Isolation and Partial Characterization of a Soybean Cystatin Cysteine Proteinase Inhibitor of Coleopteran Digestive Proteolytic Activity

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A 12 000-Da protein with cysteine proteinase inhibitory activity characteristic of cystatins was isolated from soybean seeds. The inhibitor was homologous in N-terminal amino acid sequence to rice oryzacystatin and chicken egg white cystatin. It inhibited papain and ficin but did not inhibit bromelain, bovine trypsin, bovine α-chymotrypsin, and porcine pepsin. The soybean cystatin also inhibited, to varying degrees, the proteolytic activity of crude extracts of larval midguts of several Coleopteran insects with known digestive cysteine proteinase activity. Of the insects tested, the inhibitor was most effective against extracts of the cowpea weevil, *Callosobruchus maculatus*, and the red flour beetle, *Tribolium castaneum*. The soybean cystatin inhibitor was similar in potency on a molar basis to that of the cysteine proteinase inhibitors E-64 and chicken egg white cystatin. The isoelectric point of the soybean cystatin was pH 5.3, and a glycoprotein stain was negative. The inhibitory activity of the protein was lost after heating at 100 °C for 30 min.

INTRODUCTION

It has been suggested that the endogenous resistance of stored pulse crops, such as dry beans (*Phaseolus vulgaris*) and cowpeas (*Vigna unguiculata*), to insect pests could be improved by manipulating primary gene products such as proteinase inhibitors, lectins, and α-amylase inhibitors in the pulse crops (Foard et al., 1983). Recent research has shown that proteinases of the cysteine class (EC 3.4.22) are a major component of larval digestive systems for pests of important legume food crops. Cysteine proteinases have been isolated and partially characterized from the midgut of the larval bean weevil, *Acanthoscelides obtectus* (Say) (Wieman and Nielsen, 1988), and cowpea weevil, *Callosobruchus maculatus* (F) (Gatehouse et al., 1985; Kitch and Murdock, 1986; Campos et a., 1989).

Cysteine proteinases are apparently commonly used by many beetles (Coleoptera) for dietary protein digestion (Murdock et al., 1987) and appear to be a vulnerable target for inhibitors specific to cysteine proteinases. Utilization of cysteine proteinases may be an evolutionary adaptation that enables insects to consume legume seeds and other plant materials that are naturally high in serine proteinase inhibitors (Ryan, 1990).

Artificial diet bioassays have provided evidence that these larval cysteine proteinases are essential for normal growth and development of the insect. In these studies, proteinase inhibitors specific for cysteine or serine proteinases were incorporated into artificial seed diets. Mortality and developmental time from egg to adult were monitored. The addition of E-64 [1-(L-*trans*-epoxysuccinylleucylamino)-4-guanidinobutane], a specific and potent low molecular weight cysteine proteinase inhibitor (Hanada et al., 1978), to the artificial diets had a strong negative effect on development and survival of *A. obtectus* (Hines et al., 1990), *C. maculatus* (Murdock et al., 1988), and *Leptinotarsa decemlineata* (Colorado potato beetle) (Wolfson and Murdock, 1987). These negative effects were reversed for *A. obtectus* by the addition of free amino acids to the artificial seeds, in a ratio identical with that found in *P. vulgaris* storage proteins (Hines et al., 1990).

Proteins capable of selective and specific inhibition of cysteine proteinases have been identified and isolated from a variety of animal, microbial, and plant tissues. Fossum and Whitaker (1968) isolated a protein from chicken egg white that was capable of inhibiting the plant cysteine proteinases papain and ficin but not trypsin or chymotrypsin. This inhibitor was not related to the ovomucoid and ovoinhibitor serine proteinase inhibitors found in chicken egg white. Barrett (1981) proposed naming this inhibitor "cystatin" and using this term as the name of a superfamily of cysteine proteinase inhibitors found in a variety of animal tissues and homologous with the chicken egg white cystatin (Barrett, 1987).

