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Isolation and Characterization of a Proteinase Inhibitor from Marama Beans (42184)

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Abstract. A protease inhibitor was purified from the African marama bean (*Tylosema esculenturm*). The inhibitor is present in large amounts, representing about 10.5% of the total protein. The molecular weight is slightly larger than soybean trypsin inhibitor and was estimated at 23,000 by SDS-gel electrophoresis or 24,500 by amino acid analysis. The amino acid composition was atypical of most other plant inhibitors with a cysteine content of only one or possibly two residues/mole and a blocked amino terminus. Inhibition studies indicated virtually no inhibition of chymotrypsin activity. Elastase, however, was inhibited to the same extent as trypsin, requiring about 2 moles of inhibitor for complete inhibition of the enzyme.

The occurrence of proteinase inhibitors in storage organs is widespread in the plant kingdom, occurring in relatively high levels in plants such as corn (1), potatoes (2), and many varieties of beans (3–6). In some instances, such as soybeans, the raw bean is of little nutritive value until subjected to conditions which either remove or denature the inhibitor (7). Whether the inhibitor is directly responsible for the antinutritive effect is still a matter of debate (8).

The marama bean (*Tylosema esculenturm*) is a hard shelled legume, eaten by the native people of the Kalahari Desert, that provides an excellent source of protein (32.8%) (9). Since the beans thrive in arid regions with poor soil conditions, they could possibly develop into a major protein source if cultivated by people in impoverished areas of the world. Recently it was shown that raw marama beans show no hemagglutinating activity yet contain very high levels of a trypsin inhibitor (8). It was the purpose of this study to isolate and characterize the major proteinase inhibitor found in marama beans.

Materials and Methods. The method of proteinase inhibitor extraction used was a modification of the Wagner and Riehm technique (4). Two hundred grams of shelled beans were slurried in 80% ethanol using a Waring commercial blender, diluted to 1 liter and allowed to soak for 30 min. The slurry was suction filtered and the procedure repeated two more times. The meal was then extracted in 500 ml of 0.05 *N* hydrochloric acid for 6 hr and then centrifuged (Sorval, RC-5) at 12,000 rpm and 4°C. The supernatant was then brought to 80% ammonium sulfate saturation and centrifuged at 8000 rpm and 4°C for 20 min. The precipitate was dissolved in water and dialyzed against repeated changes of distilled water and then lyophilized. The yield of freeze-dried extract was 7.2 g.

The inhibitor was further purified using trypsin–agarose affinity chromatography followed by gel filtration on G-75 Sephadex. The trypsin agarose separation technique was based on a modification of the procedure of DeLumen and Belo (5). One gram of lyophilized extract was dissolved in 50 ml of 0.05 *M* Tris, 0.10 *M* potassium chloride, 0.2 *M* calcium buffer, pH 8.0 and centrifuged at 10,000 rpm for 10 min. The supernatant was applied to a 0.9 × 20-cm trypsin–agarose column (Elastin Products Company) equilibrated with the same Tris buffer. The column was washed with the equilibration buffer until the OD returned to baseline. The inhibitor was then eluted using a 0.1 *M* sodium citrate buffer adjusted to pH 2.0. Five-milliliter fractions were collected and the optical density measured at 280 nm. Fractions containing antitryptic activity were located, pooled, dialyzed, and lyophilized. Two hundred milligrams of this product was then dissolved in 3 ml of 0.01 *M* potassium phosphate buffer pH 7.6 and applied to a 2.5 × 40-cm Sephadex G-75 column. Five-milliliter fractions were collected, assayed for antitryptic activity and the active fractions pooled, dialyzed, and freeze-dried.

Purity and molecular weight of the product were determined using SDS–acrylamide gel

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electrophoresis on 8% slab gels following the procedure of Weber and Osborn (10). Standard protein markers used were bovine serum albumin, ovalbumin, pepsin, trypsin, lysozyme, and cytochrome *c*.

