Partial Isolation and Characterization of a Cysteine Proteinase Inhibitor from Lima Bean (*Phaseolus lunatus*)

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Lima beans (*Phaseolus lunatus*) have been shown to contain cysteine proteinase inhibitor (CPI) activity, but the CPI has not been isolated or characterized. Accordingly, our objective was to isolate and partially characterize a CPI from lima bean. The isolation scheme included water extraction of lima bean flour followed by a chromatography series using DEAE Sepharose, Phenyl Sepharose, hydroxyapatite, and reversed-phase high performance liquid chromatography. This scheme resulted in the partial purification of a ~20 000-dalton protein with high inhibitory activity against papain. This isolated lima bean CPI had an N-terminal sequence homologous with other members of the cystatin class of CPIs. The protein was relatively heat labile; suggesting it could be inactivated with normal cooking, which is favorable for its use in transforming plants to create insect resistance.

Keywords: *Cysteine proteinase inhibitors; enzyme inhibitors; Phaseolus lunatus; lima bean*

INTRODUCTION

Food legume seeds are an economical and nutritious source of protein throughout the world, especially in developing countries (*1*). Farmers and consumers in developing countries who utilize dry beans and cowpeas suffer losses from destruction of the stored seeds by the bean weevil and the cowpea weevil, respectively. Numerous available insect-control methods are cost prohibitive to the small-scale farmers in third-world nations (*2*). Inhibition of the proteolytic digestive enzymes in bean weevils and cowpea weevils may be a key to controlling the destruction they cause. These insects and certain other insects of economic importance in the U.S. use a different mechanistic class of digestive enzymes than do higher mammalian species (*3*−*8*). Whereas mammals use serine proteinases in their digestive systems, many insect species belonging to the Coleopteran and other orders widely utilize cysteine proteinases in digestion. This makes cysteine proteinases a good target enzyme for inhibition of growth and development. The potential exists for naturally occurring cysteine proteinase inhibitors to be manipulated by genetic engineering to create plants that are resistant to infestation by certain classes of insects (*3*, *5*, *9*). Cysteine proteinase inhibitors (CPIs), which inhibit cysteine proteinases, are classified into three major families: stefins (type I cystatins), type II cystatins, and kininogens (*10*−*12*). Many plant-source cysteine proteinase inhibitors are cystatins. Some genetic variation exists between the cystatins found from different plant sources. This genetic variability may account for the different inhibitory potencies against specific cysteine proteinases. A wealth of research demonstrates the inhibition of insect digestive cysteine proteinases by well-studied CPIs (*3*, *5*, *6*, *7*, *13*−*19*). Research also has shown that plants can be transformed with genes that express CPIs to create insect resistance (*20*−*25*). Numerous plant-source CPIs have been isolated and tested against the digestive enzymes of insects that utilize cysteine proteinases. Results have indicated that CPIs from various sources differ in their efficacy against the cysteine proteinases of different insects (*14*, *17*, *18*, *26*−*29*). Lima beans have been shown to have high CPI activity (*30*), but a CPI from lima bean has yet to be isolated and compared to other CPIs. Therefore, the objectives of this research were to purify and partially characterize a CPI from lima bean.

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MATERIALS AND METHODS

Lima bean flour was extracted, and the extract was subjected to various chromatographic steps to partially purify a CPI. During purification the CPI was subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and to assays for protein content and CPI activity. The partially purified CPI was characterized regarding reaction with an antibody to CPI, heat stability, and N-terminal sequence analysis.

Cysteine Proteinase Inhibition Assay. The ^{3}H-methemoglobin (^{3}H-metHb) assay used by Hines et al. (*31*), which had been modified from Kitch and Murdock (*32*), was used to measure inhibitory activity during partial purification of lima bean CPI.

Protein Concentration Determination. The micro bicinchoninic acid (BCA) assay was used to determine the protein concentration of samples generated. Reagents were purchased in the Micro BCA Protein Assay Reagent Kit from Pierce (Rockford, IL).

Isolation of a Cysteine Proteinase Inhibitor. Whole lima beans var. Henderson, purchased in 50-pound bags from Burpee Seed (Warminister, PA), were coarsely broken and ground on a Buhler−Miag mill, model MLI-204, no. 1700 (Hertfordshire, England). Partially crushed lima beans were ground to a flour in a Janke and Kunkel KG (Staufen i. Breisgau, West Germany) water-cooled laboratory micro mill until a flour that could pass through a US 20-mesh screen was produced. Water extracts of lima bean flour were prepared according to the method of Hines et al. (*31*).

