

RESEARCH ARTICLE

# Isolation and partial sequence of a Kunitz-type elastase specific inhibitor from marama bean (*Tylosema esculentum*)

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## Abstract

An isolation procedure utilizing ammonium sulfate fractionation and affinity chromatography was used to purify an elastase inhibitor present in large amounts in marama beans (*Tylosema esculentum*). The protein appeared to be heterogeneous due to carbohydrate differences, demonstrating two bands on SDS gels with molecular weights of 17.8 kDa and 20 kDa. Partial sequence, derived from mass spectrometry, indicated that the protein is a Kunitz-type inhibitor distinct from other known plant serine protease inhibitors. The marama bean inhibitor is specific for elastase, with very low  $K_i$  for both pancreatic and neutrophil elastase. The quantity of elastase inhibitor present in marama beans is many times greater than in soybean or any other bean or nut source reported to date. This raises the question of why a bean found in an arid corner of the Kalahari Desert would be so rich in a very potent elastase inhibitor.

**Keywords:** Marama bean; elastase inhibitor; amino acid sequence

## Introduction

Protease inhibitors in plants play an important role in regulation of growth and development, response to stress, and defense against insects and pathogens. (To avoid confusion, the term “protease”—which includes both endopeptidases and exopeptidases—has been used throughout this report.) As early as 1947 it was observed that the larvae of certain insects could not develop normally when grown on soybean products. Subsequently, soybean trypsin inhibitor was shown to be toxic to the larvae of the flour beetle<sup>1</sup>. Since then, there have been many examples of protease inhibitors in different plant tissues, including leaves, flowers, seeds, and roots, exhibiting a protective effect against specific insect species, both *in vitro*<sup>1–6</sup> and *in vivo*<sup>7,8</sup>. These inhibitors are thought to be involved primarily in defense against herbivores, which rely on proteases to digest the proteins they consume, and for protection against wounds inflicted by insects<sup>9,10</sup>. Protease inhibitors moderate the growth and survival of many insect herbivores when included in artificial diets, and reduce insect feeding rate and performance when expressed in

transgenic plants<sup>11,12</sup>. In seeds, where protease inhibitors are often found in high concentrations, they keep the plants' own proteases in check to prevent degradation of storage proteins<sup>10</sup>. Serine proteases have been identified in the digestive tracts of insects from many families, particularly those of lepidoptera<sup>13</sup>, and many plant inhibitors have been found to be active against these gut proteases<sup>2,3</sup>. These protease inhibitors also exhibit a very broad spectrum of activity against other pathogens including: pathogenic nematodes such as *Globodera tabaccum*, *G. pallida*, and *Meloidogyne incognita*<sup>14</sup>; inhibition of spore germination and mycelium growth of *Alternaria alternata*<sup>15</sup>; and growth of many pathogenic fungi including *Trichoderma reesei*<sup>16</sup>. Wound-inducible protease inhibitor proteins are present in plant leaves and soft tissue products such as tomatoes. When ingested by polyphagous insects, these inhibitors can interact with proteases of the insect midgut and lead to an arrest of growth and development, and death<sup>9,17</sup>. Tissue engineering studies have shown that enhanced resistance to insects and pathogens results from overexpressing protease inhibitors<sup>9,18,19</sup>.

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The marama bean, *Tylosema esculentum*, is a perennial tuberous legume that is native to the arid regions of southern Africa<sup>20,21</sup>. The bean is an important supplement to the diet of tribes in the Kalahari Desert region where the bean is roasted, and then the hard, 2-mm-thick shell is removed prior to consumption, providing a valuable source of protein. The nutritional value of the marama bean is potentially quite high, being very similar to soybean<sup>22-24</sup>. There has been one study using young rats that were fed either raw or cooked marama bean meal contributing 10% of the protein in the diet<sup>24</sup>. For both groups fed marama bean meal, food consumption was poor and the animals lost weight. A possible reason for the poor nutritive value of the marama bean in rats was thought to be the unusually high trypsin inhibitor level, which has been reported to be 2–4 times higher than found in soybeans<sup>25</sup>. Subsequent studies indicated that there was also an elastase inhibitor in marama beans<sup>26</sup>, and apparently the trypsin and elastase inhibitors were separate proteins<sup>27</sup>. The marama bean elastase inhibitor is a very potent inhibitor with low  $K_i$  for pancreatic elastase from mouse, rat, and pig as well as human neutrophil elastase<sup>28</sup>.