Abe and Arai (1985) isolated a cysteine proteinase inhibitor, oryzacystatin, from rice seeds and found this inhibitor homologous with the cystatin superfamily (Abe et al., 1987). Cysteine proteinase inhibitors have been isolated from potato (*Solanum tuberosum* L.) (Rodis, 1974; Akers and Hoff, 1980; Rodis and Hoff, 1984; Brzin et al., 1988), pineapple stem bromelain powder (*Ananas cosmosus*) (Perlstein and Kezdy, 1973), cowpeas (*Vigna unguiculata*) (Rele et al., 1980), mung beans (*Phaseolus aureus*) (Baumgartner and Chrispeels, 1976), seeds of the leguminous *Bauhinia* tree (Goldstein et al., 1973), pumpkin seeds (*Cucurbita maxima*) (Zimacheva et al., 1988), maize (*Zea mays*) (Abe et al., 1980), and Brazilian beans (*Enterolobium contortisiliquum*) (Olivia et al., 1988). Learmonth (1951, 1958) identified papain inhibitory activity in soybean (*Glycine max*) flour, which he believed was concentrated in the "germ" or hypocotyl of the seed (Learmonth, 1958). Brzin et al. (1990) isolated two forms of a papain inhibitor from soybean seeds (12 000 and 13 000 Da) that were homologous to several cystatin proteins. Hines et al. (unpublished results) screened a large number of legume seeds and found soybeans, in general, to be highest of those tested in cysteine proteinase inhibitory activity. The Century and Tracy varieties of soybeans were found to be highest in cysteine proteinase inhibitory activity. Extracts from the hypocotyl of the seed contained the highest cysteine proteinase inhibitory activity relative to protein concentration.

The objective of the present research was to isolate a cysteine proteinase inhibitor from a legume seed source, characterize it, and determine through in vitro assays if

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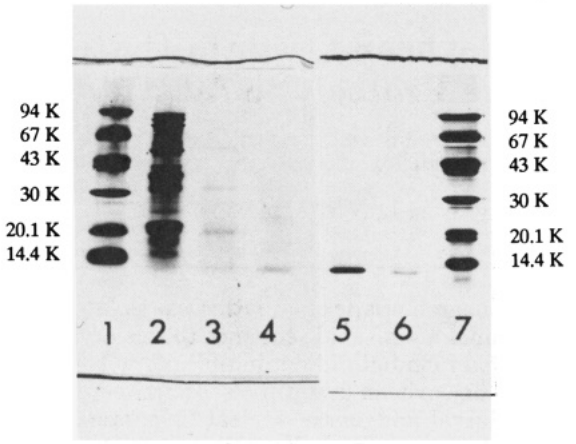

Figure 1. SDS–PAGE of isolation of cystatin cysteine proteinase inhibitor from soybean seeds. (Lanes 1 and 7) Marker proteins (14.4–94 kDa); (lane 2) water extract; (lane 3) DEAE anion-exchange "breakthrough" fraction with high papain inhibitory activity; (lane 4) affinity eluate fraction with high papain inhibitory activity; (lane 5) purified soybean cystatin; (lane 6) same sample as lane 4. The SDS–PAGE gel was 7.5–25% acrylamide and was stained with Coomassie Blue R250.

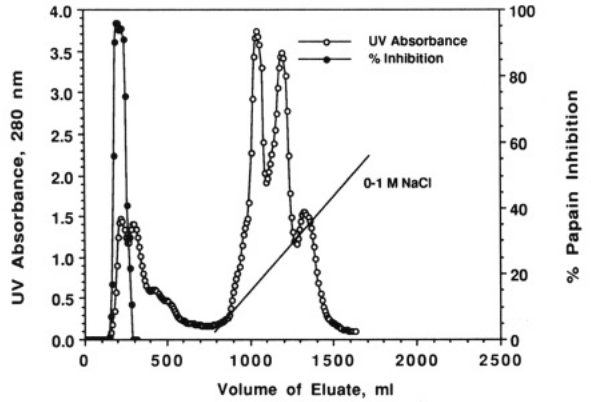

Figure 2. Chromatogram from DEAE-Sepharose anion-exchange chromatography of hypocotyl-rich soybean flour extract. The 5 × 10 cm column was equilibrated with 50 mM sodium phosphate buffer, pH 6.2. After 1.5 column volumes of 50 mM sodium phosphate buffer, pH 6.2, was allowed to pass through, a linear gradient of 0–1 M NaCl, over 1000 mL, was eluted through the column. Eight milliliters per test tube was collected at a flow rate of 90 mL/h. Contents of the tubes were assayed for percent papain inhibition and monitored for 280-nm UV absorbance.

it is effective against digestive proteinase activity in midguts of larval insects that utilize digestive cysteine proteinases. The gene coding for an effective inhibitor would be a good candidate to use in transformation of dry beans, cowpeas, and other legume seeds to confer insect resistance.

MATERIALS AND METHODS

Cysteine Proteinase Inhibition Assay. A modification of the [^{3}H]methemoglobin ([^{3}H]metHb) assay of Kitch and Murdock (1986) was used to monitor inhibitory activity during isolation of the cysteine proteinase inhibitor (CPI) from soybean seed extracts. Papain (2× crystallized and lyophilized, Sigma Chemical Co., St. Louis, MO) or a crude gut extract of *A. obtectus* was used as the cysteine proteinase source for testing inhibitory activity. The assay with papain utilized 10 μL of 1.7 μM papain in 0.2 M sodium phosphate buffer, pH 6.2, and 30 μL of the extract or fraction to be assayed. Inhibition was determined as the percent reduction in counts per minute (cpm) of digested [^{3}H]metHb, compared to a control. To verify inhibitory activity against the digestive proteolytic activity of *A. obtectus*, fractions from the isolation steps were also assayed against a crude extract of the midgut of larval *A. obtectus*, prepared as described by Wieman and Nielsen (1988).

