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Chymotrypsin Inhibitor Activity in Winged Beans (*Psophocarpus tetragonolobus*)

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Chymotrypsin inhibitor activity of winged beans (*Psophocarpus tetragonolobus*) was investigated. Extractability of the inhibitors by aqueous solution increases with increasing pH. The chymotrypsin inhibitor activity in winged bean meal was extremely resistant to dry heat treatment. Prolonged boiling (60 min) of the bean was required to destroy the inhibitor activity. Autoclaving is more effective in destroying the chymotrypsin inhibitor activity in the bean meals: 20 min of autoclaving at 120 °C, 1.05 kg/cm^2, generally destroyed more than 90% of the inhibitor activity. There are some varietal variations in the thermal stability of winged bean chymotrypsin inhibitor activity. The chymotrypsin inhibitor activity in the winged bean meal extract is heat labile.

The winged bean, *Psophocarpus tetragonolobus*, is a tropical legume that shows exceptional promise as a food crop for the humid tropics (National Academy of Sciences, 1981). It is also known as four-angled bean or kacang botol in Malaysia. All parts of the winged bean plant are edible and highly nutritious. The mature seed is very similar in composition to the soybean and is of comparable biological value (Claydon, 1975; National Academy of Sciences, 1981).

As with many legume seeds, the mature seed of the winged bean contains a variety of toxic factors (Jaffe and Korte, 1976; National Academy of Sciences, 1981; Tan et al., 1982), including proteinase inhibitors such as chymotrypsin and trypsin inhibitors. It has been established that proteinase inhibitors cause pancreatic hypertrophy in rats, chicks, and mice (Nesheim and Garlich, 1966; Rackis, 1965; Liener and Kakade, 1980) and growth inhibition has been observed in animals fed proteinase inhibitors at high levels (Liener et al., 1949; Ham et al., 1945; Westfall and Hauge, 1948). The antinutritional effects of raw winged bean seed such as growth inhibition, pancreatic hypertropy, and ultimately death are reported to be more deleterious than those of raw soybean (Jaffe and Korte, 1976).

Early studies on the winged bean have indicated the presence of trypsin inhibitor activity (Sohonie and Bhandarkar, 1954). Subsequently, several trypsin inhibitors and a chymotrypsin inhibitor were isolated and purified from winged bean (Kortt, 1979, 1980; Tan et al., 1979; Chan and De Lumen, 1982a). The chymotrypsin inhibitor isolated by Kortt (1980) has a molecular weight of approximately 21 000 and is a stable protein. The inhibitor resists digestion with pepsin at pH 2.0 (Kortt, 1981).

In this paper, we report the results of our investigation on the assay and thermal stability of chymotrypsin inhibitor activity in six varieties of winged beans grown locally.

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, Serdang, Selangor. Soybeans were obtained from local commercial outlets. Winged bean meals were prepared by grounding the mature beans manually with a mortar and pestle. The fine powder obtained was stored below 0 °C in a glass container before use. Bovine α-chymotrypsin and *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) were purchased from

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Sigma Chemicals. All other chemicals were of analytical reagent grade.

Assay of Chymotrypsin Inhibitor Activity. Chymotrypsin inhibitor activity (CIA) was determined by using a method modified from Hirado et al. (1981). Chymotrypsin inhibitor extract was prepared by extracting 100 mg of finely ground winged bean meal with 5 mL of 0.005 N sodium hydroxide (for routine assay) or with the appropriate extraction medium (for extractability studies). With raw meal the extraction time was 1 h, whereas 4 h was used for heat-treated samples.

The suspension (extract) was diluted to the point where 0.5 mL produced 30–40% chymotrypsin 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 1.0 mL with distilled water. One milliliter of α-chymotrypsin solution (0.02 mg mL^{-1} dimethylformamide) was added, followed by thorough mixing. Exactly 15 min later the reaction was terminated by adding 0.5 mL of 30% acetic acid. The mixture was then centrifuged at 10000*g* for 10 min, and absorbance of the supernatant was measured at 410 nm against a reagent blank by using a Beckman Acta III spectrophotometer. The reagent blank was prepared by adding 0.5 mL of 30% acetic acid to a test tube containing chymotrypsin and water (1 mL each) before the 0.4 mL of BTPNA solution was added. A sample blank is prepared by adding 0.4 mL of BTPNA solution to the diluted sample extract and water (0.5 mL each), incubating the mixture at 37 °C for 10 min, and then adding 0.5 mL of acetic acid followed by the addition of 1 mL of α-chymotrypsin. The blank readings for the other concentrations of the sample was computed by simple mathematical calculations.