In the present study we further characterized the protease inhibitor present in marama beans and found unusually high levels of a specific elastase inhibitor that has sequence homology to the Kunitz family of protease inhibitors.

## Materials and methods

Two sources of marama beans (*Tylosema esculentum*) were used: recently acquired samples were a generous gift from Dr. Chris Cullis at Case Western Reserve University; and 30-year-old beans from a previous study<sup>26</sup>. Other materials were obtained as follows: *N*-succinyl-(Ala)<sub>3</sub>-*p*-nitroanilide (SAPNA) and porcine pancreatic elastase, Elastin Products, Owensville, MO; *N*-benzoyl-L-arginine-ethyl-ester (BAEE), Nutritional Biochemical Corporation, Cleveland, OH; trypsin, Sigma Chemical Co., St. Louis, MO; Affigel-10, Bio-Rad Laboratories, Hercules, CA.

### Inhibitor extraction

The inhibitor was extracted from marama beans using a modified Wagner and Riehm technique<sup>29</sup>. The shell was removed from one bean, and the nut (1.7 g) was homogenized in 24 mL of 80% ethanol. The homogenate was extracted for 1 h at room temperature and the slurry centrifuged at 20,000 × *g* for 15 min. The supernatant containing the seed oil was discarded and the meal was extracted in 10 mL of 0.05 M HCl for 1 h at room temperature and then centrifuged at 20,000 × *g* for 10 min at 4°C. The extraction was repeated three times to ensure the complete removal of acid soluble proteins. The four extracts were combined and brought to 80% ammonium sulfate saturation and stirred at 4°C for 1 h. The suspension was then centrifuged for 20 min at 9000 × *g* at 4°C. The supernatant was discarded and the precipitate was then dissolved in 5 mL of water, dialyzed for 24 h with repeated changes of

water, and freeze-dried. For comparison, protease inhibitors were similarly extracted from raw soybeans, Brazil nuts, and Macadamia nuts using the same protocol.

### Affinity chromatography

Porcine pancreatic elastase was linked to Affigel-10 according to the manufacturer's instructions. Briefly, 5 mL of Affigel-10 was washed with water and reacted with 30 mg of porcine pancreatic elastase, and the gel equilibrated with 67 mM potassium phosphate buffer, pH 7.6. The freeze-dried extract from one marama bean (77 mg) was dissolved in 13 mL of the 67 mM phosphate buffer, centrifuged at 9000 × *g* for 10 min, filtered through a 0.2- $\mu$ m syringe filter, and applied to the elastase-affinity column. The column was washed with 100 mL of the 67 mM phosphate buffer followed by 10 mL of the same buffer containing 0.5 M sodium chloride. The column was then re-equilibrated with water, and proteins bound to the column were eluted with 0.1 M sodium citrate buffer, pH 2.1. Fractions (2 mL) were collected, the absorbance at 280 nm was measured, and the fractions assayed for both anti-trypsin and anti-elastase activity as described below. Soybean trypsin inhibitor (5 mg) was also fractionated using the elastase affinity column. The inhibitor was dissolved in 67 mM potassium phosphate buffer, pH 7.6, and treated as described above for the marama bean extract.

### Anti-elastase activity

Each fraction (7  $\mu$ L) was added to a 96-well microwell plate, followed by addition of 8  $\mu$ L of porcine pancreatic elastase (200  $\mu$ g/mL in 0.001 M HCl). The volume in each well was brought to 100  $\mu$ L with 0.5 M Tris-HCl buffer, pH 8.8. The mixture was pre-incubated for 10 min at room temperature and then 8  $\mu$ L of the substrate (5 mM SAPNA dissolved in 1-methyl-2-pyrrolidinone) was added. After a 15-min incubation at room temperature, absorbance at 410 nm was measured. To compare the quantity of inhibitor in extracts from different sources, we defined a unit of inhibitor as the amount that inhibits 50% of the activity of 1  $\mu$ g of porcine pancreatic elastase in the standard microwell assay described above.

