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# Comparison of Chemical Characteristics of Three Soybean Cysteine Proteinase Inhibitors

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Three recombinant soybean cysteine proteinase inhibitors (rSCPIs) L1, R1, and N2 were chemically characterized. These inhibitors have the potential to inhibit the growth and development of three major agricultural crop pests known to utilize cysteine proteinases (CPs) for protein digestion: Western corn rootworm, Colorado potato beetle, and cowpea weevil. Characterization data obtained show differences between the inhibitors and will be needed to consider the use of rSCPIs to create insect resistance in plants.

**KEYWORDS: Cysteine proteinase inhibitors; Western corn rootworm; Colorado potato beetle; cowpea weevil; insect digestive enzymes**

## INTRODUCTION

Crop damage caused by Western corn rootworms (WCR), Colorado potato beetles (CPB), and cowpea weevils (CW) is a major problem for agriculture and food industries. Failure of traditional insect control methods to reduce crop destruction has led to research seeking alternative means to manage these crop pests. Better understanding of insect digestive enzymes may be a key to making crops insect resistant. Recent work has indicated that cysteine proteinases are used by many insects for digestion (*1*). This indicates that a potential exists for naturally occurring cysteine proteinase inhibitors (CPIs) to be introduced by genetic engineering to create crop plants that resist infestation by these pests. Crop plants expressing CPI genes from other plant species would provide alternatives to the use of conventional pesticides.

Isolation in our laboratory of a native soybean CPI with inhibitory activity against insect digestive CPs (*2*) led to an effort to generate recombinant soybean CPIs (rSCPIs). Three unique cDNA clones (pL1, pR1, and pN2) that encode SCPI were isolated from an immature embryo ZAPII library by Polymerase Chain Reaction (PCR) of poly(A)+RNA from soybean (*Glycine max* L. Merr.) embryos, indicating the presence of CPI isoforms in this genotype (**Figure 1**) (*3*). The proteins encoded by these clones contained the consensus QxVxG motif and a W residue

```
      1                                                  50
L1    ..GNRDVTGSQNSVEIDALARFAVEEHNKKQNALLEFEKVVTAKQQVVSG
R1    LGGFTDITGAQNSIDIENLARFAVDEHNKKENAVLEFVRVISAKKQVVSG
N2    LGGITELTGAQNSVEINNLARFAVEEQNKRENSVLEFVRVISAKQQVVHG
      51                                                 100
L1    TLYTITLEAKDGGQKKVYEAKVWEKSWLNFKEVQEFKLVGDAPA.......
R1    TLYYITLEANDGVTKKVYETKVLEKPWLNIKEVQEFKPITVAVNPLSVTV
N2    VNYYITLEAKDGLIKNEYEAKVWVREWLNSKELIEFKPVNVSSTQ.......
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**Figure 1.** Deduced amino acid sequence of L1, R1, and N2 of the recombinant soybean cysteine proteinase inhibitors.

in the appropriate spatial context for interaction with the cysteine proteinase (CP) papain. From the amino acid sequence analyses, it was deduced that L1, R1, and N2 not only shared 60−70% sequence homology with one another but showed overlapping conserved amino acid sequences with oryzacystatin and chicken egg white cystatin. Notable differences among the rSCPIs, oryzacystatin, and the chicken egg white cystatin were that (1) the rSCPIs did not contain a N-terminal methionine, indicating that the cDNAs of the three rSCPIs did not encode for full-length open reading frames, and (2) at the N-terminal, the rSCPI N2 was one residue short, and the rSCPIs L1 and R1 were nine and seven residues short, respectively, compared to oryzacystatin. The three rSCPIs were devoid of any Cys residues, indicating that disulfide linkages did not contribute to their functional structure. The key residues in the first and second hairpin loops were conserved in all three rSCPIs, spatially located in appropriate positions downstream of the N termini (*4*).

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Analyses of kinetic data from all three rSCPIs indicated that they were noncompetitive inhibitors of papain, as indicated by the Lineweaver−Burk plots (*3*). This was contrary to the prevalent hypothesis that cystatins are reversible competitive inhibitors of papain (*5*). Zhao et al. (*3*) explained that the three rSCPIs tested are noncompetitive inhibitors on the basis of the "docking" model of CPI interaction with CPs as proposed from crystallographic data (*6*, *7*) and the inhibition kinetic data of oryzacystatin I (*8*). The inhibition constants for N2 and R1 were 57 and 21 nM, respectively, both of which were 100−500 times lower than the inhibition constant of L1 (19000 nM) (*3*). The $K_i$ values for N2 and R1 were 2.5−3 orders of magnitude lower than that of CPI L1. The $K_i$ values of N2 and R1 against papain are comparable to those of corn CPI and OC I, which are 37 and 32 nM, respectively (*8*). Although plant cystatins vary in activity against specific CPs (*3*, *9*, *12*), indicating target specificity, it has been deduced from work done by Zhao et al. (*3*) that wound-induced SCPIs exhibit greater relative inhibitory activity against both plant and insect CPs than does the constitutively expressed protein. After wounding, the soybean plants were able to continue normal growth and development (*3*).