Chymotrypsin inhibitor activity is estimated from the residual chymotryptic activity of the mixture of diluted bean extract and α-chymotrypsin. One unit of α-chymotrypsin activity was arbitrarily defined as an increase of 1 absorbance unit (at 410 nm) of the reaction mixture under the conditions used herein. Chymotrypsin inhibitor activity is defined as the number of α-chymotrypsin units inhibited (chymotrypsin inhibitor unit, CIU).

Determination of the Thermal Stability of Chymotrypsin Inhibitor Activity in Winged Bean Meals. The thermal stability of the chymotrypsin inhibitor activity in winged bean meals was determined by assaying the residual CIA in the heat-treated winged bean meals. The heat-treated winged bean meals were prepared as follows. (i) 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. (ii) 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, and 20 min, after the desired temperature had been reached. The autoclave oven was preheated before use to minimize time taken to reach the desired temperature (approximately 7 min).

Effect of Boiling on Chymotrypsin Inhibitor Activity in Whole Beans. Whole winged beans were boiled in distilled water for 10, 15, 20, 45, and 60 min, drained off their broths, and dried in an oven at 50 °C for 2 h. The moisture content of the samples were determined and appropriate weight corrections were made in the calculation of CIA inactivation to take into account the increase in moisture content of the boiled beans. The boiled beans were ground manually to yield a fine powder. Thermal stability of CIA in the whole beans was determined by analyzing the residual CIA in the bean meals.

Table I. Extraction of Winged Bean Seed Chymotrypsin Inhibitors (Variety 207)

extraction medium	chymotrypsin inhibitor activity,[a] CIU $(g \text{ of sample})^{-1}$
pH 4 (0.2 M sodium acetate buffer)	598 ± 35
pH 6 (0.2 M sodium acetate or sodium phosphate buffer)	715 ± 42
pH 8 (0.2 M sodium phosphate or Tris-chloride buffer)	744 ± 51
0.005 N sodium hydroxide	868 ± 57
distilled water	826 ± 48

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

Thermal Stability of the Chymotrypsin Inhibitor Activity in Winged Bean Meal Extracts. Thermal stability of the CIA in winged bean meal extracts was determined by assaying the residual CIA in the boiled winged bean meal extracts. To prepare the 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. Chymotrypsin inhibitor activity in the appropriately diluted suspension was then determined as described above.

Thermal Stability of the Chymotrypsin Inhibitor Activity in Soybean. For comparison, the effects of various types of heat treatments on CIA in soybean were also examined by using the method described above.

RESULTS AND DISCUSSION

Determination of Chymotrypsin Inhibitor Activity in Winged Bean Meals. Various methods of assaying CIA have been reported (Kortt, 1980; Chan and De Lumen, 1982a). The method described here is a modification of the procedure of Hirado et al. (1981), based on the procedure for the determination of trypsin inhibitor activity by Kakade et al. (1974). An uncentrifuged extract was used as this suspension generally yields a higher value of CIA. With BTPNA as the chymotrypsin substrate, the inhibition curve of bovine α-chymotrypsin by winged bean chymotrypsin inhibitors at pH 8 was linear only up to 45% inhibition of the enzyme. Thus, to obtain accurate results, it was important to ensure that the suspension was diluted to the points where 0.5 mL produced only 30–40% inhibition of chymotrypsin. On the other hand, Kortt (1980) showed that with *N*-benzoyltyrosine ethyl ester as the chymotrypsin substrate, the inhibition curve was linear up to 70% enzyme inhibition.

The extractability of the CIA increases with increasing pH (Table I). Using 0.005 N sodium hydroxide as the extraction medium, the pH of the suspension was usually between 9.5 and 9.8. At this pH region, maximum amount of CIA was extracted. Distilled water is also efficient in extracting CIA from winged bean meals (Table I).

