A plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases (DvCALs) in the larval midgut of western corn rootworm (*Diabrotica virgifera virgifera*)

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Abstract Feeding bioassay results established that the soybean cysteine proteinase inhibitor N (soyacystatin N, scN) substantially inhibits growth and development of western corn rootworm (WCR), by attenuating digestive proteolysis [Zhao, Y. et al. (1996) Plant Physiol. 111, 1299–1306]. Recombinant scN was more inhibitory than the potent and broad specificity cysteine proteinase inhibitor E-64. WCR digestive proteolytic activity was separated by mildly denaturing SDS–PAGE into two fractions and in-gel assays confirmed that the proteinase activities of each were largely scN-sensitive. Since binding affinity to the target proteinase [Koiwa, H. et al. (1998) Plant J. 14, 371–380] governs the effectiveness of scN as a proteinase inhibitor and an insecticide, five peptides (28–33 kDa) were isolated from WCR gut extracts by scN affinity chromatographic separation. Analysis of the N-terminal sequence of these peptides revealed similarity to a cathepsin L-like cysteine proteinase (DvCAL1, *Diabrotica virgifera virgifera* cathepsin L) encoded by a WCR cDNA. Our results indicate that cathepsin L orthologs are pivotal digestive proteinases of WCR larvae, and are targets of plant defensive cystatins (phytocystatins), like scN.

Key words: Cysteine proteinase inhibitor; Digestive proteolysis; Insect

1. Introduction

Western corn rootworm (WCR) (*Diabrotica virgifera virgifera*) larvae are the major pest of maize in the USA. It is estimated that 17% of potential yield is compromised by the activity of WCR [3], primarily from damage inflicted by larvae feeding on the roots of immature plants [4]. To date, genetic host plant resistance to WCR has been identified only in *Tripsacum dactyloides*, a perennial relative of maize [5,6]. So, there seems to be a formidable taxonomic constraint to the genetic introgression of WCR resistance into maize. Consequently, biotechnology strategies, which utilize plant defensive molecules (e.g. proteinase inhibitors), are being considered as a means to increase host plant resistance against this pest [7].

Unlike mammals and most insects that utilize serine proteinases for digestion, digestive proteolysis by many coleopteran (including WCR) and hemipteran insects, and nematodes is predominantly due to cysteine proteinase activity [8–11]. About 90% of the proteolytic activity in WCR third instar larval guts is attributable to cysteine proteinases [1]. Presumably, digestive cysteine proteinases are synthesized in WCR gut epithelial cells [12] and then secreted into the lumen of the midgut where they facilitate amino acid assimilation by hydrolyzing dietary proteins [13,14]. Available biochemical and molecular data indicate WCR midgut proteinases are similar to mammalian cathepsin-like lysosomal cysteine proteinases, particularly, cathepsin B, H and L [15–17].

To date, there are no reported data that directly link cathepsin-like proteinases to digestive proteolysis required for growth, development and fecundity of WCR. Also, there are no results from which it can be concluded that these hydrolytic enzymes are the principal targets that make WCR vulnerable to plant defensive cysteine proteinase inhibitors (phytocystatins). Here, we present direct evidence that cathepsin-like proteinases are, indeed, targets for and thus determine insecticidal activity of soyacystatin N (scN). WCR larval growth was inhibited substantially by scN in feeding bioassays. Affinity chromatographic purification of scN-binding proteins from WCR gut extracts, and amino acid analysis of these proteins, revealed that five major scN-sensitive digestive proteinases are isoforms of DvCAL1, a WCR cathepsin L-like proteinase.

2. Materials and methods

2.1. Recombinant cysteine proteinase inhibitor production

The *Nco*I–*Sal*I fragment of pSSNM$^{8-103}$ [2] was ligated into pET28a and the plasmid transformed into BL21(DE3) for expression of His-tagged scN protein. Tagged scN was then either immobilized on or purified with Ni^{2+}-chelating Sepharose according to the manufacturer's (Pharmacia) protocol.