In this paper we have partially characterized the rSCPIs on the basis of heat denaturation, optimal pH activity, and sensitivity to various human digestive enzymes, to enhance the understanding of the recombinant CPIs from a human safety point of view. In addition, we have evaluated the inhibitory efficacy of the rSCPIs on the basis of the retention of their inhibitory activity when exposed to crude gut extracts of WCR, CPB, and CW against reference E-64 at specific concentrations.

## MATERIALS AND METHODS

**Isolation of Three Recombinant CPIs.** Recombinant *Escherichia coli* DH5α, containing PCR products of open reading frames of L1, R1, and N2 in frame with the glutathione-*S*-transferase (GST) gene, was used to express the three recombinant proteins. The method of Guan and Dixon (*10*) was adopted to express, isolate, and purify the rSCPIs (*3*, *11*). The N2 also was produced using Phenyl-Sepharose and DEAE-Sepharose column chromatography as described by Koiwa et al. (*13*).

**[$^3$H]Methemoglobin Assay for pH and Heat Stability Tests.** *Assay Conditions*. The [$^3$H]metHb assay (*14*) was used to monitor (a) the CP activity of papain and crude plant extracts, (b) the effect of inhibitors on the CP activity in crude gut extracts of larval WCR, CPB, and CW, (c) the purification of rSCPIs, (d) the effect of pH on the inhibitory activity of the rSCPIs, and (e) the heat stability of rSCPIs. Determinations for all five types of assays were made in triplicate.

*Effect of pH on Inhibitory Activity of rSCPIs.* The activity dependence of the rSCPIs on pH was determined using the general [$^3$H]-metHb assay. rSCPIs were allowed to equilibrate in buffers ranging from pH 3.5 to 10.0. The buffers used for the blanks and samples were pH 3.5−6.0, 0.2 M sodium acetate; pH 6.5−8.0, 0.2 M PIPES; and pH 8.5−10.0, 0.2 M Tris. All of the buffers were adjusted to the same conductivity using NaCl. A 10-μg aliquot (from 1 mg/mL stock) of each rSCPI was analyzed per assay, and the assay was performed in triplicate with three batches of rSCPIs.

*Heat Stability of rSCPIs.* A 60-μL aliquot of the three rSCPIs (1 mg/mL) was pipetted into a 1-mL microcentrifuge tube and heated in a water bath maintained at 100 °C (*15*, *16*). The microcentrifuge tubes were removed at 1, 3, 5, 10, 15, 30, and 60 min and immediately placed in a trough of ice. The extent of denaturation of the three rSCPIs as a function of time was determined against the plant CP, papain (Sigma) (10 μg/assay), and the mammalian CP, cathepsin B (Sigma) (10 μg/assay). The three rSCPIs were assayed for residual activity by the general [$^3$H]metHb assay to obtain a time course heat denaturation profile. Three different preparations of rSCPI were each assayed in triplicate.

**Isoelectric Focusing (IEF).** IEF of the rSCPIs was performed to determine their isoelectric points. An IEF gel was used with a pH range of 3.5−10.0, using the procedure described in LKB Application Note 250 (Pharmacia-LKB Biotechnology).

**Digestion of rSCPIs by Trypsin, Chymotrypsin, and Pepsin.** rSCPIs were dissolved in appropriate buffer at 1 mg/mL, and aliquots of 20 μL were reacted with trypsin, chymotrypsin, or pepsin (*15*, *16*). The buffer systems selected for the different proteases were 50 mM HCl for pepsin and 50 mM Tris-HCl, pH 8.1, containing 20 mM $CaCl_2$ for trypsin and chymotrypsin. Heated and unheated rSCPIs (1 mg/mL) were digested at 37 °C with these enzymes after they had been solubilized in the appropriate buffer. Heat treatment of the rSCPIs was done in sealed tubes at 99 °C for 30 min. Unheated rSCPI digestion assays were conducted at ratios of 10:1; 25:1; 50:1; 75:1, and 100:1 rSCPI/enzyme (w/w), whereas ratios of 75:1; 100:1; 250:1; 500:1, and 1000:1 were used for all heated samples. Each of the rSCPI digestions was done for 1 min and for 10 min for every specified rSCPI/enzyme ratio. For each condition, assays were done in triplicate. At the end of digestion, the reaction was terminated by adding SDS-PAGE sample buffer in equal volume. All digests were boiled for 5 min before ~10 μg of protein was loaded on a 16.5% Tricine SDS-PAGE gel. The results were visualized using a standard Coomassie Brilliant Blue stain.