Anion-exchange chromatography was performed on a DEAE Sepharose Fast Flow column (Pharmacia-LKB Biotechnology,

10.1021/jf0007333 CCC: $20.00
Published on Web 02/02/2001

Table 1. Specific Activity and Purification Factor throughout Partial Purification of Lima Bean Cysteine Proteinase Inhibitor

fraction	protein conc. (μg/mL)	protein[a] content for listed activity (μg)	% papain inhibition[a]	protein for 50% inhibition (μg)	specific activity[b]	purification factor[c]	cumulative[d] purif. factor
water extract	10237	307	75%	205	0.00244		
DEAE[e] pooled fractions	2042	61	95%	32	0.0155	6	6
HIC[f] pooled fractions	157	4.7	93%	2.5	0.1979	13	81
hydroxyapatite pooled fractions	27	0.82	86%	0.47	1.0552	5	432
HPLC fractions	0.99	0.030	94%	0.016	31.6498	30	12971

[a] Protein content and papain inhibition for 30 μL of test solution used in ^{3}H-methemoglobin assay described in Materials and Methods. [b] Specific activity is defined as % papain inhibition achieved by a given amount (μg) of protein divided by that given amount of protein. [c] Purification factor is defined as the improvement in the amount of protein required for 50% inhibition of papain. [d] Cumulative purification factor through the individual steps is based on the increase in specific activity. [e] Diethylamine ethyl, an anion exchange chromatography resin. [f] Hydrophobic interaction chromatography, through use of a Phenyl Sepharose column.

Piscataway, NJ) with column dimensions of 2.5 × 34.5 cm. The column was equilibrated with 20 mM Tris, pH 8.0. Flow rate was controlled at 1.5 mL/min using a LKB model 2132 Microperex peristaltic pump (Pharmacia-LKB Biotechnology, Piscataway, NJ). Fractions of 5.25 mL were collected with a LKB model 2112 RediRac fraction collector. Approximately 30 to 40 mL of lima bean water extract was applied to the anion-exchange column. The column was subsequently washed with 525 mL of the Tris buffer. A gradient of 0 to 0.5 M NaCl in the Tris buffer, over 525 mL, was used to elute the protein. Fractions were measured for absorption at 280 nm to monitor protein content using a Perkin-Elmer Coleman model 571 spectrophotometer (Perkin-Elmer, Oakbrook, IL). Fractions were assayed for CPI activity using the ^{3}H-metHb/papain assay. To prepare for loading on the hydrophobic interaction column, fractions with papain inhibitory activity greater than 80% were pooled and dialyzed against a buffer of 0.75 M ammonium sulfate in 50 mM sodium phosphate, pH 6.2.

Hydrophobic interaction chromatography was performed on a Phenyl Sepharose 6 Fast Flow (low sub) column (Pharmacia Biotech, Piscataway, NJ) with column dimensions of 1.5 × 20 cm. The column was equilibrated with 0.75 M ammonium sulfate in 50 mM sodium phosphate, pH 6.2. Flow rate was controlled at 1.5 mL/min, and 1.5-mL fractions were collected. The dialyzed active DEAE fractions (about 30 mL of material) were applied to the column. The column was subsequently washed with 75 mL of the ammonium sulfate/sodium phosphate buffer. A gradient of 0.75 M to 0 M ammonium sulfate in a 50 mM sodium phosphate buffer, pH 6.2, over 75 mL, was used to elute the protein. The column was washed with 75 mL of 50 mM sodium phosphate, pH 6.2, containing no ammonium sulfate. Fractions were measured for absorption at 280 nm to monitor protein content and assayed for CPI activity using the ^{3}H-metHb/papain assay. Fractions from one peak with papain inhibitory activity greater than 80% were pooled.

