIA). This is consistent with other studies which have demonstrated that trypsin inhibitors of legumes are generally composed of several protein components. For example, the trypsin inhibitor fraction from lima beans (Fraenkel-Conrat et al., 1952; Jones et al., 1963; Haynes and Feeney, 1967), navy bean (Bowman, 1971), soybean (Liener and Kakade, 1969) and *Lathyrus sativus* seed (Roy, 1980) contains six, five, eight, and five protein components respectively. A unique feature of this study is that, without further column chromatographic separations, the identity of each of the protein bands was established by chromatic staining for TIA and CIA. Our results show that all the protein bands possess TIA, with two bands exhibiting both TIA and CIA and that none of the proteins were inactive with respect to TIA or CIA. This is an important consideration since we are developing a method to produce sufficient quantities of biologically active compounds for animal feeding. That is, proteins that are biologically inactive and would perhaps have contributed as a protein source in the animal diet are not present in the inhibitor fraction.

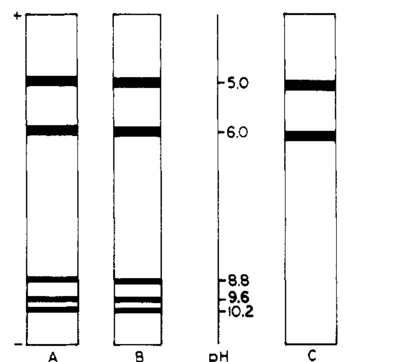

Figure 3. Electrofocusing of WBTI. The WBTI was run on a ready-made ampholine plate with a pH range of 3.5–9.5. Twenty milliamperes was applied per tube for 3.5 h. (A) Trypsin inhibitory activity. (B) Protein bands. (C) Chymotrypsin inhibitory activity. The bands in gels A and C actually represent the absence of staining.

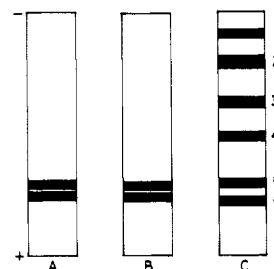

Figure 4. Sodium dodecyl sulfate electrophoresis of WBTI. A phosphate buffer system, pH 7.0, was used for electrophoretic running of the slab gel. (A) Mercaptoethanol was added to the system. (B) Mercaptoethanol was not added. (C) Protein standards were applied: (1) phosphorylase *b* (94 000); (2) albumin (67 000); (3) ovalbumin (43 000); (4) carbonic anhydrase (30 000); (5) soy bean trypsin inhibitor (20 100); (6) α-lactalbumin (14 400).

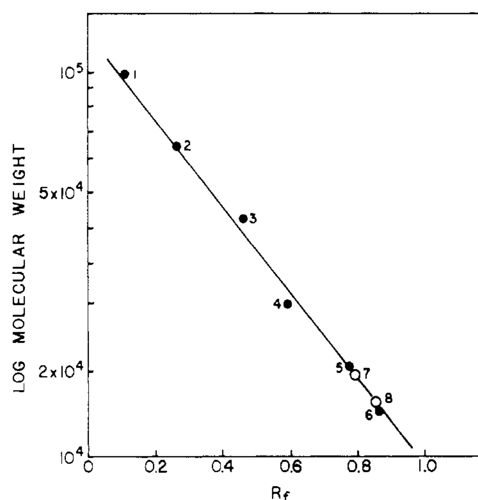

Figure 5. R_fs (relative mobility) of protein standards plotted against the log of the molecular weight. Numbers 7 and 8 correspond to the two protein bands displayed on the gel when $NaDodSO_4$ electrophoresis was performed on the WBTI.

With electrofocusing, the WBTI fraction was separated into five components, each exhibiting TIA. The two protein components which contained TIA and CIA had isoelectric points at pH 5.0 and 6.0 whereas the three protein bands which contained only TIA had isoelectric points at pH 8.0, 9.6, and 10.2 (Figure 3). The dual-activity components had lower isoelectric points and thus are more acidic than the components containing only TIA.

Once denaturated by $NaDodSO_4$, the heterogeneity of WBTI is reduced to two homogeneous bands with mo-

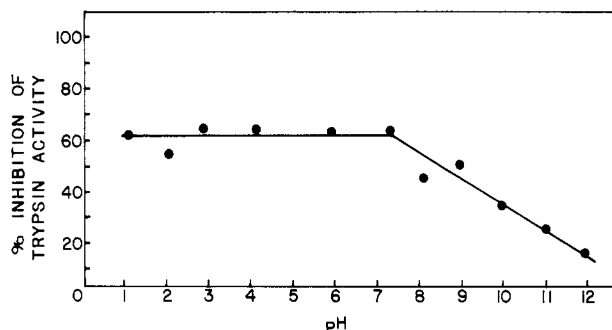

Figure 6. Effects of pH on trypsin inhibitory activity of the WBTI. The WBTI solution was incubated in different pHs from 1 to 12 for 20 min at 25 °C, after which the TIA of the solution was assayed.

lecular weights 20 900 and 16 000, regardless of whether mercaptoethanol was added or not (Figures 4 and 5). This suggests that the eight protein component of WBTI belong to either of two classes of isoinhibitor: one with a molecular weight of 20 900 and the other with a molecular weight of 16 600. These molecular weights are rather large compared to those of the majority of plant proteinase inhibitors which have molecular weights between 8000 and 10 000 (Richardson, 1981). The eight soybean TIs are also large and can be grouped into two molecular weight categories of 20 000–24 000 and 14 000–18 000, indicating the possibility that, in terms of molecular weight, the TI fraction of winged bean resembles that of soybeans.