Chymotrypsin Inhibitor Activity in Winged Beans. There is 2-fold variation of CIA in the six varieties of winged beans examined (Table II). Chymotrypsin inhibitor activity in soybeans was, however, much lower than that in winged bean. The major soybean trypsin inhibitors inhibit both trypsin and chymotrypsin. In winged bean, however, it has been reported (Kortt, 1980) that the major chymotrypsin inhibitor is strictly specific for chymotrypsin. The major chymotrypsin inhibitor comprises about 1.5% of the extractable seed protein. On the other hand, Chan

Table II. Effect of Dry Heat Treatments on Chymotrypsin Inhibitor Activity in Winged Bean Meals

winged bean varieties	native source	chymotrypsin inhibitor activity,[a] CIU (g of sample)$^{-1}$ raw	120 °C, 2 h
207	Malaysia	868 ± 57	854 ± 49
046	New Guinea	1112 ± 82	1100 ± 91
185	Thailand	1613 ± 97	1528 ± 85
100	Indonesia	1142 ± 75	1142 ± 79
141	Indonesia	805 ± 60	799 ± 71
095	New Guinea	1329 ± 91	1287 ± 88
soybean		192 ± 21	191 ± 18

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

Table III. Effects of Autoclave Treatments on Chymotrypsin Inhibitor Activity in Winged Bean Meals

winged bean varieties	inactivation of chymotrypsin inhibitor activity by autoclave treatments, 120 °C, 1.05 kg/cm^2, % chymotrypsin inhibitor activity destroyed[a] 5-min autoclave	10-min autoclave	15-min autoclave	20-min autoclave
207	70 ± 6	88 ± 8	88 ± 9	89 ± 8
046	65 ± 7	75 ± 7	82 ± 8	93 ± 6
095	55 ± 5	87 ± 7	88 ± 7	93 ± 8
185	80 ± 6	87 ± 9	90 ± 9	90 ± 7
100	66 ± 7	66 ± 5	84 ± 7	89 ± 6
141	70 ± 6	78 ± 7	82 ± 6	88 ± 6
soybean	56 ± 4	56 ± 4	62 ± 5	71 ± 8

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

and De Lumen (1982a) demonstrated the presence of several minor winged bean trypsin inhibitors that also inhibit chymotrypsin.

Thermal Stability of Chymotrypsin Inhibitor Activity in Winged Bean Meals. Dry heat at 120 °C for 2 h inactivated less than 5% of the CIA in winged bean meals (Table II). Autoclave treatment is, however, much more effective in inactivating the CIA in the meals (Table III). Twenty minutes of autoclave treatment generally destroyed 90% of the CIA in winged bean meals. Results in Table III also show that autoclave treatments destroyed winged bean CIA more effectively than soybean CIA. There are, however, some varietal variations in the thermal stability of CIA when the meals were subjected to autoclave treatments. For example, even though 10 min of autoclaving destroyed 87–88% of the CIA in winged bean varieties 207, 095, and 185, the same treatment inactivated only 66% of CIA in variety 100.

Effect of Boiling on the Chymotrypsin Inhibitor Activity of Winged Beans. The effects of boiling on the CIA of the whole winged beans (variety 207) are shown in Table IV. Boiling the beans for 20 min reduced the CIA by only 40%. In contrast, 5 min of autoclaving destroyed 70% of CIA of variety 207. Prolonged boiling (60 min) was required to inactivate 90% of the CIA.

Thermal Stability of Chymotrypsin Inhibitor Activity in Winged Bean Meal Extracts. Table V shows that boiling the winged bean meal extracts for 5 min reduced the CIA substantially (83–91%). This finding is in agreement with the observation of Kortt (1981), who reported that the purified winged bean chymotrypsin inhibitor was stable only to 70 °C at pH 4–8. On the other hand, boiling the soybean meal extract destroyed only 4% of the CIA. Similar differences in thermal stability of trypsin inhibitor activity in winged bean and soybean extracts have been reported (Tan and Wong, 1982). The

Table IV. Effect of Boiling on Chymotrypsin Inhibitor Activity in Whole Winged Beans (Variety 207)

duration of treatment, min	% inactivation of chymotrypsin inhibitor activity by boiling[a]
10	5 ± 2
15	29 ± 4
20	40 ± 5
45	70 ± 6
60	90 ± 6

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

Table V. Thermal Stability of Chymotrypsin Inhibitor Activity in Winged Bean Meal Extracts

winged bean varieties	% inactivation of chymotrypsin inhibitor activity in winged bean meal extract by 5-min boiling[a]
207	88 ± 7
046	89 ± 6
095	91 ± 7
185	83 ± 8
100	87 ± 5
141	86 ± 6
soybean	4 ± 1

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

difficulties in destroying the CIA in winged bean meals by dry heat treatment and inactivating the CIA in whole beans by boiling may be due to the inefficiency of heat penetration through the bean meals or beans.