2.2. WCR feeding bioassay

Feeding bioassays of WCR neonates on artificial diet were conducted in plastic trays (C-D International, NJ, USA) having 32 sets (columns) of 4 wells/set. Each 4 well set was a treatment block in a randomized complete block design. The basal diet was a modification of the diet described by Rose and McCabe [18] and was purchased from BioServ, NJ, USA. Test compounds were mixed with basal diet prior to loading into each well. To facilitate feeding of larvae, slits were sliced into the diet with a sterile micro-spatula. For larval infestation, the eggs of WCR (French Ag. Research, MN, USA) were surface-sterilized and incubated at 26°C for 2 days. As hatching progressed, three neonates were transferred to each well. To determine

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the WCR larval response, the mean larval weight and mortality for each treatment were determined 12 days post-infestation, and were regressed against compound dose.

2.3. In-gel proteinase analysis of WCR gut extract

Zymographic analysis was performed as described by Gillikin et al. [16]. Guts from 25 third instar larvae were homogenized in 100 μl of extraction buffer and the suspension was filtered through a 0.45 μm PDVF filter. The filtered extract (15 μg of protein) was mixed either with water (control), or 20 μg of BSA or scN and incubated for 5 min on ice. At the end of incubation, an equal volume of 2× non-reducing SDS–PAGE loading buffer [19] was added to the mixture. The proteins in the extract were separated in 10% SDS–PAGE gels that had been co-polymerized with gelatin (0.25%) as a substrate for proteolysis. After electrophoresis at 4°C, the gel was incubated in 2.5% Triton X-100 for 30 min at room temperature, and subsequently in assay buffer (0.1 M MES, 10 mM cysteine, 10 μM Cbz-Phe-Arg-Mec, pH 6.0) for 3 h at 37°C. Fluorescent bands, indicative of amidolytic hydrolysis of Cbz-Phe-Arg-Mec, were detected with UV illumination. Gelatin was detected in the gel by staining with CBB-R250 with cleared zones indicative of proteolytic activity.

2.4. Purification of cysteine proteinases from WCR gut extract

Two hundred third instar WCR larvae were dissected under a stereo microscope to isolate midguts [20]. The gut tissue was washed and homogenized in extraction buffer (0.1 M Tris–HCl, 0.2 M NaCl, 1 mM imidazole, pH 7.0). After the extract was clarified by centrifugation, 100 μg of His-tagged scN in 50 μl of buffer was added to the 300 μl of extract (3 mg of gut protein). The suspension was incubated for 10 min on ice, and then added to 100 μl of chelating Sepharose loaded with Ni^{2+}. This suspension was incubated for 5 min at room temperature with constant agitation, and then centrifuged. The pellet was washed three times with extraction buffer, three times with wash buffer (10 mM Tris–HCl, 0.25 M NaCl, 60 mM imidazole, pH 7.8) and then once with extraction buffer. Proteinases were eluted with 0.1 M NaOH. The eluate was immediately neutralized with 0.1 volume of 1 M Tris–HCl (pH 7.0) and analyzed by SDS–PAGE. For N-terminal sequencing, the sample was blotted onto PVDF membrane and analyzed by automated Edman degradation (Applied Biosystems).