The WBTI fraction isolated by Kortt (1979) also yielded large molecular weight trypsin inhibitors. The WBTI fraction, however, was not isolated from the whole bean but rather from a protein fraction recovered by various isoelectric precipitations of the winged bean extract (Gillespie, and Blagrove, 1978). From the fraction, Kortt purified three trypsin inhibitors: WBTI-1, WBTI-2, and WBTI-3. WBTI-2 and WBTI-3 are the major inhibitors, accounting for 60% of the protein in the TI fraction. They are basic proteins with molecular weights of 20 500. WBTI-1, the minor inhibitor, comprising about 10% (w/w) of the WBTI fraction, is an acidic protein with a molecular weight of 10 500. All three of the isolated trypsin inhibitor fraction (WBTI-1, WBTI-2, and WBTI-3) exhibit both TIA and CIA. When $NaDodSO_4$ electrophoresis was performed on the Kortt trypsin inhibitors, additional minor bands were observed in the presence of mercaptoethanol.

In this study, however, this is not the case. The number and the migration pattern of the bands, when $NaDodSO_4$ electrophoresis was run on WBTI, are the same regardless of whether a reducing agent, mercaptoethanol, has been added. This observation would indicate that there are no subunits in WBTI which are linked by disulfide bridges. If this was not the case, the addition of mercaptoethanol would increase the number of protein bands. Furthermore, the contribution of disulfide bridges to the tertiary structure of WBTI apparently is minor. If there was a relative abundance of disulfide linkages in the tertiary structure, reduction would cause a change in the migration pattern independent of any increase in the number of bands.

Effects of pH on WBTI Activity. Under the described conditions, WBTI was stable at acidic conditions but becomes labile in alkaline conditions, beginning at pH greater than 8.0 (Figure 6). Figure 6 shows that optimal pH for the inhibitor ranges from pH 1 to pH 7.5. Denaturation of the inhibitor may have occurred in the low pH range. This phenomenon, however, may have been reversed or masked when the system was adjusted back to pH 8.1 in order to eliminate the effects of pH on the TAME assay. In this study, the WBTI was incubated in a certain pH solution for 20 min at 25 °C. Varying these parameters can change the shape of the curve. Longer incubation periods and higher temperature would enhance the effect of pH on TIA.

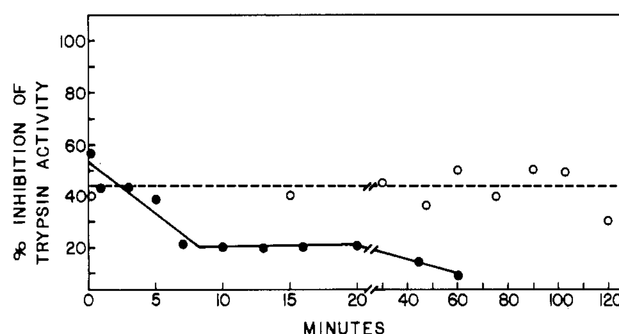

Figure 7. Effects of temperature on WBTI. Two-milliliter aliquots of 0.002 mg/mL WBTI were immersed in a 60 °C water bath for 0–120 min (○) and in 100 °C for 0–60 min (●).

Table I. Amino Acid Composition of WBTI and Winged Bean Seed Protein

amino acid	winged bean trypsin inhibitor, mg/g of N	winged bean[a] seeds, mg/g of N
isoleucine	171	306-350
leucine	723	462-564
lysine	699	413-600
methionine	118	75-87
cystine	*b*	73
phenylalanine	34	214-362
tyrosine	506	195-200
threonine	231	269-287
tryptophan	*b*	47-63
valine	190	242-306
arginine	434	400-440
histidine	89	169-183
alanine	325	230-269
aspartic acid	882	719-781
glutamic acid	1142	856-1154
glycine	445	259-269
proline	776	425-431
serine	602	306-327

[a] Data from Cerny (1978). [b] Not determined in this analysis.

Many inhibitors are known to be stable under low pH but a few are quickly destroyed (Richardson, 1977). The ability of inhibitors to remain active during passage through the human stomach determines to a large extent whether it would have adverse physiological effects in the intestine. Our data indicate that WBTI can survive acidic conditions, but whether it is resistant to pepsin hydrolysis is not known.

Thermal Stability of WBTI. The WBTI is stable at 60 °C. There was no significant loss of TIA even after 120 min of heating at this temperature (Figure 7). However, at 100 °C, the TIA decreased with time in a triphasic pattern. During the first 7 min of heating, TIA decreased with time, leveled off between 10 and 20 min of heating, and gradually decreased again thereafter. The shape of the temperature-stability curve would indicate that the eight protein components of WBTI possess varying heat stabilities. Overall, the WBTI is less stable to heat as compared to other plant proteinase inhibitors which remain essentially unaltered after heating at 80–100 °C for

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.

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

Jones Chan[1] and Benito O. de Lumen*

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.

Department of Nutritional Sciences, University of California, Berkeley, California 94720.

[1]Present address: Shaklee Corporation, Research Center, Hayward, CA 94545.

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