Hydroxyapatite chromatography was performed on a Macro-Prep Ceramic Hydroxyapatite, Type I, media column (Bio Rad Laboratories, Hercules, CA) with column dimensions of 0.7 × 21 cm. The column was equilibrated with 10 mM potassium phosphate, pH 6.8. Flow rate was controlled at 0.75 mL/min, and 2.25-mL fractions were collected. Active HIC pooled fractions were dialyzed against the potassium phosphate buffer to prepare for application to the hydroxyapatite column. The column was subsequently washed with 112 mL of potassium phosphate buffer. A gradient of 10 mM potassium phosphate, pH 6.8, to 400 mM potassium phosphate, pH 8.0, over 225 mL, was used to elute the protein. Fractions were measured for absorption at 280 nm to monitor protein content and assayed for CPI activity using the ^{3}H-metHb/papain assay. The fractions from the one peak with papain inhibitory activity greater than 50% were pooled.

In preparation for loading on the HPLC reversed-phase column, the pooled active fractions from the hydroxyapatite column described previously were concentrated from 2 mL to approximately 200 μL using a YM-3 Centricon from Millipore Corporation (Bedford, MA). A Vydac (Hesperia, CA) reversed-phase C18 column, size 2.1 × 250 mm, was used. A variable wavelength absorbance detector (Applied Biosystems, Foster City, CA) was set at 214 nm. Following are the final parameters that were used: injection volume, 214 μL; Solvent A, 0.1% trifluoroacetic acid; Solvent B, 70% acetonitrile; flow rate, 175 μL/min; gradient profile, time 0 to 5 min, 0% Solvent B; 5 to 15 min, 0−48% Solvent B; 15 to 75 min, 48−58% Solvent B. Protein peaks were collected and assayed for papain inhibition in the ^{3}H-methemeglobin assay.

Characterization of the Isolated Cysteine Proteinase Inhibitor. Protein samples throughout partial purification of lima bean CPI were analyzed for cross reactivity with antibodies raised against the N_2 recombinant soybean CPI by the method reported by Towbin et al. (*33*).

The molecular weight of the isolated CPI from lima bean was determined by SDS−PAGE using low-molecular-weight markers of 19 200−104 000 daltons (Pharmacia-LKB).

The N-terminal amino acid sequence of the CPI was determined from a sample of approximately 50 pmol. The sequence was determined by the Purdue Laboratory for Macromolecular Structure using an Applied Biosystems protein sequencer, model 473A (Applied Biosystems, Foster City, CA). This sequencer utilizes Edman degradation chemistry in the gas phase. Twenty-two cycles were analyzed with the sequencer.

Heat stability of the CPI activity was determined with the ^{3}H-MetHb assay after 0.06 μg of the lima CPI in 10 mM potassium phosphate, pH 6.8, was heated to 100 °C for 30 min in sealed Eppendorf microcentrifuge tubes.

RESULTS AND DISCUSSION

A purification scheme for lima bean CPI, including water extract of lima bean flour, DEAE Sepharose chromatography, Phenyl Sepharose chromatography, hydroxyapatite chromatography, and reversed-phase HPLC, resulted in the partial purification of a ~20 000-dalton protein with high papain inhibitory activity. Chromatograms from anion exchange, hydrophobic interaction, hydroxyapatite, and reversed-phase HPLC chromatographies are shown in Figures 1 and 2. A 12 971-fold purification from the extract to the partially purified lima bean CPI was achieved (Table 1). The reversed-phase HPLC column resulted in the greatest purification at 30-fold. The molecular weight was estimated to be 20 400 Da by SDS−PAGE (Figure 3). The active fraction from the reversed-phase HPLC revealed a major doublet band with two minor contaminating lower-molecular-weight bands. The major doublet was believed to be the lima CPI, because of the results of the Western blot visualization of the interaction with antibodies raised against the N_2 recombinant soybean CPI (data not shown). The doublet was the only protein that reacted with the antibodies in the Western blot of the reversed-phase HPLC fraction. Therefore, this protein was recognized by an antibody raised against a

