Insecticidal activity of a basement membrane-degrading protease against Heliothis virescens (Fabricius) and Acyrthosiphon pisum (Harris)

Huarong Li
_Iowa State University_

Hailin Tang
_Iowa State University_

S. Sivakumar
_Iowa State University_

Judith Philip
_Durham University_

Robert L. Harrison
_United States Department of Agriculture_

Follow this and additional works at: [http://lib.dr.iastate.edu/ent_pubs](http://lib.dr.iastate.edu/ent_pubs)

Part of the _Entomology Commons_

The complete bibliographic information for this item can be found at [http://lib.dr.iastate.edu/ent_pubs/21](http://lib.dr.iastate.edu/ent_pubs/21). For information on how to cite this item, please visit [http://lib.dr.iastate.edu/howtocite.html](http://lib.dr.iastate.edu/howtocite.html).
Insecticidal activity of a basement membrane-degrading protease against *Heliothis virescens* (Fabricius) and *Acyrthosiphon pisum* (Harris)

Huarong Li\(^a\), Hailin Tang\(^a\), S. Sivakumar\(^a\), Judith Philip\(^b\), Robert L. Harrison\(^c\), John A. Gatehouse\(^b\), Bryony C. Bonning\(^a\,*

\(^a\)Department of Entomology, Iowa State University, 418 Science II, Ames, IA 50011-3222, USA
\(^b\)School of Biological and Biomedical Sciences, Durham University, South Road, Durham DH1 3LE, UK
\(^c\)Invasive Insect Biocontrol and Behavior Laboratory, USDA Agricultural Research Service, Plant Sciences Institute, 10300 Baltimore Avenue, Beltsville, MD 20705, USA

Received 26 November 2007; received in revised form 7 February 2008; accepted 7 February 2008

Abstract

ScathL is a cathepsin L-like cysteine protease derived from the flesh fly *Sarcophaga peregrina* that functions in basement membrane (BM) remodeling during insect development. A recombinant baculovirus expressing ScathL (AcMLF9.ScathL) kills larvae of the tobacco budworm, *Heliothis virescens*, significantly faster than the wild-type virus. Here, we show that the occurrence of larval melanization prior to death was closely associated with the onset of high cysteine protease activity of ScathL in the hemolymph of fifth instars infected with AcMLF9.ScathL, but not with AcMLF9.ScathL.C146A, a recombinant baculovirus expressing a catalytic site mutant of ScathL. Fragmented fat body, ruptured gut and malpighian tubules, and melanized tracheae were observed in AcMLF9.ScathL-infected larvae. Phenoloxidase activity in hemolymph was unchanged, but the pool of prophenoloxidase was significantly reduced in virus-infected larvae and further reduced in AcMLF9.ScathL-infected larvae. The median lethal dose (LD\(_{50}\)) for purified ScathL injected into fifth-instar *H. virescens* was 11.0 \(\mu\)g/larva. ScathL was also lethal to adult pea aphids, *Acyrthosiphon pisum* with a similar loss of integrity of the gut and fat body. Injection with purified ScathL.C146A or bovine trypsin at 20 \(\mu\)g/larva did not produce any effect in either insect. These results illustrate the potent insecticidal effects of ScathL cysteine protease activity and the potential for use of ScathL in development of insect resistant transgenic plants when combined with an appropriate delivery system.

\(\odot\) 2008 Elsevier Ltd. All rights reserved.

Keywords: Cathepsin L; Cysteine protease; Basement membrane; Insecticidal protein; Tobacco budworm; Pea aphid; Baculovirus; Melanization

1. Introduction

Chemical control of insect pests of agricultural crops is plagued with problems such as insecticide resistance and environmental contamination (Clark and Yamaguchi, 2002; Li et al., 2003). To address some of the shortcomings associated with synthetic pesticides, transgenic crops expressing insecticidal proteins derived from *Bacillus thuringiensis* (Bt) have been adopted to improve crop resistance to insect infestation (Shelton et al., 2002). Transgenic Bt technology is now a primary tool for lepidopteran and coleopteran pest control on cotton and corn in the United States and elsewhere (Bates et al., 2005; Lawrence, 2005). However, evolution of resistance to Bt toxins in insect pest populations remains a concern (Heckel et al., 2007; Li et al., 2005).