**Degradation of rSCPIs over Time by the Crude Gut Extracts of WCR, CPB, and CW.** *SDS-PAGE Visualization of the Degradation of the rSCPIs.* Cleaved and purified recombinant proteins were reacted with the crude gut extracts of WCR, CPB, and CW over time, and the degradation of the three rSCPIs was followed by Tricine SDS-PAGE (*14*). Digestive tracts from larvae (third instar of WCR; third and fourth instar of CPB; third instar of CW) were dissected into 100 μL of 0.2 M sodium acetate (pH 5.0) in a 1.5-mL Eppendorf microcentrifuge tube. The whole guts were homogenized using a tight-fitting tissue homogenizer while being held at 4 °C. The homogenate was centrifuged at 12800*g* for 5 min at 23 °C. The supernatant was diluted appropriately to give a 3-fold difference in counts between the blanks and the controls (i.e., normalized to show a 3-fold spread) with 0.2 M sodium acetate buffer (pH 5.0), for measurement of CP activity in the crude gut extract by the [$^3$H]metHb assay (*14*).

A 60-μL aliquot of each rSCPI at a concentration of 1 mg/mL (in 0.2 M sodium acetate, pH 5.0) was individually allowed to react with 100 μL of the crude gut extract of WCR, CPB, or CW and 20 μL of 50 mM cysteine. This reaction mixture was incubated at 37 °C. A 15-μL aliquot was removed at 0, 1, 3, 5, 10, and 20 h and then added to 15 μL of SDS-PAGE sample buffer solution. These samples were boiled for 5 min and cooled before application to a 16.5% Tricine SDS-PAGE gel. The gel was electrophoresed at 100 V for 90 min and stained with standard Coomassie Brilliant Blue R250 stain.

*Degradation of rSCPIs Monitored by the [$^3$H]metHb Assay.* The effect of exposure time of the rSCPI to the crude gut extract of WCR, CPB, or CW on residual CPI activity was monitored using the [$^3$H]-metHb assay at the end of the specified time intervals of 0, 1, 3, 5, 10, and 20 h.

## RESULTS AND DISCUSSION

**IEF of the rSCPIs L1, R1, and N2.** The isoelectric points of recombinant rSCPIs were as follows: (1) L1, pH 8.8 (major band), (2) R1, pH 7.4 (major band) and 6.3 (minor band), and (3) N2, pH 8.4 (major band) and 7.9 (minor band) (**Figure 2**). Each of these rSCPIs exhibited more than one distinct band upon isoelectric focusing, indicating that there could be more than one species of each protein. Sequence analysis of the recombinant proteins was consistent with the view that the two major species for each of L1, R1, and N2 are merely homologous proteins with differences in their N-terminal sequence regions.

In contrast to the isoelectric point of the native soybean CPI (SCPI) (*2*) and the soybean CPI identified by Brzin et al. (*17*), both of which were approximately pH 5.3, all three rSCPIs had significantly higher isoelectric points. R1, which closely resembles the native SCPI, had a higher isoelectric point (pH 7.4

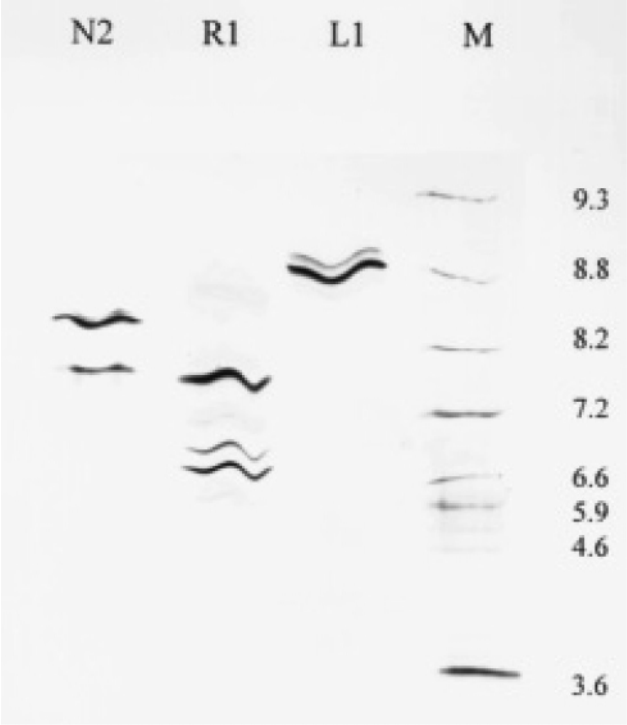


**Figure 2.** Isoelectric focusing (IEF) gel of recombinant soybean cysteine proteinase inhibitors (rSCPIs) N2, R1, and L1. Ten micrograms was used for each protein. Prestained IEF markers (p*I* range between 3.5 and 9.3) are shown.

and 6.3) than the pH 5.3 for SCPI (*2*). Brzin et al. (*18*) reported that three potato CPIs with molecular masses of 25, 22, and 22 kDa focused predominantly in the alkaline part of the gel at pH 6.6, 8.3, and 9.4, respectively. A chestnut seed cystatin with a molecular mass of 11 kDa and sharing a 70% sequence identity to a phytocystatin from cowpeas exhibited a p*I* of 6.9. Its amino acid sequence included all three motifs that are thought to be essential for inhibitory activity (*19*). The isoelectric points of two CPIs isolated from sunflower seeds, Sea and Scb, were 5.6 and 9.6, respectively (*20*).