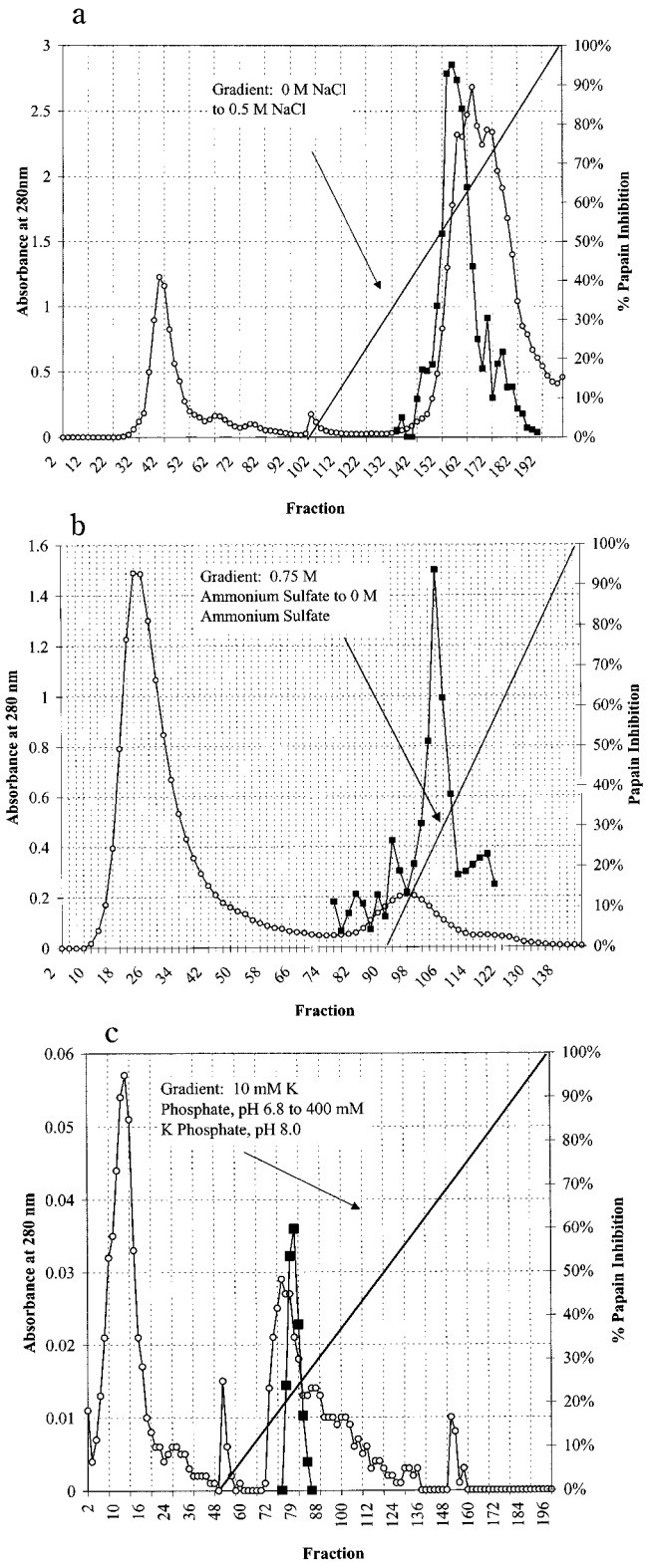

Figure 1. Chromatogram from lima bean cysteine proteinase inhibitor (CPI) isolation. (A) DEAE Sepharose chromatography of aqueous extraction of lima bean flour, (B) hydrophobic interaction chromatography (HIC) using DEAE Sepharose chromatography fractions with CPI activity, and (C) hydroxyapatite chromatography using HIC fractions with CPI activity. Details are given in Materials and Methods. ○, absorbance at 280 nm; ■, % inhibition.

CPI with very similar amino acid sequence and function. The doublet witnessed in the SDS−PAGE was similar to the results found by both Brzin et al. (*34*) and Hines et al. (*31*) for native soybean CPIs that each isolated.