### Anti-trypsin activity

Each fraction (7  $\mu$ L) was added to a 96-well microwell plate, and then 1  $\mu$ L of trypsin (1 mg/mL in 0.001 M HCl) was added to each well. The volume in each well was brought to 100  $\mu$ L with 67 mM potassium phosphate buffer, pH 7.6. The mixture was pre-incubated for 10 min and then 4.6  $\mu$ L of BAEE (10 mM in phosphate buffer) was added. Absorbance was measured at 253 nm in 30-s intervals for 5 min to assess the rate of proteolysis.

### Gel electrophoresis

The isolated marama bean elastase inhibitor (10  $\mu$ g) was dissolved in loading buffer<sup>30</sup>, reduced with  $\beta$ -mercaptoethanol, and applied to an 8–12% gradient SDS (sodium dodecyl sulfate) gel. A molecular weight standard

mixture (SeeBlue Plus2; Invitrogen Corp.) was run with each gel. Proteins were visualized with Coomassie blue. The bands at apparent molecular weights of 17.8 kDa and 20 kDa were excised and eluted with water for mass spectrometry analysis.

### Mass spectrometry

Proteins in the gel bands were digested *in situ* with trypsin (Promega modified) in 40 mM  $\text{NH}_4\text{HCO}_3$  at 37°C for 4 h. The digests were analyzed by MALDI-TOF-MS (matrix-assisted laser desorption/ionization-time of flight-mass spectrometry) and HPLC-ESI-MS/MS (high performance liquid chromatography-electrospray ionization-tandem MS). MALDI-TOF mass spectra were obtained on an Applied Biosystems Voyager-DE STR with positive ion detection in reflectron mode and  $\alpha$ -cyano-4-hydroxycinnamic acid as matrix. HPLC-ESI tandem mass spectra were acquired on a Thermo Fisher LTQ linear ion trap mass spectrometer fitted with a New Objective PicoView 550 nanospray interface. On-line HPLC separation of the digests was accomplished with an Eksigent NanoLC micro HPLC: column, PicoFrit™ (New Objective; 75  $\mu\text{m}$  i.d.) packed to 10 cm with C18 adsorbent (Vydac; 218MSB5, 5  $\mu\text{m}$ , 300 Å); mobile phase A, 0.5% acetic acid (HAc)/0.005% trifluoroacetic acid (TFA); mobile phase B, 90% acetonitrile/0.5% HAc/0.005% TFA; gradient 2–42% B in 30 min; flow rate, 0.4  $\mu\text{L}/\text{min}$ . MS conditions were: ESI voltage, 2.9 kV; isolation window for MS/MS, 3; relative collision energy, 35%; scan strategy, survey scan followed by acquisition of data dependent collision-induced dissociation (CID) spectra of the seven most intense ions in the survey scan above a set threshold. The peptide mass maps and uninterpreted CID spectra were searched against the NCBI nr database by means of Mascot (Matrix Science). Manual *de novo* sequencing of the tandem mass spectra was then conducted because no high-confidence identifications were found by the Mascot searches.

## Results

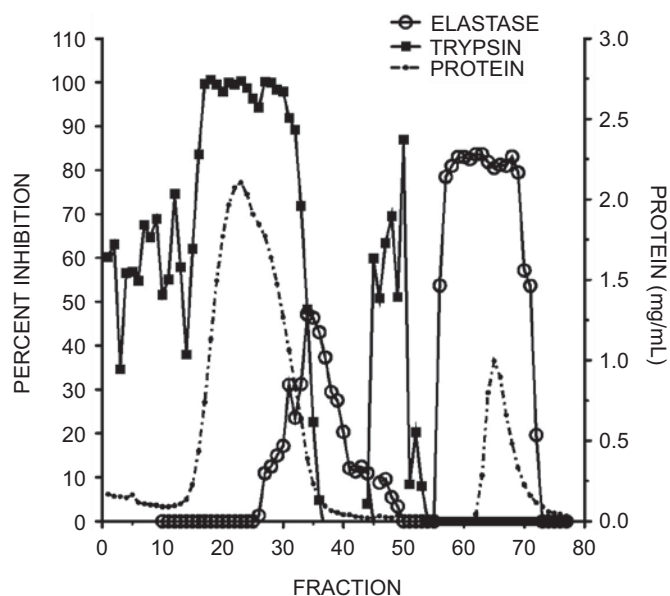
Both sources of marama beans gave identical results during purification through the affinity step and appearance on SDS gels. Storage for 30 years at room temperature had no apparent ill effects on the structure or function of the elastase inhibitor. However, the data shown are from the fresher marama beans supplied by Dr. Cullis.