**Heat Stability of rSCPIs L1, R1, and N2.** The rSCPIs L1, R1, and N2 are heat labile to varying degrees, with L1 being more heat stable than R1 and N2 (**Figure 3A−C**). When exposed to 100 °C for 60 min, R1 and N2 (10 $\mu$g) had no detectable inhibitory activity against the CPs papain and cathepsin B. With no heat exposure, R1 and N2 (10 $\mu$g) showed nearly 100% inhibitory activity against papain and cathepsin B. When R1 and N2 were exposed to moist heat for increasing periods of time, there was an exponential decrease in inhibitory activity against both papain and cathepsin B. Less than 10% inhibitory activity was detected after 15 min at 100 °C for both R1 and N2.

In contrast to R1 and N2, L1 (10 $\mu$g) when heated at 100 °C initially increased in activity against papain and then gradually decreased to 74% inhibition at 60 min. L1 inhibition of cathepsin B remained low throughout the heat treatment. The increase in inhibitory activity of L1 against papain after 1 min of heating suggested the possibility that L1 unfolds to a form that has higher inhibitory activity, by uncovering inhibitory sites inaccessible in the native form. A second experiment performed to assess the inhibitory activity of L1 with moist heat showed a gradual decrease in activity after 50 min at 100 °C (50% inhibitory activity) and 0% inhibition after 2 h (data not shown).

The heat-labile nature of the rSCPIs, especially N2 and R1, has favorable implications in transforming legume seeds with

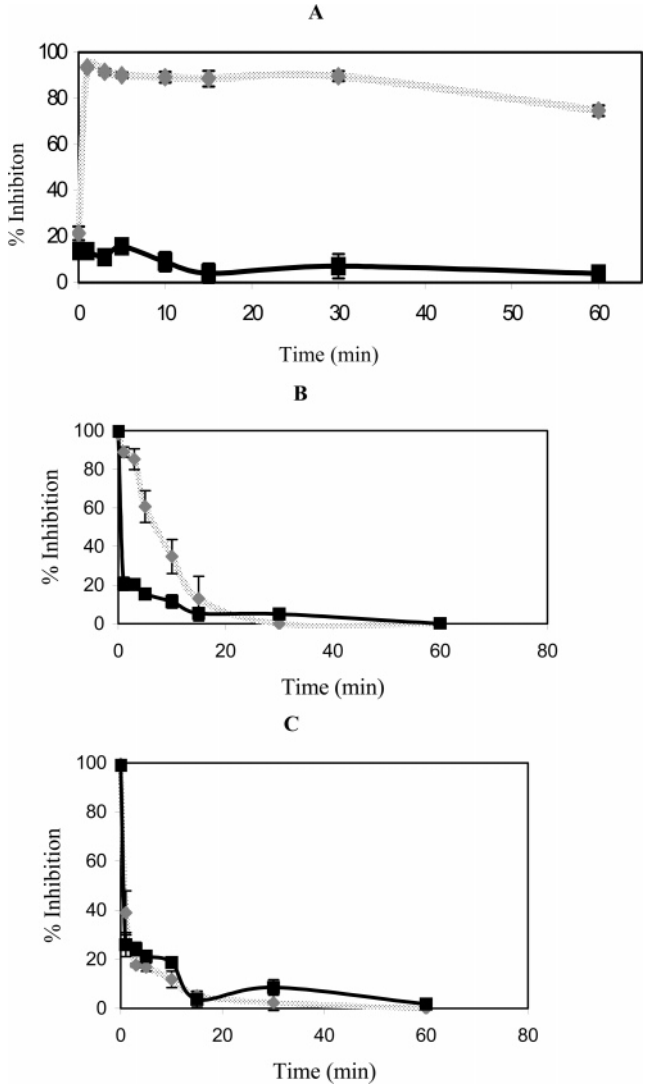


**Figure 3.** Time course heat inactivation profile of soybean cysteine proteinase inhibitors (rSCPIs) L1 (**A**), R1 (**B**), and N2 (**C**) against papain (gray symbols) and cathepsin B (black symbols). Mean and standard deviation of determinations on four batches of each purified rSCPI, assayed in triplicate, are shown.

CPIs, because heat lability would reduce any effect CPIs might have on the mammalian digestive systems. The native SCPI lost all inhibitory activity when exposed to 100 °C for 15 min (*2*). Like the native SCPI, there was no evidence based on the heat treatment data that the three rSCPIs will produce a negative effect at elevated levels in a human diet.

**pH Stability of rSCPIs.** The pH profiles for inhibitory activity of the three rSCPIs are shown in **Figure 4**. The pH had little effect on N2 inhibitory activity against papain, with 90−100% inhibition in the pH range 3−10. Unlike for N2, both R1 and L1 inhibitions of papain were affected by pH. Optimal inhibitory activity of R1 was detected at pH 3.0−5.0, at pH 7.0, and at pH 9.0−10.0. At pH 5.5−6.5 and 7.5−8.5, lower inhibitory activity was detected for R1. L1 showed multiple activity maxima, at pH 6.0−7.5 and 9.5.