The index used for determining relative purity of the CPI during isolation was the amount of protein required for 50% inhibition of papain in the assay described above. Up to 10 different concentrations of the samples from the isolation procedure were assayed against papain, and the data from percent inhibition vs protein quantity were used to obtain regression equations. Protein concentration was determined according to the Lowry method (Lowry et al., 1951) or a microBCA method (Pierce, Rockford, IL).

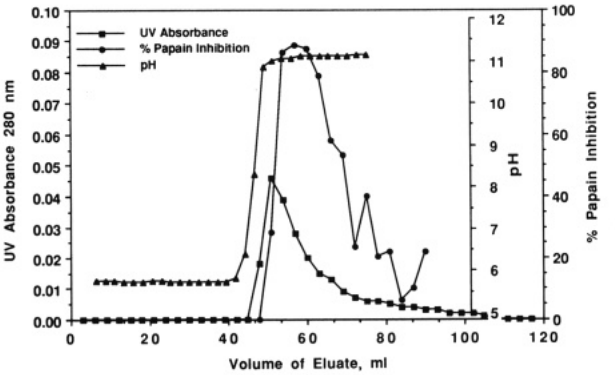

Figure 3. Chromatogram from carboxymethylated (CM) papain Sepharose affinity chromatography of DEAE-Sepharose fraction containing papain inhibitory activity. The fraction represented by eluate volume 150–300 mL (Figure 2) was applied to the affinity media in a 2-L Erlenmeyer flask in 0.2 M sodium phosphate, pH 6.2, with 0.1% Triton X-100, 0.5 M NaCl, and 10 mM L-cysteine (all concentrations represent final volume) added. This was mixed gently for 3 h at 20 °C on a rotary shaker and poured into a 2.5 × 10 cm column. The column was washed with 200 mL of 0.2 M sodium phosphate buffer, containing 0.1% Triton X-100 and 0.5 M NaCl, followed with 200 mL of 0.2 M sodium phosphate buffer, pH 6.2. The column was eluted with 0.2 M sodium phosphate, pH 11.8, at a flow rate of 60 mL/h, collecting 3 mL per test tube. Contents of the test tube were assayed for percent papain inhibition and monitored for 280-nm UV absorbance and pH.

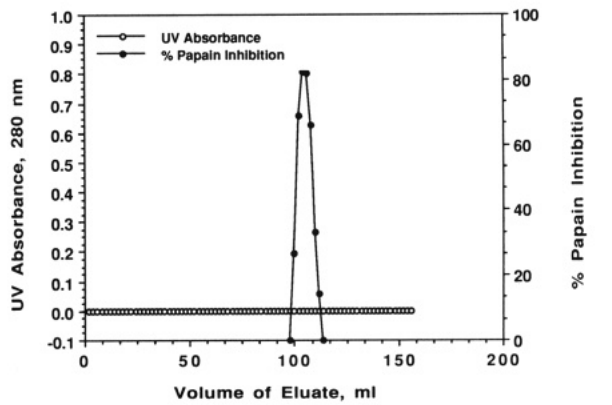

Figure 4. Chromatogram from size exclusion chromatography of CM-papain Sepharose affinity column fraction containing papain inhibitory activity (Figure 3). Pooled fractions containing papain inhibitory activity from eight CM-papain affinity column runs were concentrated to 0.5 mL and applied to the size exclusion chromatography column. The three 3-mL eluates containing the highest papain inhibitory activity of each affinity column run were pooled and concentrated. The size exclusion chromatography column was of Sephacryl S-200 HR media and in a 1.6 × 87 cm column. The column was equilibrated and run with 20 mM sodium phosphate buffer, pH 6.2, with 2% (v/v) glycerol and 0.04% sodium azide (w/v) and run at a flow rate of 30 mL/h with 2 mL collected per test tube. The contents of each tube were assayed for percent papain inhibition and monitored for 280-nm UV absorbance.