Marama beans are similar to soybeans in that they contain over 30% protein by weight (Table 1). This is in

accordance with the observations of other investigators<sup>22–24</sup>. Macadamia and Brazil nuts were comparable in texture to the marama bean yet contained much lower levels of protein. The quantity of protein in the ammonium sulfate fraction (containing the inhibitor) and the total units of elastase inhibitor were much greater in the marama bean than the Brazil and Macadamia nuts and soybeans used for comparison (Table 1). Even though the elastase inhibitor was present in relatively high concentrations, the trypsin inhibitor still represented 90% of the protein present in the ammonium sulfate fraction.

The protein and activity profiles of the trypsin and elastase inhibitors eluting from the affinity column in fractions 1–50 in the equilibration buffer are shown in Figure 1. The majority of the protein eluted as a broad peak coinciding with the anti-trypsin activity. A small peak with elastase inhibitory activity eluted immediately following the anti-trypsin fractions. Subsequent elution with pH 2.2-citrate buffer (fractions 51–77) yielded a single, broad peak with anti-elastase activity and no detectable anti-trypsin activity. Fractions 60–70 were pooled, dialyzed against water, and freeze-dried, yielding approximately 10 mg of elastase-specific inhibitor.

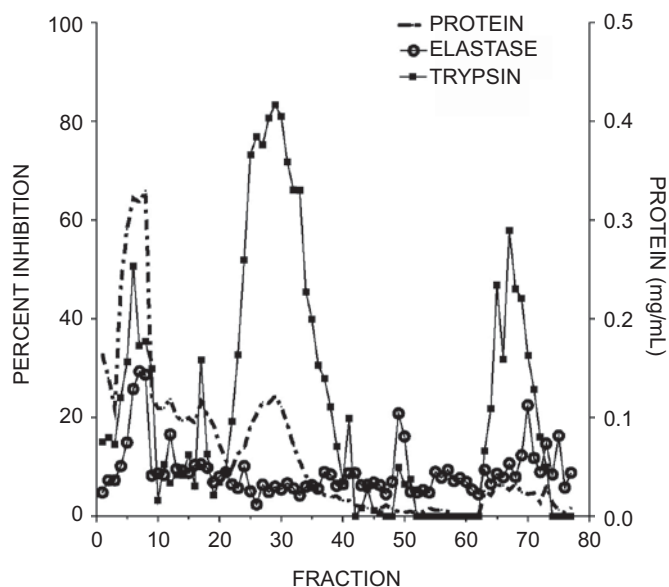
Soybean trypsin inhibitor applied to the elastase affinity column yielded a different separation pattern (Figure 2). A large peak of protein with low anti-trypsin and anti-elastase activity eluted early followed by a broad peak containing high levels of anti-trypsin activity but no detectable elastase inhibition. Elution with pH 2.2-citrate buffer resulted in a low-intensity, broad peak that exhibited low levels of both trypsin and elastase inhibitory activity.



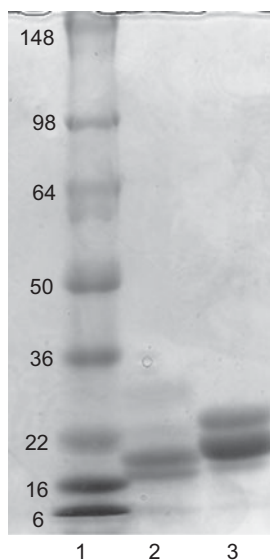
**Figure 1.** Elution profile of elastase and trypsin inhibitory activity from marama bean extracts using an elastase affinity column. The column wash included fractions 1–50 and the citrate elution fractions 51–77. Protein quantity measured at 280 nm is illustrated by the dotted line, trypsin inhibitory activity by squares, and elastase inhibition by open circles. Each fraction was 2 mL. Inhibitory activity was expressed as a percent of the control with no added inhibitor.