In vitro studies to determine the inhibition profiles of the three rSCPIs to CPs were carried out at pH 5.0, or in some cases at pH 6.5 (in the case of testing for the presence of CPIs in isolation and purification steps) (Lalitha et al., submitted for publication). This was done to detect inhibition of the CPs at the optimal pH of the CPs of the three crop pests, WCR, CPB, and CW. Any variation from pH 5.0 in the optimal inhibitory activity of the

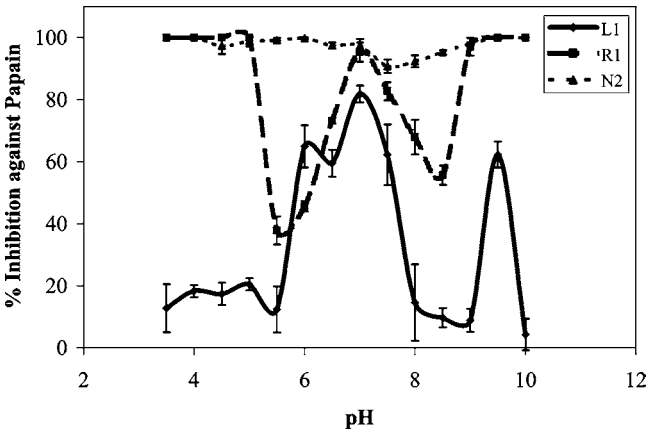


**Figure 4.** Effect of pH on inhibitory activity of recombinant cysteine proteinase inhibitors (rSCPIs) L1, R1, and N2 against papain. The rSCPIs were held at 4 °C for 15 h at the pH indicated prior to assay at that pH. Points are means of determinations on five batches of purified rSCPIs L1, R1, and N2, each assayed in triplicate, and standard deviation.

rSCPIs may have skewed the observations. For example, the low inhibitory effect of L1 on crude gut proteinases of WCR, CPB, and CW may be due to an inappropriate pH chosen for the activity assays. A mildly acidic pH range was shown to be an ideal range to test the activity of crude gut proteinases of WCR, CPB, and CW for inhibition with appropriate CPIs (Lalitha et al., submitted for publication).

**Enzymatic Hydrolysis of rSCPIs.** The enzymatic hydrolysis patterns of the three rSCPIs L1, R1, and N2 subjected to the digestion by human digestive enzymes trypsin, chymotrypsin, and pepsin were visualized by Tricine SDS-PAGE (**Figure 5**). The enzymatic digestions were carried out with both heated and unheated rSCPIs. On the basis of the amino acid sequences of the rSCPIs (*3*), it was predicted that the three rSCPIs would be susceptible to the digestive enzymes to varying degrees.

Tryptic digestion of the three unheated rSCPIs (**Figure 5A**) showed that all three rSCPIs were almost completely digestible at high concentrations of trypsin, with a gradual decrease in susceptibility at lower trypsin concentrations. There was little digestion at concentration ratios of 75:1 and 100:1 of CPI/enzyme (w/w) for all three rSCPIs regardless of the time period of digestion tested. Unheated R1 and N2 showed greater susceptibility to trypsin than did L1. Digestion of L1 to a low molecular weight peptide was noted only at the lower ratios of 50:1 and 75:1 (CPI/enzyme, w/w) compared to R1 and N2. In the case of the tryptic digestion of heated rSCPIs, lower CPI/enzyme ratios (i.e., lower concentrations of enzyme relative to rSCPI) were used because of the higher susceptibility of the proteins to digestion, due to conformational changes caused by thermal denaturation. Among all three rSCPIs, vulnerability to trypsin was highest at the highest enzyme concentrations and decreased with lower concentrations of trypsin relative to the CPI. As expected, for all enzyme−CPI combinations, the extent