Nutritional Significance of the Chymotrypsin Inhibitor Activity in Winged Beans. It has been demonstrated that winged bean trypsin inhibitors cause pancreatic hypertrophy and are partly responsible for the growth inhibition (Chan and De Lumen, 1982b). The antinutritional effect of the winged bean chymotrypsin inhibitors has not been elucidated. The effects of heat treatments on chymotrypsin inhibitor activity and trypsin inhibitor activity of winged beans are similar (Tan and Wong, 1982). Both chymotrypsin and trypsin inhibitor activity in the meals are resistant to dry heat treatment but could be inactivated by moist heat treatment, particularly 15–20 min of autoclaving. It is important to note, however, that while the level of trypsin inhibitor activity in winged bean is comparble to that in soybean, the chymotrypsin inhibitor activity in winged bean is 4–8 times higher than that in soybean. Thus, even though 15–20-min autoclave treatment destroyed 82–93% of the chymotrypsin inhibitor activity in winged beans, the residual chymotrypsin inhibitor activity of the autoclaved winged bean samples may still be comparable to the chymotrypsin inhibitor activity in raw soybean. Thus, the nutritional significance of the residual CIA in the moist heat treated winged bean meals warrants further studies.

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Fate and Effect of [^{14}C]Fenvalerate in a Tidal Marsh Sediment Ecosystem Model

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The fate and effect of [^{14}C]fenvalerate in a tidal marsh sediment ecosystem model were examined. Two dosages (0.2 and 1.0 ppm) of [^{14}C]fenvalerate were uniformly applied to the sediment. Additionally, a third treatment (1.0 ppm) was applied to the top centimeter of a 3-cm column in order to monitor the vertical movement of the compound (plus metabolites). No adverse effects of [^{14}C]fenvalerate on heterotrophic microorganisms were observed after 7 days at any treatment level, as measured by plate counts and substrate degradation assays (starch, cellulose, and protein). The half-lives of [^{14}C]fenvalerate in the 0.2- and 1.0-ppm noncolumn treatments were 6.3 and 8.9 days, respectively. The lower half-life (3.2 days) observed in the 1.0-ppm column treatment was attributed to a higher leaching rate in that treatment. TLC analysis of sediment extracts revealed the presence of three identifiable metabolites in all treatments after 4 days of incubation.

In the past 25 years, considerable ecological research has been performed concerning energy flow, productivity, and nutrient cycling in salt marsh environments. The high productivity of salt marshes has been attributed to the vertical mixing of water, which produces a nutrient trap that is comparable to land under intensive agriculture (Edwards and Davis, 1975; Pomeroy et al., 1972; Teal, 1962). Microbial degradation of plant material serves as the principal link between primary and secondary production in salt marsh environments. Only small amounts of the predominant marsh grasses (i.e., *Spartina alterniflora*) are consumed while living. Decaying plant material from marsh and terestrial sources provided carbon and energy for the microflora of the marsh sediments (Haines, 1977; Maccubbin and Hodson, 1980). Subsequently, the sediment microflora enrich the nutritive value of the detritus as food for a variety of grazers (Haines and Hansen, 1979; Tenore, 1977). It has recently been determined that heterotrophic bacteria are the most important component in salt marsh sediment in regards to the mineralization process (Fallon and Pfaender, 1976).

Xenobiotics, including many pesticides, enter estuaries as a result of erosion, agricultural runoff, industrial effluents, or by direct application in or near salt marsh environments. The same tidal action that functions as a nutrient trap may cause pesticides to be swept back and forth through the salt marsh at concentrations possibly injurious to productivity. Concern for possible hazards to nontarget species, including disruption of the heterotrophic decomposition process in salt marshes, has prompted studies to determine the fate and effects of these pesticides (Bourquin et al., 1976). In this paper, we report the development and use of a model salt marsh ecosystem to evaluate the fate and effect of a pesticide under simulated salt marsh conditions. [^{14}C]Fenvalerate [cyano(3-phenoxyphenyl)methyl 4-chloro-α-(1-methylethyl)benzeneacetate] was used as the reference pesticide in this study. Fenvalerate is a pyrethroid insecticide that has great potential for control of a wide range of insect pests in agriculture, because it combines outstanding insecticidal activity, moderate mammalian toxicity, and adequate stability in the field. Only a few studies have been performed with the aim of examining the metabolic pathway of fenvalerate in the environment (Ohkawa et al., 1978; Mikami et al., 1980).

MATERIALS AND METHODS

Tidal Marsh Sediment Ecosystem. Eight 10-L capacity glass ecosystem tanks (operating volume = 7 L) were built (Figure 1a). Two equal-sized chambers were formed in each tank by using a glass partition fitted with a capillary drain hole. Sediment containers (six per tank) were placed on a 9-cm glass platform located in one chamber

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