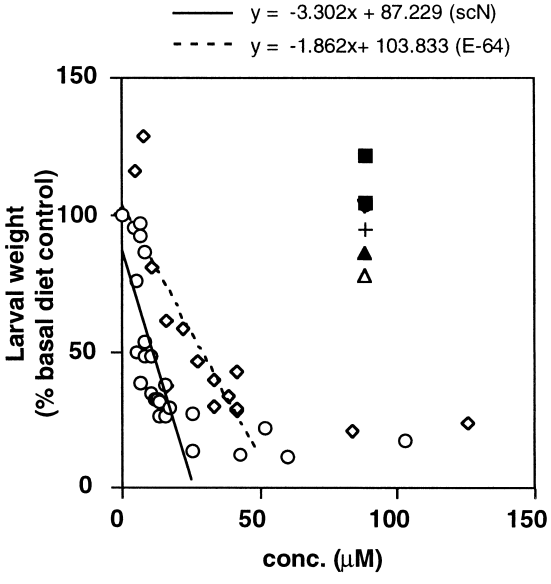

Fig. 1. scN substantially inhibits WCR larval growth. Larvae were placed on artificial diet containing various amounts of scN (○) or E-64 (◇), a cysteine proteinase inhibitor with broad specificity. Control treatments included basal artificial diet or diet with 1000 ppm of total protein extract (equivalent to 85 μM of scN) from seeds of various legumes: *Callandra eriophylla* (■), *Acacia romeriana* (●), *Sutherlandia frutescens* (♦), *Pithecelobium flexicaule* (▼), *Sophora arizonica* (+), *Coursetia glandulosa* (▲) and *Parkinsonia aculeata* (△). Larval weight was determined 12 days after insect egg hatch and the data illustrated are averages of >12 larvae/treatment from four separate experiments.

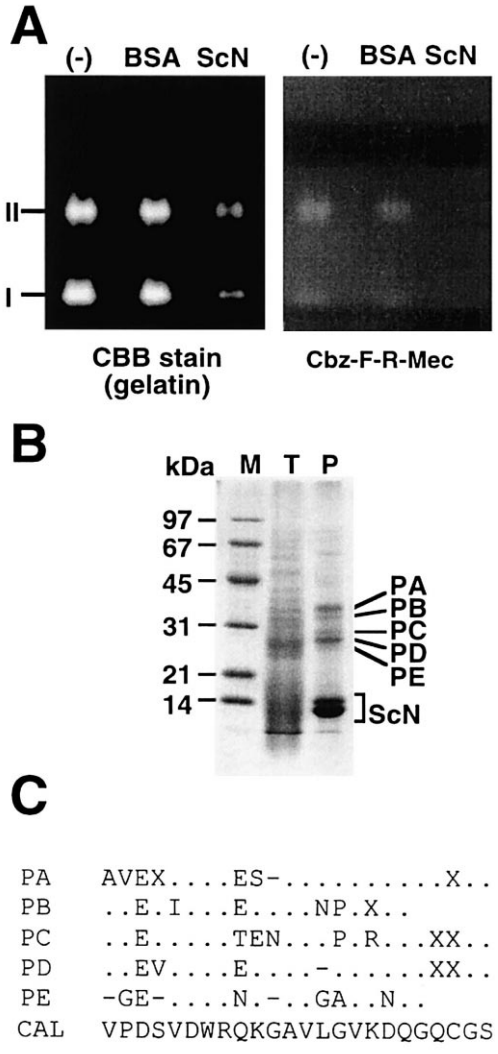

Fig. 2. DvCAL1-like proteinases are targets for inhibition by scN that attenuates WCR larval growth and development. (A) In-gel proteinase activities after mildly denaturing SDS–PAGE separation. Proteolytic activities were visualized either: left, by staining gelatin co-polymerized in the gel with CBB-R250 (cleared zones arised from hydrolysis of gelatin), or right, UV excitation of the fluorescent substrate Cbz-Phe-Arg-Mec; (−) w/o protein, w/BSA or w/scN. Proteinase activity was determined at pH 6.0. (B) scN-binding WCR proteinases isolated from gut extracts by affinity chromatographic separation by binding to immobilized scN, and separated by SDS–PAGE. Total gut extract (T) was incubated with His-tagged scN immobilized on Ni^{2+}-charged chelating Sepharose. Bound proteinases (P) were eluted with 0.1 M NaOH. scN protein detected in the eluant may be due to partial integrity loss of the affinity matrix at alkaline pH; (M) molecular weight marker. (C) N-terminal sequence of major proteinases (PA–PE) aligned with deduced amino acid sequence of DvCAL1 (CAL1). Residues identical to DvCAL1 are indicated as dots, and sequence gaps by hyphens.