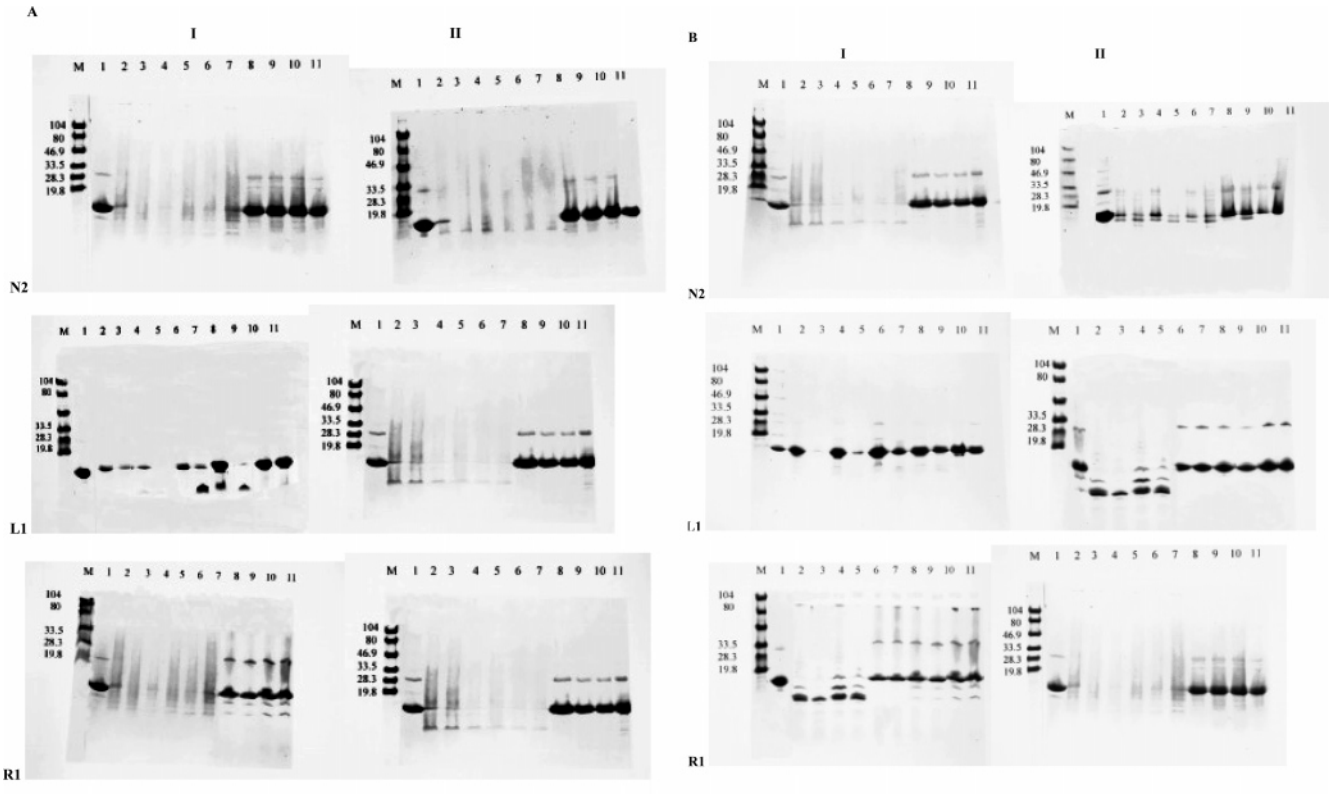


**Figure 5.** SDS-Tricine PAGE (16.5%) visualization of tryptic (**A**) and chymotryptic (**B**) digestion of unheated and heated recombinant soybean cysteine proteinase (rSCPI) N2, L1, and R1 (10 $\mu$g) digested at 37 °C for various time periods and at various R1/enzyme concentration ratios. Gel I, digestion of unheated N2, L1, and R1: lane M, prestained molecular weight marker (kilodaltons); lane 1, unheated N2, L1, and R1 (10 $\mu$g); lane 2, R1/enzyme (R1:E) of 10:1 (w/w), 1 min; lane 3, R1:E of 10:1, 10 min; lane 4, R1:E of 25:1, 1 min; lane 5, R1:E of 25:1, 10 min; lane 6, R1:E of 50:1, 1 min; lane 7, R1:E of 50:1, 10 min; lane 8, R1:E of 75:1, 1 min; lane 9, R1:E of 75:1, 10 min; lane 10, R1:E of 100:1, 1 min; lane 11: R1:E of 100:1, 10 min. Gel II, digestion of heated (held at 99 °C for 30 min prior to digestion) N2, L1, and R1: lane M, prestained molecular weight marker (kilodaltons); lane 1, heated R1 (10 $\mu$g); lane 2, R1:E of 75:1 (w/w), 1 min; lane 3, R1:E of 75:1, 10 min; lane 4, R1:E of 100:1, 1 min; lane 5, R1:E of 100:1, 10 min; lane 6, R1:E of 250:1, 1 min; lane 7, R1:E of 250:1, 10 min; lane 8, R1:E of 500:1, 1 min; lane 9, R1:E of 500:1, 10 min; lane 10, R1:E of 1000:1, 1 min; lane 11, R1:E of 1000:1, 10 min.

of enzymatic hydrolysis was greater at 10 min of digestion compared to 1 min of digestion by the enzyme.

Chymotryptic digestion of the three rSCPIs (**Figure 5B**) showed a different pattern than with trypsin. At a concentration of 10:1 (L1/enzyme, w/w) for unheated L1, complete digestion occurred at 10 min, whereas little digestion was seen after digestion for only 1 min. At lower concentrations of enzyme relative to CPI, no noticeable digestion of L1 occurred regardless of experimental time. R1 and N2 exhibited greater susceptibility to chymotrypsin than did L1. At the 10:1 and 25:1 (w/w) concentrations of CPI/enzyme, both R1 and N2 showed protein breakdown into peptides of lower molecular weight. Accumulation of cleaved product peptides of lower molecular mass was observed on the Tricine SDS-PAGE gels. Heat-treated L1 was more readily digested by chymotrypsin than was unheated L1. Heat-denatured R1 showed susceptibility to chymotrypsin as a function of enzyme concentration. Exposure of thermally denatured N2 to chymotrypsin resulted in smaller peptides at 10:1 and 25:1 (w/w) concentrations of N2/enzyme.