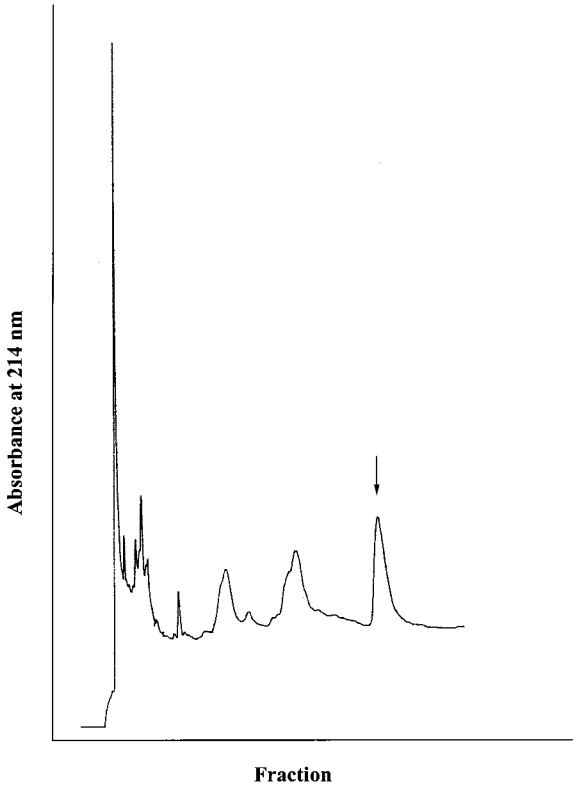

Figure 2. Chromatogram of reversed-phase HPLC chromatography using hydroxyapatite chromatography fractions with cysteine proteinase inhibitor activity (details given in Materials and Methods). The arrow identifies the peak with CPI activity.

The native soybean CPIs isolated by Brzin et al. (*34*) and Hines et al. (*31*) have identical N-terminal sequences. The two bands in the doublet observed in Figure 3 are likely the same protein, with the lower-molecular-weight band having a minor modification during purification (i.e., possible cleavage of a portion of the N-terminus). The two minor bands that contaminated the major protein of interest, as visualized in Figure 3, were estimated to constitute less than 20% of the total protein content. This was suggested by the ability to achieve a single N-terminal amino acid sequence from the mixture.

An N-terminal amino acid sequence based on 22 cycles was obtained for the lima bean CPI (Table 2). The positively identified residues showed high homology with the native soybean CPI isolated by Hines et al. (*31*) and the cowpea recombinant CPI isolated by Fernandes et al. (*35*). In fact, of the 20 positively identified residues in the lima bean CPI, there is 75% conservation as compared to the cowpea CPI. Homology also is seen with the rice oryzacystatin I and II isolated by Abe et al. (*12*) and Kondo et al. (*28*), respectively, soyacystatin isolated by Misaka et al. (*36*), and corn cystatin isolated by Abe et al. (*37*). A characteristic peptide sequence (leu, ala, arg, phe, ala, val) found in many cystatins including the cowpea CPI and soybean CPI, oryzacystatin I and II, soyacystatin, and corn cystatin, is conserved in residues 12 through 17 of the lima bean CPI. This is clear evidence that the protein partially purified and sequenced which shows high papain-inhibitory activity most likely belongs to the cystatin class of CPIs. Another characteristically conserved pentapeptide found in cystatins, gln-val-val-ala-gly (*10*), found approximately between residues 45 and 57, was not observed for the