Isolation of a Cysteine Proteinase Inhibitor. A soybean hypocotyl-rich flour was prepared by coarsely grinding whole Century soybeans (Agricultural Alumni Seed Improvement Association, Romney, IN) through an Allis-Chalmers 6 × 6 roller mill (Allis-Chalmers, Milwaukee, WI). The roller mill used had Dawson-type rollers with eight corrugations per inch, a spiral of 3/8 in./ft, operating at 600 rpm. A gap between the two rollers was 4 mm. The gap was adjusted to coarsely crush the soybeans,

Table I. Summary of Purification of a Cysteine Proteinase Inhibitor from Soybean Hypocotyl-Rich Fraction

purif step	total protein,[a] mg	protein for 50% inhibition,[b] μg	purif factor[c]	CPI,[d] mg	CPI units[e] (×10³)	CPI yield,[f] %	sp act.[g]	cum purif[h]
extract	6300	83.9		6.01	75.1	100	0.0119	
AEC[i]	281	8.5	9.9	2.64	33.0	43.9	0.118	9.9
affinity	0.288	0.18	48.4	0.128	1.6	2.1	5.56	467
SEC[j]	0.026	0.08	2.25	0.026	0.33	0.4	12.5	1050

[a] Total protein of eight accumulated runs for one size exclusion chromatography (SEC) run. [b] Protein for 50% inhibition of papain in [³H]metHb/papain assay. This was the index for papain inhibition. [c] Purification factor. The purification factor was defined as the improvement in the amount of protein required for 50% inhibition of papain. For example, 83.9 to 8.5 μg was a 9.9-fold purification factor. [d] Cysteine proteinase inhibitor. Quantity of CPI was estimated by assuming that the 0.026 mg of CPI isolated after the SEC step was 100% pure. The percent of specific activity from the previous step was the factor of purification. For example, 0.08 μg from 0.18 μg was 44%. This percent was assumed to be the percent of protein in the previous step that was CPI. Forty-four percent of 0.288 mg was 0.128 mg. [e] Cysteine proteinase inhibitor units. A CPI unit was defined as the amount of inhibitor (mg) that inhibited 0.17 μM (0.4 μg) of papain 50% in the [³H]metHb/papain assay. [f] Cysteine proteinase inhibitor yield. This was the accumulated recovery calculated as percent of CPI recovered from the extract purification step. [g] Specific activity. Defined as CPI units divided by protein (mg). [h] Cumulative purification through the individual steps, based on increase in specific activity. [i] Anion-exchange chromatography. [j] Size exclusion chromatography.

Table II. Inhibition of the Proteolytic Activity of Proteinases and Insect Larval Crude Gut Extracts by a Cystatin Cysteine Proteinase Inhibitor Isolated from Soybean Seeds[a]

enzyme	molar concn, μM	control cpm[b] av[d]	control cpm[b] SD[e]	CPI[c] cpm av	CPI[c] cpm SD	blank cpm av	blank cpm SD	% inhibition
		0.2 μg of Soybean Cystatin						
papain[f]	0.17	2980	36.1	420	16.2	178	4.4	91.4
ficin[f]	0.17	3190	46.3	560	54.8	178	4.4	87.2
bromelain[f]	0.17	2260	94.1	2110	40.5	178	4.4	7.0
trypsin[g]	0.17	2560	64.0	2460	NA	153	5.3	4.1
α-chymotrypsin[g]	0.17	2300	NA[i]	2240	92.3	153	5.3	2.4
pepsin[h]	0.17	4340	104.6	4440	NA	143	0.0	2.3
		0.4 μg of Soybean Cystatin						
papain	0.17	2650	46.2	348	NA	180	10.4	93.2
A. obtectus[j]	–[k]	2880	79.1	2180	60.1	180	10.4	26.0
C. maculatus	–	2470	112	436	7.9	180	10.4	88.8
Z. subfaciatus	–	2520	13.1	1960	109	180	10.4	24.1
T. castaneum	–	3430	85.2	857	57.5	172	NA	79.0
E. varivestis	–	3180	17.8	1390	45.2	172	NA	58.5

[a] Inhibitor assayed against [³H]methemoglobin in assay with enzyme specified. [b] Counts per minute. [c] Cysteine proteinase inhibitor. [d] Average. [e] Standard deviation. [f] Buffer was 0.2 M sodium phosphate, pH 6.2. [g] Buffer was 0.2 M Tris-HCl, pH 8.2, with 20 mM $CaCl_2$. Trypsin and α-chymotrypsin were of bovine source. [h] Buffer was 0.2 M sodium citrate, pH 3.1. Pepsin was of porcine source. [i] NA, not available because less than three samples were available. [j] All gut extracts were assayed with [³H]metHb assay described under Materials and Methods, using 0.2 M sodium phosphate buffer, pH 6.2. *Acanthoscelides obtectus* (common bean weevil). *Zabrotes subfaciatus* (Mexican bean weevil). *Callosobruchus maculatus* (cowpea weevil). *Tribolium castaneum* (red flour beetle). *Epilachna varivestis* (Mexican bean beetle). [k] The molar level for gut extracts is unknown.