When unheated rSCPIs L1, R1, and N2 were subjected to pepsin treatment, total digestion of the rSCPIs did not occur at the low enzyme concentrations (gels not shown). Even at higher enzyme concentrations, the rSCPIs showed more resistance to pepsin hydrolysis than to trypsin or chymotrypsin digestion. This may be attributed to the fact that there are fewer pepsin-sensitive sites present compared to enzyme sites for trypsin and chymotrypsin. Digestion by pepsin of all three rSCPIs was increased with enzyme concentration and time of reaction.

Interestingly, the proteolytic patterns visualized on the Tricine gel after tryptic, chymotryptic, or peptic digestion differed despite the close homology in the amino acid sequences of L1, R1, and N2. The inhibitory activity of the segments of the three rSCPIs obtained upon digestion by trypsin, chymotrypsin, and pepsin remains to be determined. Such experiments have been conducted with the protease hydrolysis of oryzacystatin I (OC I) and oryzacystatin (OC II) by Michaud et al. (*21*). Papain inhibitory activity assays of OC I and OC II segments cleaved by the digestive proteinases trypsin, chymotrypsin, and pepsin could not be carried out in their studies due to experimental constraints, but activity was tested using selected digestive proteinases. Tryptic and chymotryptic segments of OC I and OC II were reported to remain active even after 30 min of digestion, as indicated by their activity against the digestive proteinases of CPB and black vine weevil (*21*−*23*). These results indicate high conformational stability near the active site of OC I and OC II. Orr et al. (*24*) concluded that tryptic fragments of the potato multicystatin effectively inhibited the gut CP activity of WCR but did not inhibit growth of WCR.

Although remains to be determined if rSCPIs L1, R1, and N2 have inhibitory activity after enzymatic digestion, it is reasonable to conclude that enzymes involved in the human digestive system are capable of digesting the three rSCPIs to varying degrees.

**Enzymatic Digestion of rSCPIs by Crude Gut Extracts of WCR, CPB, and CW.** Susceptibility of the rSCPIs L1, R1, and N2 to proteolysis by proteases in the crude gut extracts of WCR, CPB, and CW was assessed. The extent of proteolytic digestion was monitored visually on 16.5% Tricine SDS-PAGE gels over a period of 20 h, and any reduction in the inhibitory activity was measured (**Figure 6**). Total proteolytic activities of crude gut extracts of WCR, CPB, and CW, as measured by the [$^3$H]MetHb assay using papain as the test proteinase, were monitored in parallel over time of exposure to the rSCPIs (**Figure 7**). The objective of this experiment was to evaluate

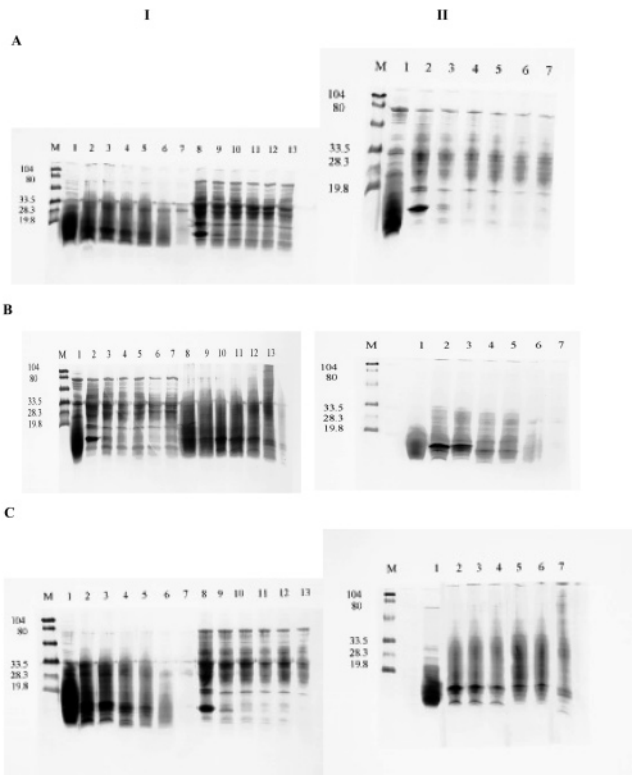


**Figure 6.** Time course digestion of recombinant soybean cysteine proteinase inhibitors N2, R1 (gel I), and L1 (gel II) by crude gut extract of Western corn rootworm (WCR) (**A**), Colorado potato beetle (**B**), and cowpea weevil (**C**). Gel I, N2 and R1: lane M, marker; lane 1, crude gut extract; lane 2, lane 1 + 5 $\mu$g of N2 at 0 h; lane 3, lane 2 at 1 h; lane 4, lane 2 at 3 h; lane 5, lane 2 at 5 h; lane 6, lane 2 at 10 h; lane 7, lane 2 at 20 h; lane 8, CGE of WCR + 5 $\mu$g of R1 at 0 h; lane 9, lane 8 at 1 h; lane 10, lane 8 at 3 h; lane 11, lane 8 at 5 h; lane 12, lane 8 at 10 h; lane 13, lane 8 at 20 h. Gel II, L1: lane M, marker; lane 1, crude gut extract; lane 2, lane 1 + 5 $\mu$g of L1 at 0 h; lane 3, lane 2 at 1 h; lane 4, lane 2 at 3 h; lane 5, lane 2 at 5 h; lane 6, lane 2 at 10 h; lane 7, lane 2 at 20 h.