**Table 1.** Protein content and elastase inhibitor content of Macadamia nut, Brazil nut, soybean, and marama bean.

Source	% Protein	% Protein in $\text{NH}_4\text{SO}_4$ fraction	Elastase inhibitor (U/mg protein)	Inhibitor total units
Macadamia	14.0	1.5	0	0
Brazil	15.8	1.3	7.4	3.5
Soybean	39.5	4.6	66	1036
Marama bean	30.8	27	258	6991



**Figure 2.** Elution profile of elastase and trypsin inhibitory activity from soybean trypsin inhibitor using an elastase affinity column. The column wash included fractions 1–50 and the citrate elution included fractions 51–77. Protein measured at 280 nm is illustrated by the dotted line, trypsin inhibitory activity by squares, and elastase inhibition by open circles. Each fraction was 2 mL. Inhibitory activity was expressed as a percent of the control with no added inhibitor.



**Figure 3.** SDS PAGE of purified elastase inhibitor compared with soybean trypsin inhibitor. Lane 1, standard proteins with molecular mass shown; lane 2, two intensely-staining bands of apparent 17.8 kDa and 20 kDa in a reduced sample of the marama bean extract purified through the affinity column; lane 3, separation of reduced soybean trypsin inhibitor indicating major bands at 23.1 kDa and 24.0 kDa.

An image of the SDS PAGE (polyacrylamide gel electrophoresis) separation of proteins in the pooled fractions of marama bean extract that eluted from the elastase affinity column with pH 2.2-citrate buffer is shown in Figure 3. Two major bands can be seen, with apparent molecular weights of 17.8 kDa and 20 kDa. The bands were excised and the protein eluted from the gel with water and assayed

**Table 2.** *De novo* sequences obtained from HPLC-ESI-MS/MS analysis of the elastase-specific inhibitor in marama bean.

Peptide	RT (min) <sup>a</sup>	Sequence <sup>b</sup>
1	20.0	AVVXNPHN
2	20.0	AVVXNPH
3	22.9	LM <sub>ox</sub> YYPDSN
4	23.5	LM <sub>ox</sub> YYPDS
5	24.3	QVDVSPXGNNNQTK + glycan (most likely on D)
6	24.6	LMYYPDSN
7	25.1	LMYYPDS
8	26.7	AVVXNPHNTF
9	29.8	VVDTDGYPVGNHGQYXXPV <sub>--</sub>
10	30.2	VVDTDGYPVGDGHGQYXXPV <sub>--</sub>
11	31.2	XPYYFAPFQVHDAEK
12	33.6	XPYYFAPFQVHD
13	35.1	AVVXNPHNTFGXPVXFR

<sup>a</sup>Retention time in HPLC-ESI-MS/MS analysis.

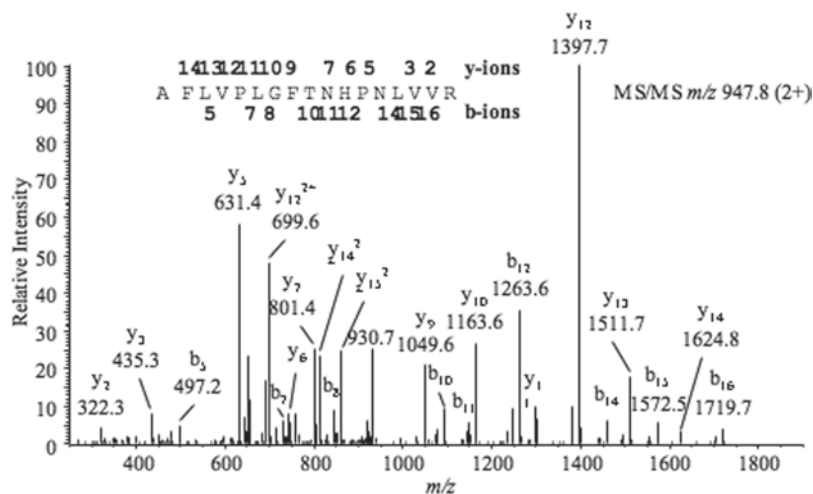
<sup>b</sup>X, I, or L; M<sub>ox</sub>, oxidized Met; --, the identity of these residues was not able to be determined.

for anti-trypsin and anti-elastase activity. The proteins that eluted from both of the major protein bands excised from the marama bean extract expressed elastase inhibitory activity, while no trypsin inhibition was detected in either band. In a previous report<sup>28</sup> from our laboratory, the  $K_i$  data for the elastase inhibitor from marama beans were derived from an impure ammonium sulfate fraction that we now know contains approximately 80% trypsin inhibitor and 20% elastase inhibitor. This indicates that the  $K_i$  values for the marama bean elastase inhibitor are actually four-fold lower than our published values. Re-calculation of the marama bean elastase inhibitor  $K_i$  using the corrected value for the protein content indicates that the  $K_i$  values are comparable to those obtained for other specific elastase inhibitors and correspond to uniformly strong inhibition of all sources of elastase we investigated.