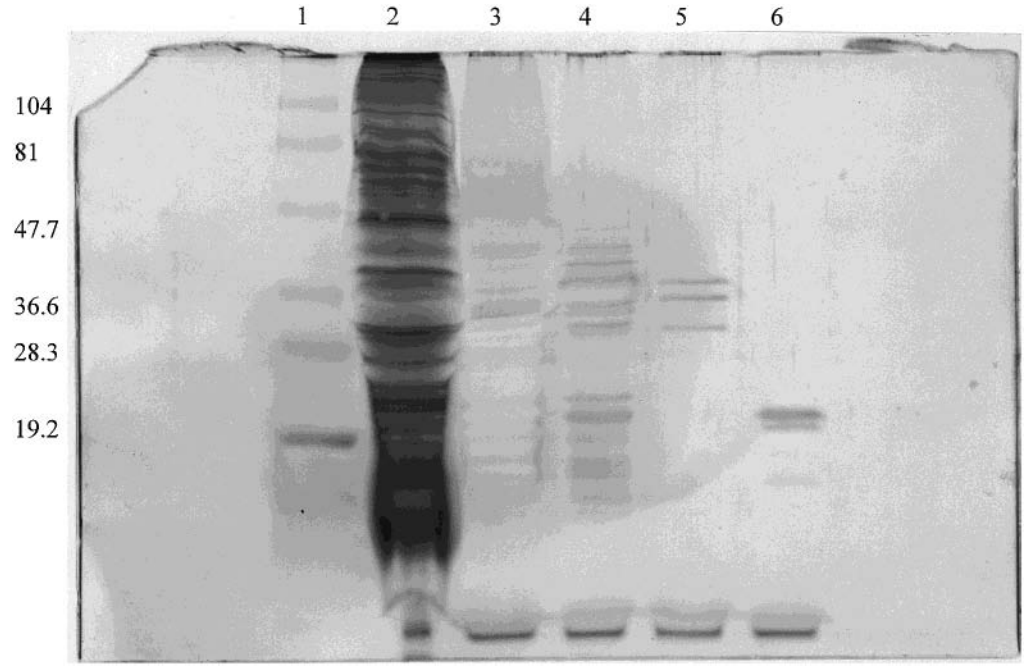

Figure 3. SDS−PAGE visualization of samples throughout partial purification of lima bean cysteine proteinase inhibitor (visualized by silver stain procedure). Lane 1, marker proteins; lane 2, lima bean flour water extract; lane 3, fractions of peak papain inhibition from DEAE Sepharose anion exchange chromatography; lane 4, pooled fractions of peak papain inhibition from Phenyl Sepharose hydrophobic interaction chromatography; lane 5, pooled fractions of peak papain inhibition from hydroxyapatite chromatography; lane 6, pooled fractions of peak papain inhibition from reversed-phase HPLC chromatography.

Table 2. Comparison of an N-terminal Amino Acid Sequence of a Cysteine Proteinase Inhibitor (CPI) from Lima Bean with Those of Other Plant Cystatins[a]

numbering for lima bean CPI	lima bean CPI	cowpea CPI[a] (*35*)	soybean CPI (*31*)	soya-cystatin[b] (*36*)	oryza-cystatin I[b] (*12*)	oryza-cystatin II[b] (*28*)	corn cystatin[b] (*37*)
		M			G		
		A			P		
		A			V		
		L			L		
		G			G		
		G			G		
		N	Q or V	L	V		I
		R	?	R	E	R	K
		D	F	D	P	Q	D
		V	I	S	V	P	V
1	L	A	T	Q	?	A	P
2	L	G	G	G	G	Q	A
3	V	N	A	S	N	R	N
4	**Q**	**Q**	**Q**	**Q**	E	E	E
5	**N**	**N**	**B or D**	**N**	**N**	**N**	**N**
6	**S**	**S**	?	**S**	D	D	D
7	**L**	**L**	I	V	**L**	**L**	**L**
8	**E**	**E**	?	Q	H	T	Q
9	**I**	**I**	**I**	T	L	T	L
10	**E**	D	**E**	**E**	V	V	Q
11	**S**	**S**	B or D	A	D	E	E
12	**L**	**L**	**L**	**L**	**L**	**L**	**L**
13	**A**	**A**	**A**	**A**	**A**	**A**	**A**
14	?	**R**	?	**R**	**R**	**R**	**R**
15	**F**	**F**	**F**	**F**	**F**	**F**	**F**
16	**A**	**A**	**A**	**A**	**A**	**A**	**A**
17	**V**	**V**	**V**	**V**	**V**	**V**	**V**
18	?	E	D	D	T	A	N
19	**E**	**E**		**E**	**E**	**E**	**E**
20	L	H		H	H	H	H
21	**N**	**N**		**N**	**N**	**N**	**N**
22	L	K		K	K	S	Q
		K		K	K	K	K
		Q		Q	A	A	A

[a] Bolding of amino acid residues in table denotes homology with lima bean CPI sequence. [b] Full known sequence for cowpea CPI, soyacystatin, oryzacystatin I and II, and corn cystatin not reported.

lima bean CPI because of the inability to continue N-terminal sequence analysis on a larger number of residues. The lima bean CPI N-terminal sequence also showed high homology with the sequences of three

Table 3. Comparison of an N-terminal Amino Acid Sequence of a Cysteine Proteinase Inhibitor (CPI) from Lima Bean with Those of Soybean Native and Recombinant Cystatins[a]

numbering for lima bean CPI	lima bean CPI	soybean CPI (*31*)	R1 soybean CPI (*15*)	N2 soybean CPI (*15*)	L1 soybean CPI (*15*)
		Q or V			
		?			
		F			
		I	I	V	V
1	L	T	T	H	T
2	L	G	G	G	G
3	V	A	A	A	S
4	**Q**	**Q**	**Q**	A	**Q**
5	**N**	**B or D**	**N**	**N**	**N**
6	**S**	?	**S**	**S**	**S**
7	L	I	I	V	V
8	**E**	?	D	**E**	**E**
9	**I**	**I**	**I**	**I**	**I**
10	**E**	**E**	**E**	N	D
11	S	B	N	N	A
12	**L**	**L**	**L**	**L**	**L**
13	**A**	**A**	**A**	**A**	**A**
14	**?**	**?**	**R**	**R**	**R**
15	**F**	**F**	**F**	**F**	**F**
16	**A**	**A**	**A**	**A**	**A**
17	**V**	**V**	**V**	**V**	**V**
18	?	D	D	E	E
19	**E**		**E**	**E**	**E**
20	L		H	Q	H
21	**N**		**N**	**N**	**N**
22	L		K	K	K
			K	R	K
			E	E	Q
			N	N	N