Amino acid analysis was performed on a Beckman 6300 amino acid analyzer using standard procedures. Cysteic acid and methionine sulfoxide were determined following performic acid oxidation (11) and tryptophan content was determined following methane sulfonic acid hydrolysis (12). N-terminal sequencing was performed using a Beckman 890C sequencer.

For the enzyme-inhibitor studies trypsin (2× crystallized, Sigma) was first repurified by affinity chromatography to separate inactive from active enzymes. Two milligrams of trypsin was dissolved in 2 ml of 0.02 *M* phosphate buffer pH 7.0 and applied to a small (0.7 × 5 cm) column of agarose covalently linked to soybean trypsin inhibitor. The bound enzyme was washed with 5 column vol of phosphate buffer and the active trypsin eluted with 0.2 *M* sodium citrate buffer pH 2.0. Fractions containing the enzyme were combined and dialyzed against 0.0025 *M* HCl. Trypsin concentration was calculated using $E^{1\%}_{280} = 15.4$.

Antitryptic activity was assessed using a modification of the procedure of Eriksson (13). *N*-2-benzoyl-DL-arginine-*p*-nitroanilide HCl (BAPNA) dissolved in distilled water (0.45 mg/ml) was used as the substrate. Assays were conducted in 96-well microtiter plates. Trypsin (20 μg) was combined with varying amounts of inhibitor (5–80 μg) and 0.1 *M* Tris buffer, pH 8.2 containing 0.02 *M* $CaCl_2$ was added to bring the volume to 150 μl. Substrate (150 μl) was added to each well to initiate the reaction which was conducted at room temperature for 9 min. Controls consisted of the inhibitor being replaced by buffer and a blank containing no enzyme. Optical density was determined at 405 mu on a Titertek Multiskan.

Antielastase activity was determined colorimetrically in a similar manner to the trypsin assay except filterable millititer plates (Millipore Corp.) containing hydrophobic bottoms were used. Elastase (Elastin Products Co.) 2 μg in 0.0025 *M* HCl, was combined with varying amounts of inhibitor (0.5 to 8 μg) and the volume adjusted to 200 μl with 0.1 *M* Tris buffer pH 8.2 containing 0.02 *M* $CaCl_2$. After incubation for 10 min 150 μl of elastin–rhodamine substrate (Elastin Products Co.; 20 mg/ml) was added to initiate the reaction. Controls with no inhibitor or no enzyme were carried through the procedure. After 20 min incubation at room temperature the plates were filtered directly into a 96-well microtiter plate. Exactly 200 μl of each filtrate was transferred to another well and the OD at 540 mu measured on the Titertek Multiskan.

Antichymotryptic activity was determined using benzoyl-L-tyrosine ethyl ester (BTEE; Sigma) as the substrate (14). Twenty micrograms of α-chymotrypsin (Worthington) was combined with varying amounts of inhibitor (5 to 80 μg) and the total volume brought to 1.5 ml with 0.08 *M* Tris buffer pH 7.8 containing 0.1 $CaCl_2$. To initiate the reaction 1.5 ml of substrate was added and the change in OD at 256 nm recorded after 3 min at 25°C.

All enzyme assays were linear over the course of the reaction for the enzyme concentrations used.

Results and Discussion. Extraction of the marama beans and subsequent ammonium sulfate precipitation yielded 3.6 g of freeze-dried protein per 100 g of original beans. This represents 10.5% of the total protein content, recoverable as the inhibitor at this stage of purity. Although this appears high, other plants such as barley (15) and potatoes (16) have been shown to contain this level of inhibitors.