releasing the hypocotyls from most seeds but allowing most of the cotyledons to stay in fragments large enough to be retained by the 5-, 7-, and 10-mesh screens. The partially crushed soybeans were sifted with a Smico laboratory model sifter (Smico Corp., Oklahoma City, OK) for 2 min through the following screens: U.S. 5, 7, 10, and 14. The screening was performed to collect a fraction with the highest concentration of the hypocotyls of the soybean seed. Most of the hypocotyls passed through the 5-, 7-, and 10-mesh screens but were retained by the 14-mesh screen. The fraction collected from the 14-mesh screen was aspirated by a Bates laboratory aspirator (Rapsco Co., Brookshire, TX) to remove hull fragments and then screened for 2–5 min on a 12-mesh screen with the 14-mesh screen below to further refine the "hypocotyl-rich" fraction. The crushed hypocotyl-rich fraction was ground in a Janke and Kunkel KG Model A10 laboratory micromill (IKA Werk, Staufen i. Breisgau) until fine enough to pass through a U.S. 20-mesh screen. The flour was then defatted by *n*-hexane, mixing the flour with 5–10 volumes of hexane (v/v) for 5–10 min. This mixture was filtered through Whatman No. 1 filter paper and the sediment washed three to five times with hexane to remove residual soybean oil. The defatted flour was air-dried in a laboratory hood until dry and free of hexane odor.

Defatted hypocotyl-rich soybean flour (4.5 g) was extracted with 50 mL of deionized, distilled (dd) water for 30 min at room temperature. The mixture was filtered through cheesecloth, strained by hand to obtain all liquid, and centrifuged at 12800*g* for 30 min at 20 °C in a Beckman J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA). The supernatant obtained was filtered through Whatman No. 1 filter paper. This supernatant was then used for isolation of a CPI by a sequence of anion-exchange, affinity, and size exclusion chromatography columns.

Anion-exchange chromatography was performed on a DEAE Fast Flow Sepharose column (Pharmacia-LKB Biotechnology, Piscataway, NJ), with column dimensions of 5 × 10 cm. The column was equilibrated with 50 mM sodium phosphate buffer, pH 6.2, prior to sample application. Approximately 35–40 mL of soybean flour extract, prepared as described previously, was applied to the anion-exchange column. A linear gradient of 0–2 M NaCl in 50 mM sodium phosphate buffer, pH 6.2, was used to elute the anion-exchange column. Flow rate was 90 mL/h, and 8 mL per test tube was collected. Eluates were monitored by UV absorbance at 280 nm and assayed for papain inhibition.

An affinity column of carboxymethylated (CM) papain Sepharose was prepared by inactivating free papain (2× crystallized and lyophilized, Sigma) and attaching to cyanogen bromide (CNBr) activated Sepharose (Pharmacia-LKB), according to the procedure of Anastasi et al. (1983). A 50-mL quantity of wet affinity medium, equilibrated in 0.2 M sodium phosphate buffer, pH 6.2, was mixed with the "breakthrough" DEAE fraction of approximately 80 mL with high papain inhibitory activity. Triton X-100 (Sigma) (0.1% v/v) and NaCl (0.5 M, final concentration) were added to the mixture. The affinity medium mixture was gently shaken on a Hoefer Model PR-70 red rotor rotary laboratory shaker (Hoefer Scientific Instruments, San Francisco, CA) for 3 h at room temperature. The mixed affinity medium was packed into a 2.5 × 10 cm column and washed with 200 mL of 0.2 M sodium phosphate buffer, pH 6.2, containing 0.1% Triton-X 100 and 0.5 M NaCl, and then with 200 mL of 0.2 M sodium phosphate buffer, pH 6.2. The

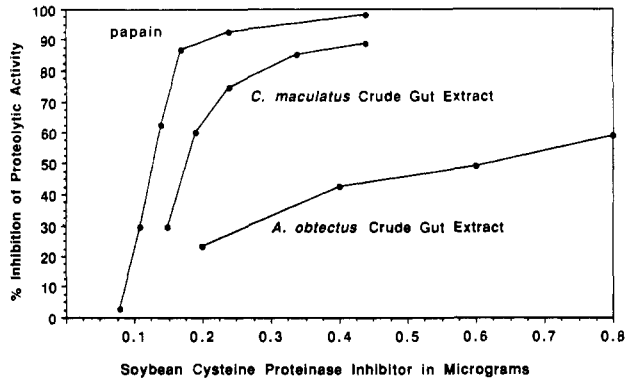

Figure 5. Inhibition of papain and crude gut extracts (CGE) of *A. obtectus* and *C. maculatus* at different levels of soybean cysteine proteinase inhibitor in [^{3}H]methemoglobin/papain and [^{3}H]-methemoglobin/CGE assays.

cysteine proteinase inhibitors were eluted with 0.2 M sodium phosphate buffer, pH 11.8. The flow rate for the pH 11.8 buffer was 60 mL/h, and 2-mL fractions were collected.