the stability of the rSCPIs when incubated with crude gut extracts of WCR, CPB, and CW. Because the rSCPIs could play an important role in inhibiting the growth and development of the WCR, CPB, and CW, CPIs would ideally be reasonably stable to any protease activity from the insect guts. The study also was intended to provide insight into whether the insects cope with inhibitors by digesting the protease inhibitors or by adapting in some other way.

The CPI digestion observed on Tricine SDS-PAGE gels indicated that all three rSCPIs were completely hydrolyzed when incubated individually for 20 h with crude gut extracts of WCR, CPB, and CW (**Figure 6A**,**B**,**C**, respectively). Each of the three rSCPIs was hydrolyzed ~60% after 10 h of incubation. The [$^3$H]metHb assay indicated that N2 retained >20% of its inhibitory activity when exposed to the crude gut extract of WCR for 10 h, whereas R1 and L1 showed <10% inhibitory activity (**Figure 7**). The reduction in inhibitory activity of N2 and R1 was orders of magnitude larger than that of L1, although the activities of N2 and R1 were not completely eliminated in 20 h. Results obtained from the [$^3$H]metHb assay confirmed that the breakdown products of N2 and R1 still inhibited CPs native to the gut extract of WCR. N2 and R1 inhibited proteolytic activity present in the gut extract of CPB and CW to <10% at the end of 20 h. The stability of L1 was the least for all three gut extracts, followed by R1. Among the three

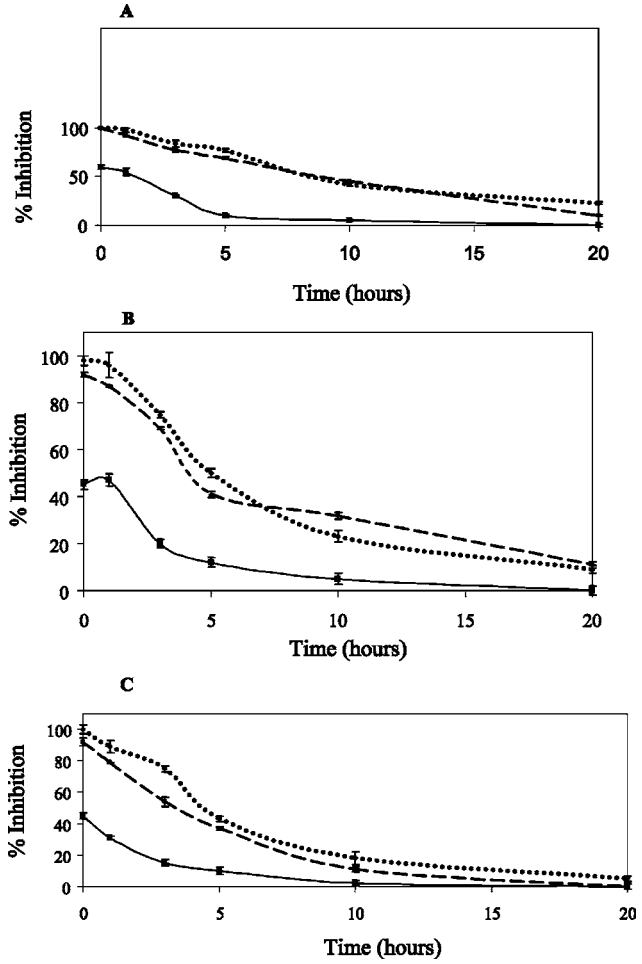


**Figure 7.** Time course stability of recombinant soybean cysteine proteinase inhibitors (rSCPIs) L1 (—), R1 (- - -), and N2 (•••) exposed to gut extract of Western corn rootworm (WCR) (**A**), Colorado potato beetle (CPB) (**B**), and cowpea weevil (CW) (**C**), measured with [$^3$H]metHb as the substrate. Points are mean and standard deviation of determinations of five batches of crude gut extract, each assayed in triplicate.

rSCPIs, N2 was found to be the most stable in all three gut extracts tested.

Orr et al. (*24*) stated that the effectiveness of the CPIs could be attributed largely to the biochemical stability of the CPIs in the insect guts. Differential inhibition and stability patterns suggest that (1) the three rSCPIs target certain CPs over others when exposed to the crude gut extract of the three select pests and (2) N2 and, to a lesser extent, R1 contain amino acid sequences that are not potentially sensitive to the complex mixture of gut proteases of the three selected pests.

On the basis of the tests of stability of rSCPIs to proteases in crude gut extracts, it appears that N2 is a better choice in conferring host plant resistance against WCR than are L1 or R1. These results concur with the in vitro and in vivo inhibition patterns of the gut extracts of WCR, CPB, and CW, which indicate that N2 is the most potent inhibitor of the three at the concentrations tested (Lalitha et al., submitted for publication).

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