The purity of the marama inhibitor was determined by SDS–acrylamide electrophoresis (Fig. 1). Surprisingly, the inhibitor was virtually a single band after only $(NH_4)_2SO_4$ precipitation. With significant overloading a faint smear was seen in front and behind the main band. It is of some concern that the inhibitor band was so broad on the SDS gels. Even with low levels of inhibitor, the band was still diffuse. The possibility exists that more than one form of the inhibitor is present, differing slightly in carbohydrate or amino acid content. Analysis of the inhibitor following affinity chromatography or gel filtration showed no further gains in purification. In fact, following affinity chromatography on agarose–trypsin, the smaller molecular weight components were even more evident and may represent breakdown products of the inhibitor. Molecular weight estimates with SDS gels were dif-

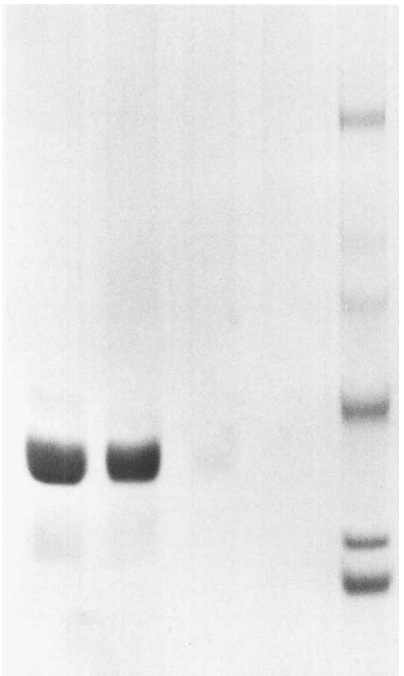

FIG. 1. SAS–acrylamide gel electrophoresis of the marama bean inhibitor following $(NH_4)_2SO_4$ precipitation. The four lanes on the left show the inhibitor at different concentrations. Standards (far right) were BSA, ovalbumen, pepsin, trypsin, lysozyme, and cytochrome *c*. (64,400; 45,000; 35,000; 23,300; 14,400; 11,700, respectively)

ficult to interpret. When compared directly on the same gel with soybean inhibitor (mol wt 21,500), the marama bean inhibitor migrated on the gel slower with an apparent molecular weight of 23,000. However, when compared to molecular weight protein standards, the apparent molecular weight was considerably smaller (18,000). It has been observed previously (17) that soybean inhibitor migrates on SDS gels as a molecule considerably smaller than its known molecular weight. It is quite conceivable that the marama inhibitor exhibits the same anomalous behavior.

The amino acid analysis of the marama inhibitor is shown in Table I. Perhaps the most notable difference in the marama inhibitor is the unusually low cysteine content, representing either one or two residues per mole. The majority of trypsin inhibitors of plant origin which have been isolated previously average from 10 to 15 residues per mole (18). Notable exceptions are the soybean inhibitor,

TABLE I. AMINO ACID COMPOSITION OF MARAMA BEAN INHIBITOR

Amino acid	Residues per molecule[a]
Asparatic acid	24.8 ± 0.7
Threonine	8.2 ± 0.5
Serine	19.3 ± 0.4
Glutamic acid	17.6 ± 0.3
Proline	14.6 ± 0.2
Glycine	25.2 ± 0.3
Alanine	11.5 ± 0.5
Valine	13.4 ± 0.7
Cysteine	1.4 ± 0.5
Methionine	2.8 ± 0.1
Isoleucine	12.6 ± 0.6
Leucine	15.8 ± 0.3
Tyrosine	13.0 ± 0.4
Phenylalanine	9.8 ± 0.3
Tryptophan	0.7 ± 0.6
Lysine	16.7 ± 0.4
Histidine	6.0 ± 0.4
Arginine	8.3 ± 0.7
Molecular weight	24,500
Amino terminus	Blocked

[a] Mean of six analyses with the standard deviation.

which contains 4 cysteines per mole and members of the potato 1 family which contain 2 or less (19). The minimum molecular weight calculation determined from the amino acid analysis, assuming methionine is present as three residues, was 24,500. End group analysis using both the manual method and the automated sequencer was negative suggesting a blocked N terminal. As far as we know this is the only plant inhibitor for which this is true.