The fractions of papain inhibitory activity from four to eight affinity column runs were pooled and concentrated to 0.5 mL with Centricon 3 microconcentrators (Amicon Division, W. R. Grace & Co., Danvers, MA). The Centricon 3 microconcentrators were loaded to 2 mL and centrifuged in a JA-20 rotor in a J2-21 Beckman centrifuge at 7000*g* at 20 °C and pooled and recentrifuged until concentrated to 0.5 mL. This concentrated retentate was then applied to the size exclusion chromatography (SEC) column.

Sephacryl S-200 HR media (Pharmacia-LKB) was used in a 1.6 × 100 cm column for the SEC isolation step. The SEC media was equilibrated with degassed 20 mM sodium phosphate buffer, pH 6.2, containing 2% (v/v) glycerin and 0.04% sodium azide (w/v). Flow rate was 30 mL/h; eluates of 2 mL per test tube were collected, and the contents of each test tube were monitored for 280-nm UV absorbance and percent papain inhibition.

Tubes with the highest papain inhibitory activity were pooled and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fling and Gregerson, 1986). A Bio-Rad Mini Protean I gel electrophoresis system (Bio-Rad, Richmond, CA) was used for electrophoresis, and the gels were prepared with a gradient of 7.5–25% acrylamide. The gels were stained with Coomassie Brilliant Blue R250 (Sigma).

Characterization of the Isolated Cysteine Proteinase Inhibitor. The molecular weight of the isolated CPI was determined by SDS–PAGE using low molecular weight markers of 14 400–94 000 (Pharmacia-LKB). Also, a SEC column calibrated with bovine serum albumin, carbonic anhydrase, cytochrome *c*, and aprotinin was used to determine the molecular weight of the CPI. Elution volume was determined for DNP-glycine (total volume) and blue dextran (void volume). All were obtained from Sigma.

The N-terminal amino acid sequence of the CPI was determined from a sample of approximately 800 pmol. The sequence was determined at the Purdue Laboratory for Macromolecular Structure by using automated Edman degradation chemistry.

Aliquots of 0.2 μg (0.17 μM) of the isolated CPI were tested against 0.17 μM papain, ficin, bromelain, bovine trypsin, bovine α-chymotrypsin, and porcine pepsin by using the [^{3}H]metHb/papain assay described previously but with the following modifications: for ficin and bromelain, the enzymes and [^{3}H]metHb were dissolved in 0.2 M sodium phosphate, pH 6.2; for trypsin and chymotrypsin, the enzymes and [^{3}H]metHb were dissolved in 0.2 M Tris-HCl, pH 8.1, with 10 mM $CaCl_2$; and for pepsin, the enzyme and [^{3}H]metHb were dissolved in 0.2 M sodium citrate, pH 3.1. All enzymes tested (10-μL aliquots) were substituted for papain in the [^{3}H]metHb/papain assay.

The isolated CPI was assayed for inhibitory activity against larval gut extracts of five insect species known to have cysteine proteinase activity. These extracts were prepared, according to the method of Wieman and Nielsen (1988), and assayed by using [^{3}H]metHb as substrate. Extracts were made of guts from *A. obtectus*, *C. maculatus*, *T. castaneum* (red flour beetle), *E. varivestis* (Mexican bean beetle), and *Z. subfaciatus* (Mexican bean weevil). All these insects have been reported to utilize a cysteine digestive proteinase (Murdock et al., 1987). The gut extracts were diluted until all were similar in relative proteolytic activity against [^{3}H]metHb, with a target cpm of 2500–2900. The buffer used for all extract dilutions and assays was 0.2 M sodium phosphate, pH 6.2.