3. Results

3.1. scN inhibits WCR gut proteolysis and larval growth

Previously, we determined that scN inhibits greater than 90% of the proteolytic activity in guts of WCR third instar larvae [1]. Consistent with abrogation of gut proteolysis, scN substantially attenuated growth of neonate larvae when included in an artificial diet (Fig. 1). Growth inhibition of WCR larvae by the soyacystatin was even greater than by

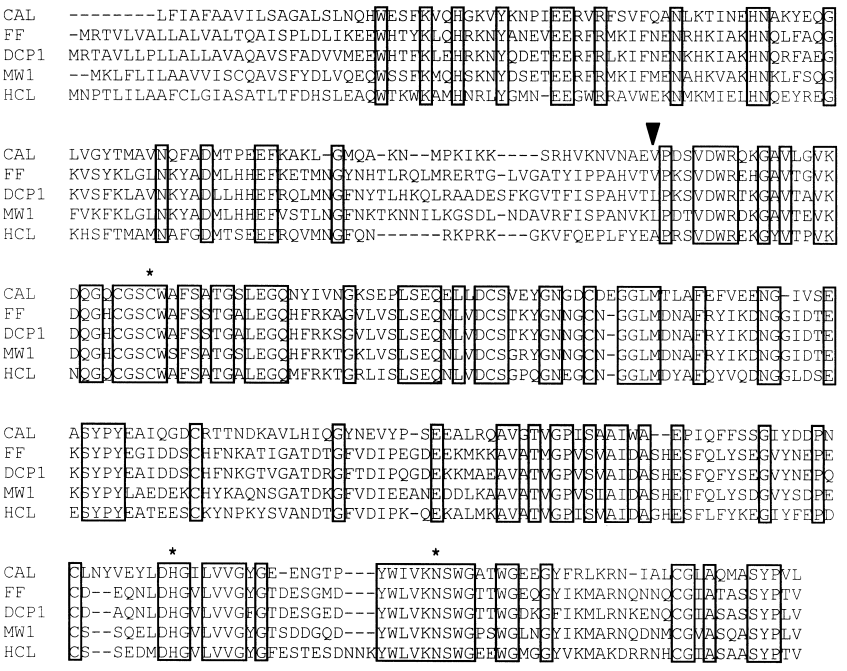

Fig. 3. DvCAL1 has sequence similarity to cathepsin L-like proteinases. The aligned amino acid sequence of DvCAL1 (CAL: GenBank accession number AF190653), and those of cathepsin L-like cysteine proteinases from maize weevil (MW1) [17], fresh fly (FF) [33], fruit fly (DCP1) [34] and human (HCL) [35]. Residues conserved in all homologues are boxed. Asterisks indicate residues for conserved catalytic triad of cysteine proteinases (Cys, His, Asn). Arrowhead identifies the position of the N-terminus determined for the purified gut proteinases (DvCALs), respectively.

E-64, a potent irreversible cysteine proteinase inhibitor that forms an ether linkage between its C-2 atom and the active site Cys residue in the proteinase. Total protein extracts from five other legumes were compared with scN but none was effective at deterring WCR larval growth.

3.2. *scN-binding proteins in WCR gut extracts are cathepsin L-like proteinases*

Zymographic analysis of WCR larval gut extracts separated on polyacrylamide gels, co-polymerized with gelatin (protease substrate) [16], resolved only two major bands of proteinase activity that were essentially all scN-sensitive (Fig. 2A). The midgut of coleopteran insects is slightly acidic, thus zymograms where visualized at pH 6.0 in order to assess activity of WCR proteinases at a physiologically relevant pH. Cysteine proteinases associated with both bands exhibited amidolytic activity as detected after cleavage of the Cbz-Phe-Arg-Mec fluorescent substrate (Fig. 2A). Hydrolysis of this substrate is mediated by proteinases with cathepsin L-like activity [21], biochemically implicating that the WCR proteolytic enzymes, that are targets of dietary scN, are cathepsin L orthologs.