[a] Bolding of amino acid residues in table denotes homology with the lima bean CPI sequence.

Table 4. Papain Inhibitory Activity of the Lima Bean Cysteine Proteinase Inhibitor after Heating at 100 °C for 30 Minutes

time of heat exposure[a]	CPM mean[b]	standard deviation	percent of control	papain inhibitory activity (%)
0 min	456	42.4	8.8	91.2
30 min	1758	295.6	84.9	15.1
control	2016.5	74.2	100	
blank	305.5	50.2		

[a] Exposure at 100 °C. [b] CPM, counts per minute. Lima cysteine proteinase inhibitor assayed for % papain inhibition as described in Materials and Methods.

recombinant CPIs: R1, N2, and L1, from soybean (Table 3) (*15*). In fact, of the 20 positively identified residues in the lima bean CPI, there is 60% conservation as compared to both R1 and L1 soybean CPI and 55% conservation as compared to N2 soybean CPI. Based on the sequence homology with many plant-source cystatins and its ability to inhibit papain, the lima bean CPI is seemingly a member of the cystatin superfamily.

Upon heating at 100 °C for 30 min, the papain inhibitory activity of the lima bean CPI was greatly reduced (Table 4). This heat labile characteristic is favorable for its use in transforming other legume plants to introduce insect resistance. Intact legume seeds would normally be cooked to a greater extent than 100 °C for 30 min. to reduce the toxicity from the lectins present (*38*). Even though this in vitro trial may not directly correlate to stability within a seed, it is likely that the lima CPI would be inactivated with normal cooking of intact legume beans, thereby conferring a greater assurance of safety for the mammalian digestive system. However, heating for 30 min. at 100 °C is a rather harsh treatment that would inactivate the biochemical activity of most proteins. Therefore, it would be desirable to determine the minimum time and temperature at which the lima bean CPI loses the papain inhibitory activity. Further heat stability was not pursued as part of this study because of insufficient quantities of lima bean CPI available at the end of the established purification sequence. It is recommended that further investigation of the heat stability issues could be accomplished in conjunction with efforts to isolate a recombinant lima CPI.

Given the sequence homology with other well studied CPIs and the comparatively high papain inhibitory activity, the lima bean CPI is a good candidate for further studies of its action on insect midgut digestive proteases and development of plants with improved insect resistance. Theoretically, plants could be transformed with the genes encoding for the lima bean CPI. When insects that depend on cysteine proteinases for protein digestion consume the expressed lima bean CPI as part of their dietary protein source, their mortality rate, growth rate, and fecundity should be adversely affected.

ABBREVIATIONS USED

BCA, bicinchonic acid; °C, degrees Celsius; cm, centimeter; CPI, cysteine proteinase inhibitor; CPM, counts per minute; Da, dalton; dd water, deionized distilled water; DEAE, diethyl amino ethyl; ^{3}H, tritium; ^{3}H-metHB, tritiated methemoglobin; HIC, hydrophobic interaction chromatography; HPLC, high-performance liquid chromatography; μL, microliter; M, molar; mM, millimolar; min, minute; mL, milliliter(s); mm, millimeter(s); nm, nanometer(s); SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

ACKNOWLEDGMENT

This is paper number 16331 of the Purdue University Agricultural Research Programs. We thank Dr. Larry Murdock of Purdue University for preparation of the [^{3}H]-methemoglobin. We also thank Kathy Sagarsee, Ana Patricia Rodriguez Burger, and Karrie Westerberg for their help, dedication, and attention to detail in completing the laboratory work.

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Received for review June 19, 2000. Revised manuscript received November 29, 2000. Accepted December 1, 2000. This research was supported in part by the USDA NRICGP award grant 91-33707-6443.

JF0007333