Table III. Amino Acid Sequence of the N Terminus of a Cysteine Proteinase Inhibitor (CPI) from Soybean Seeds[a]

numbering for soybean CPI	chicken egg white cystatin[b]	rice oryza-cystatin[c]	soybean CPI	soybean CPI[d]
	Ser			
	Glu			
	Asp			
	Arg			
	Ser	Met		
	Arg	Ser		
	Leu	Ser		
	Leu	Asp		
	Gly	Glu		
	Ala	Gly		
	Pro	Pro		
	Val	Val		
1	Pro	Leu	Gln or Val	
2	Val	Gly	?[e]	Gly
3	Asp	Gly	Phe	Phe
4	Glu	Val	Thr	Thr
5	Asn	Glu	Asp	Asp
6	Asp	Pro	Ile	Ile
7	Glu	Val	Thr	Thr
8	**Gly**	**Gly**	**Gly**	**Gly**
9	–[f]	Asn	Ala	Ala
10	–	Glu	Gln	Gln
11	–	**Asn**	**Asn or Asp**	**Asn**
12	–	Asp	?	Ser
13	–	Leu	Ile	Ile
14	–	His	?	Asp
15	Leu	Leu	Ile	Ile
16	Glu	Val	Glu	Glu
17	Arg	Asp	Asp or Asn	Asn
18	Ala	**Leu**	**Leu**	**Leu**
19	Leu	**Ala**	**Ala**	**Ala**
20	Glu	**Arg**	?	**Arg**
21	**Phe**	**Phe**	**Phe**	**Phe**
22	**Ala**	**Ala**	**Ala**	**Ala**
23	Met	**Val**	**Val**	**Val**
24	Ala	Thr	Asp	Asp
25	**Glu**	**Glu**		**Glu**
26	Tyr	His		His
27		**Asn**		**Asn**
28		**Lys**		**Lys**
29		**Lys**		**Lys**
30		Ala		Glu
31		**Asn**		**Asn**

[a] Sequence is compared to rice oryzacystatin 1, chicken egg white cystatin, and a soybean papain inhibitor isolated by Brzin et al. (1990). [b] Schwabe et al. (1984). [c] Abe et al. (1987). [d] Brzin et al. (1990). [e] ?, residue not positively identified. [f] Gap in sequence.

The soybean CPI was compared to E-64 (Sigma) (Barrett et al., 1982) and chicken egg white cystatin (Sigma) (Barrett, 1981) for inhibition against papain and the crude gut extracts of *A. obtectus* and *C. maculatus*. Equimolar quantities of the three CPIs were assayed against the three proteinase preparations, using the [^{3}H]metHb/papain and [^{3}H]metHb/crude gut extract assays.

A periodic acid stain (Dubray and Bezard, 1982) was performed on the soybean CPI electrophoresed on a 7.5–25% acrylamide SDS–PAGE gel (Fling and Gregerson, 1986) to determine if the inhibitor was a glycoprotein. The glycoprotein ovalbumin, in a low molecular weight SDS–PAGE marker protein set (Pharmacia-LKB), was used to confirm the stain.

Isoelectric focusing was performed to determine the isoelectric point (p*I*). A gel of pH range 3.5–9.5 was prepared according to the procedure of LKB Technical Bulletin 250 (Pharmacia-LKB) but modified by doubling the quantities of acrylamide and bis(acrylamide) and reducing glycerol from 7 to 4 mL.

Heat stability of the cysteine proteinase inhibitory activity was determined with the [^{3}H]metHb/papain assay after 0.4 μg

Table IV. Comparison of the Inhibitory Activity of E-64, Chicken Egg White Cystatin, and Soybean Cysteine Proteinase Inhibitor against the Proteolytic Activity of Papain and Crude Gut Extracts of *A. obtectus* and *C. maculatus*[a,b]

CPI[c]	molar strength, μM	CPI cpm[d] av[e]	CPI cpm[d] SD[f]	control cpm av	control cpm SD	blank cpm av	blank cpm SD	% inhibition
			Inhibition of Papain					
E-64	0.4	268	31.0	3240	122	169	21.6	96.8
	0.2	402	41.8	3240	122	169	21.6	92.4
CEW cyst[g]	0.4	350	10.3	3240	122	169	21.6	94.1
	0.2	864	97.4	3240	122	169	21.6	77.4
SBCPI[h]	0.4	290	5.6	3240	122	169	21.6	96.1
	0.2	481	70.9	3240	122	169	21.6	89.9
			Inhibition of *A. obtectus* Crude Gut Extract					
E-64	0.4	997	5.8	2830	56.8	154	2.1	68.5
	0.2	1260	7.8	2830	56.8	154	2.1	58.8
CEW cyst	0.4	843	7.7	2830	56.8	154	2.1	74.2
	0.2	1980	113	2830	56.8	154	2.1	31.8
SBCPI	0.4	1510	128	2830	56.8	154	2.1	49.4
	0.2	2010	99.9	2830	56.8	154	2.1	30.7
			Inhibition of *C. maculatus* Crude Gut Extract					
E-64	0.4	1370	42.5	3180	82.2	162	12.5	60.1
	0.2	1890	85.8	3180	82.2	162	12.5	42.6
CEW cyst	0.4	518	55.0	3180	82.2	162	12.5	88.2
	0.2	2560	132	3180	82.2	162	12.5	20.4
SBCPI	0.4	474	1.2	3180	82.2	162	12.5	89.7
	0.2	914	119	3180	82.2	162	12.5	75.1