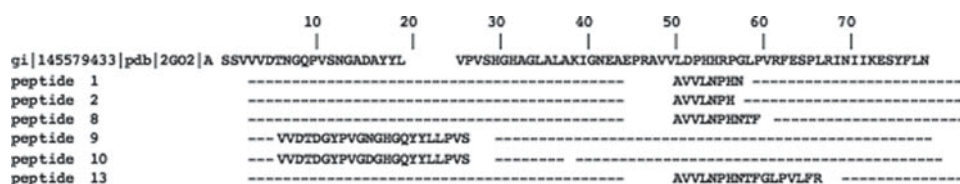
The results obtained by MALDI-TOF-MS and HPLC-ESI-MS/MS analyses indicated that the same protein was present in the two major bands of the marama bean elastase inhibitor. However, no high-confidence assignments were obtained by database searching of the tandem mass spectra, indicating a lack of sufficient identity with protein sequences in the NCBI database. As such, *de novo* analysis was undertaken of the tandem mass spectra, resulting in the series of amino acid sequences shown in Table 2. Several peptides appeared to be glycosylated, as fragments corresponding to the loss of sugar units were observed. Other peptides that were not glycosylated yielded more complete sequences. The tandem mass spectrum for peptide AVVXNPHNTFGXPVXFR is shown in Figure 4. BLAST (Basic Local Alignment Search Tool) searches (NCBI) indicated that several peptides had homology to Kunitz-like serine protease inhibitors, as shown in Figure 5.

## Discussion

We have isolated a protease inhibitor from marama bean that is specific for elastase and distinct from the marama



**Figure 4.** Tandem mass spectrum of a 1893.1-Da tryptic peptide from the marama bean elastase inhibitor; assigned b-series (*N*-terminal) and y-series (*C*-terminal) are indicated above the corresponding peaks and on the peptide sequence.



**Figure 5.** Sequence alignment of gi145579433 (Bbki, a Kunitz-type Kallikrein inhibitor, chain A) with peptides from the marama bean elastase inhibitor. Initial BLAST search indicated homology with gi145579433. A search of the peptides against Pfam family PF00197 using the Sequence Alignment Modeling system (SAM<sup>31,32</sup>) indicated that the peptides shown are significant matches.

bean trypsin inhibitor. The elastase inhibitor represents around 14 % of the total acid-extractable protein from the marama bean. It was detected as two separate, low-molecular-weight proteins with apparently identical amino acid sequences. The difference in migration of these proteins on SDS PAGE is most likely due to differences in attached carbohydrates.

BLAST searches of the sequences determined by *de novo* analysis of the tandem MS data indicated homology with Kunitz-like serine protease inhibitors. The Kunitz-type soybean trypsin inhibitor (STI) family consists mainly of protease inhibitors from *Leguminosae* seeds and contains from 170 to 200 amino acid residues and one or two intra-chain disulfide bonds. The highest level of conservation has been found in the *N*-terminal regions of these proteins. The Kunitz-type inhibitors exhibit activity against trypsin and subtilisin, thiol proteases, aspartic proteases, and elastase.

Soybean protease inhibitors have understandably received more attention than other plant inhibitors due to their widespread availability and contribution to the human diet. There has, however, been renewed interest in the marama bean due to its potential as a food source in arid regions. It is quite interesting that the marama bean contains significantly higher levels of elastase inhibitor than other known beans or nuts. The marama bean is native to only a small area of the world in or around the Kalahari Desert. If these protease inhibitors serve as a protective mechanism

against insects or other predators, it seems likely that there would be a predator that is unique to this area that contains high levels of elastase.

In summary, we have isolated and partially characterized a Kunitz-type serine protease inhibitor from marama beans. The inhibitor is present in two molecular weight forms with apparent differences in glycosylation. The inhibitor is present in unusually large quantities and is specific for inhibition of pancreatic and neutrophil elastase with no activity toward trypsin or chymotrypsin.

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