Since inhibition by cystatins depends on tight physical interaction with the proteinase [22], the target proteinases of scN were isolated from WCR gut extracts by affinity chromatographic purification using a matrix of the immobilized phytocystatin. Five major peptides, between M_r 28 and 34 kDa, were eluted from the scN affinity column and resolved by SDS–PAGE (Fig. 2B). The N-terminal sequences of these scN-binding peptides were similar to each other, indicating that the digestive proteolytic system of WCR is derived from products of a multigene family. Furthermore, these peptides had high sequence similarity to DvCAL1 (Fig. 2C), the deduced translation product of a cDNA clone isolated from a WCR cDNA library (Koiwa et al., unpublished results). The translation product of DvCAL1 has a conserved catalytic triad (Cys^{25}, His^{163}, Asn^{183}), and overall sequence similarity to cathepsin L-like proteinases from insects and mammals (Fig. 3), which further indicates the WCR digestive enzymes as cathepsin L-like cysteine proteinases. Together, these results identify cathepsin L family proteinases (DvCALs) as the principal targets in the WCR gut that are inhibited by the plant defensive protein scN sufficiently to impair larval growth of this insect pest.

4. Discussion

Previously, it was established that digestive proteolysis in WCR midguts is attributable primarily to cysteine proteinase activity, although the enzymatic entities were not identified [1,16]. Following mildly denaturing, non-reducing SDS–PAGE, zymogram analysis revealed that two major cysteine proteinase activities are present in the luminal contents of WCR. These activities have been resolved into 15 different fractions by ion exchange chromatography combined with SDS–PAGE [16]. However, it was not determined if these fractions were composed of unique gene products or of fewer enzymes that were modified post-translationally so they resolved as if a greater number of proteins. Herein, we establish that the two digestive proteinase activities identified on the zymogram are attenuated effectively by scN, indicating that a strong and stable molecular interaction occurs between the soyacystatin and the major digestive proteinases of WCR. Subsequently, five cathepsin L-like proteinases (DvCALs) were identified, by affinity purification using immobilized scN, as the enzymes responsible for the bulk of digestive pro-

teolytic activity in WCR midguts. Since SDS–PAGE analysis resolves denatured DvCALs as ca. 30 kDa peptides, the activities detected in the zymogram presumably represent the different oligomeric status of native cathepsin L-like proteinases or their denaturation products [23].

The numerous cysteine proteinases that comprise the steady-state digestive proteolytic system and inhibitor-insensitive proteinases that are produced in response to an inhibitor challenge represent the enzyme diversity whose activity must be attenuated for effective pest control [24–26]. Digestive proteolysis mediated by cathepsin L-like proteinases, that occurs in certain phytophagous insects, is proposed to be a recent evolutionary adaptation in response to plant defensive serine proteinase inhibitors [15]. So, it is possible that these insects still retain the capacity to express and utilize serine proteinases for digestion. Furthermore, aspartic and metallo proteinase activities have been identified in insect guts [15,27,28]. The slightly acidic pH of the coleopteran insect midguts is an environment favorable to cysteine proteinases so it is probable that insensitive isoforms of these enzymes are produced in response to a specific phytocystatin. The digestive proteinase complexity in the midgut likely will require an inhibitor cocktail or the expression of a chimeric peptide(s) with multiple or multifunctional inhibitor domains for abrogation of proteolytic activity. Today, molecular diversity for proteinase inhibitors can be derived from genetic pools or through directed molecular evolution [2,29]. The molecular identification of the DvCALs principally responsible for proteolytic digestion of WCR defines the major targets for the rational design and in vitro combinatorial molecular evolution of cystatins as pesticides [2].

Expression of cystatins in transgenic plants to increase host pest resistance has been marginally successful, as has been the experience when most plant defensive proteins are ectopically expressed [30,31]. Low phytocystatin expression in transgenic plants is implicated as the cause of minimal resistance levels that are achieved. High accumulation of defensive phytocystatins in the cytosol may attenuate proteolysis that is essential for metabolic function. Cystatins with greater affinity for pest rather than host plant proteinases would reduce the threshold expression level required for inhibition of pest growth and development with minimal attenuation of native proteinase function [32]. Alternatively, a cystatin could be expressed as an inactive proprotein with a specific recognition site that can be targeted for hydrolysis by an insect digestive proteinase, rendering an active inhibitor. Conditioning cystatin transgene expression in response to an inducer(s) that is specific to insect attack or targeting the protein for secretion or compartmentation are additional strategies to exploit the insecticidal potential of these cysteine proteinase inhibitors.

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