[a] *Acanthoscelides obtectus* (common bean weevil). *Callosobruchus maculatus* (cowpea weevil). [b] Assays used were [^{3}H]metHb/papain and [^{3}H]metHb/crude gut extract assays. Buffer used was 0.2 M sodium phosphate, pH 6.2. [c] Cysteine proteinase inhibitor. [d] Counts per minute. [e] Average. [f] Standard deviation. [g] CEW Cyst, chicken egg white cystatin. [h] SBCPI, soybean cysteine proteinase inhibitor.

of the soybean CPI in 30 μL of water was heated at 100 °C for 30 min in sealed Eppendorf microcentrifuge tubes.

RESULTS AND DISCUSSION

A protein of approximately 12 000 molecular weight was isolated from soybean seed hypocotyl-rich extract. Chromatograms from affinity and SEC chromatographies are shown in Figures 2–4. Molecular weight was estimated to be 12 000 by SDS–PAGE (Figure 1). The isolation of the soybean CPI was achieved with a 1050-fold purification from the extract to the purified protein (Table I). Yield of the inhibitor was poor and was estimated to be approximately 0.4% of the original quantity of CPIs in the extract. The carboxymethylated papain Sepharose affinity column gave the greatest purification at 47-fold.

The soybean CPI inhibited papain and ficin nearly 100% but not the cysteine proteinase bromelain or bovine trypsin, bovine α-chymotrypsin, and porcine pepsin (Table II). It inhibited in vitro, to varying degrees, the proteolytic activity of crude gut extracts of five insect larvae known to have digestive cysteine proteinase activity (Table II). Most strongly inhibited were extracts from the cowpea weevil and red flour beetle. Extracts of the common bean weevil and Mexican bean weevil were inhibited much more weakly. It is unknown if the variability in the amount of inhibition by the soybean CPI was due to differences in binding affinities of the putative cysteine proteinases in the midguts of the insect larvae or if other proteases exist that supplement the putative cysteine proteinases.

Dose–response assays of papain and the crude gut extracts of *A. obtectus* and *C. maculatus* with the soybean CPI showed that papain and the crude gut extract of *C. maculatus* were both affected strongly by the inhibitor and in a concentration-dependent manner (Figure 5). The inhibition of the crude gut extract of *A. obtectus* was directly proportional to the concentrations tested, but the level of inhibition was much lower than that for papain or the crude gut extract of *C. maculatus*.

The N-terminal amino acid sequence of the soybean CPI (Table III) showed homology to rice oryzacystatin (Abe et al., 1987) and chicken egg white cystatin (Schwabe et al., 1984) and was identical with the papain inhibitor identified by Brzin et al. (1990). Cysteine proteinase inhibitors of the cystatin superfamily are evolutionarily related and generally inhibit papain and ficin but not bromelain (Barrett, 1987). On the basis of its sequence homology with rice oryzacystatin and chicken egg white cystatin and its ability to inhibit papain and ficin but not bromelain or the other proteinases tested, the soybean CPI is apparently a member of the cystatin superfamily.

The isoelectric point of the soybean cystatin was determined to be pH 5.3, on the basis of its position relative to the p*I* markers carbonic anhydrase I (pH 6.6) and carbonic anhydrase II (pH 5.9) (gel not shown). This is also in agreement with a major isoelectric variant identified by Brzin et al. (1990). A periodic acid glycoprotein stain was negative for the soybean cystatin.

There was no papain inhibitory activity with the soybean cystatin after 30 min of heating at 100 °C. Although this was an in vitro treatment and its correlation to stability within a seed is unknown, the complete loss of activity suggests that this inhibitor would be readily destroyed by normal cooking procedures of legume seeds. Combined with the lack of inhibitory action on trypsin, α-chymotrypsin, and pepsin, it is not likely that this inhibitor would affect human digestive proteinases if included in the human diet.

The soybean cystatin was similar to E-64 and chicken egg white cystatin in inhibiting papain and the crude gut extracts of *A. obtectus* and *C. maculatus* (Table IV). These results, along with the inhibition of the crude gut extracts of five insect larvae with recognized digestive cysteine proteinases (Table II), suggest that the soybean cystatin is a good candidate for further studies toward the development of dry beans and other legume seeds with improved insect resistance. Dry beans, cowpeas, and other legume seeds

could be transformed with the genes that encode the soybean cystatin. If insects that depend upon cysteine proteinases for dietary protein digestion consume these transformed seeds, their rates of growth, fecundity, or survivability should be negatively affected.

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