The results of the inhibition studies are shown in Figs. 2–4. For each of the enzymes studied, soybean and marama inhibitor were

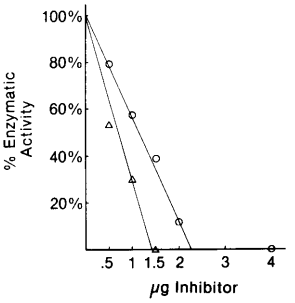

FIG. 2. Trypsin inhibition with soybean (△) and marama bean (○) inhibitors. The amount of trypsin in each assay was 2 μg.

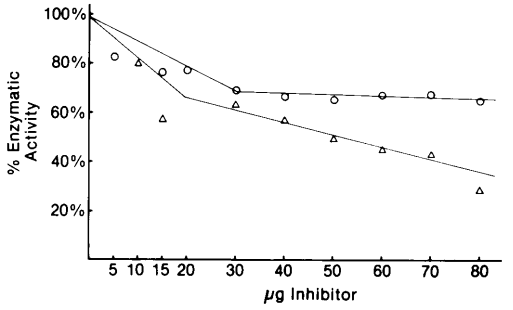

FIG. 3. Chymotrypsin inhibition with soybean (△) and marama bean (○) inhibitors. The amount of chymotrypsin in each assay was 20 μg.

compared for the effectiveness of inhibition. Trypsin inhibition by the soybean inhibitor showed the anticipated 1:1 molar ratio for complete inhibition (Fig. 2). The marama inhibitor on the other hand was somewhat less effective, requiring a 2:1 ratio of inhibitor to enzyme for complete inhibition. Inhibition of chymotrypsin by soy inhibitor was as expected much less effective than for trypsin (Fig. 3). The marama inhibitor was even less effective. With elastase, the soybean inhibitor was an ineffective inhibitor as has been indicated before (20). The marama bean inhibitor, however, was quite a good elastase inhibitor, requiring about 2 moles of inhibitor per mole of elastase for complete inhibition. The other plant inhibitor we are aware of that has been shown to be an effective elastase inhibitor is the kidney bean (21).

The effectiveness of the marama inhibitor for elastase can be illustrated by mixing equal

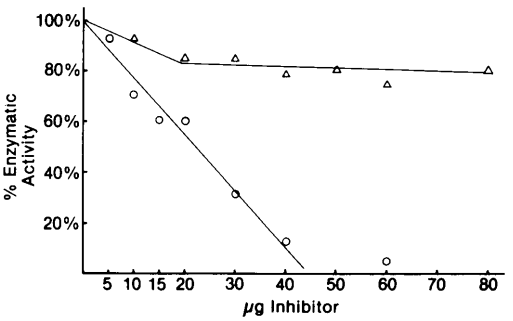

FIG. 4. Elastase inhibition with soybean (△) and marama bean (○) inhibitors. The amount of elastase in each assay was 20 μg.

TABLE II. EFFECT OF SOYBEAN AND MARAMA BEAN INHIBITORS ADDED TO AN EQUAL MIXTURE OF TRYPSIN AND ELASTASE

Inhibitor[a]	% Inhibition	
Soybean	Trypsin	95
	Elastase	0
Marama bean	Trypsin	93
	Elastase	80

[a] Inhibitors (40 μg) were added to a mixture of trypsin (20 μg) and elastase (20 μg).

quantities of elastase and trypsin with either soybean or marama inhibitor or both (Table II). When soybean inhibitor is added to a mixture of both enzymes only the trypsin is inhibited. Conversely, when the marama inhibitor is added to the enzymes the elastolytic as well as the tryptic activity is inhibited.

The question of why such high levels of trypsin inhibitors exist in plants has caused much speculation (22). The presence of such an effective elastase inhibitor in the marama bean could possibly relate to a protective role directed toward certain insects or bacteria that contain proteolytic enzymes with elastase-like specificities.

The marama inhibitor may prove to be a very useful tool in studies dealing with elastin metabolism and pathogenesis of connective tissue related diseases in experimental animals and man.

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