The use of other insecticidal proteins for development of insect resistant transgenic crops is a solution to the potential evolution of Bt resistance in the field. A number of alternative insecticidal proteins have been found, including lectins (Gatehouse et al., 1997), cholesterol oxidases (Linder and Bernheimer, 1984), chitinases (Ding et al., 1998; Kramer and Muthukrishnan, 1997), avidin (Kramer et al., 2000), and protease inhibitors (Hilder et al., 1987). Some of the genes encoding these insecticidal proteins have been engineered into plants (Johnson et al., 1989). However, successful commercial application has not
been widely achieved because of low efficacy or other difficulties.

The basement membrane (BM) has been identified as a potential target for insect pest management purposes (Harrison and Bonning, 2001; Liu et al., 2006b). BM are extracellular protein sheets surrounding all tissues and function in cell adhesion, cell signaling, and maintenance of tissue structure (Yurchenco and O’Rear, 1993). In insects, the BM must be remodeled during embryonic development, tissue and cell differentiation, and metamorphosis (Page-McCaw et al., 2003). This remodeling involves enzymes that specifically digest components of the BM, including cathepsins (Homma and Natori, 1996; Homma et al., 1994) and matrix metalloproteases (MMP) (Llano et al., 2000; Maeda et al., 2001; Mott and Werb, 2004; Srivastava et al., 2007). Expression and activation of these BM-degrading enzymes are tightly regulated to avoid uncontrolled and potentially fatal damage to other tissues (Llano et al., 2002). Insects may use MMP activators and inhibitors to mediate regulation of metalloprotease activity. The tight regulation of insect MMP and other BM-degrading proteases suggests that delivery of sufficient protease into the hemocoel may result in unregulated degradation of BM. Such damage may impair insect physiological processes and kill the insect (Liu et al., 2006b; Tang et al., 2007). Hence, enzymes that degrade BM have potential as intrahemocoelic toxins for use in insect pest management.

A cathepsin L-like protease (ScathL, EC 3.4.22.15) from the flesh fly, Sarcophaga peregrina, was isolated from the culture medium of an embryonic S. peregrina cell line (Homma et al., 1994). This protease is secreted by imaginal discs when cultured in the presence of ecdysone, and is thought to be involved in differentiation by selectively hydrolyzing two proteins of BM of various tissues, including imaginal discs (Homma and Natori, 1996). The BM appears to act as a barrier to dissemination of some viruses within an infected insect (Engelhard et al., 1994; Hess and Falcon, 1987; Smith-Johannsen et al., 1986). On this basis, a recombinant baculovirus (AcMLF9.ScathL) that expresses ScathL was constructed to enhance the efficiency of baculovirus infection (Harrison and Bonning, 2001). AcMLF9.ScathL killed larvae of the tobacco budworm, Heliothis virescens, significantly faster than the wild-type virus and triggered melanization of larvae shortly before death. Because BM is a potential barrier to the spread of baculovirus infection to other tissues within a host, we tested the hypothesis that the rapid death of insects infected with AcMLF9.ScathL was caused by accelerated secondary infection resulting from the degradation of host BM by ScathL (Li et al., 2007). Viruses expressing catalytically active and inactive ScathL were used to examine the effects of ScathL activity on budded virus release into the hemocoel during infection, the production of polyhedra in infected larvae, and the rate of infection of the gut, trachea, hemocytes, fat body, and malpighian tubules. We concluded that the enhanced insecticidal efficacy of the recombinant baculovirus that expresses ScathL does not result from altered tissue tropism or accelerated systemic infection.

In the present study, we report: (1) the insecticidal action of ScathL when delivered by a recombinant baculovirus against larvae of H. virescens, and when ScathL was purified from a yeast expression system and delivered by intrahemocoelic injection to H. virescens and adult pea aphids, Acrystosiphon pisum (Harris); (2) the correlation between insect mortality and cysteine protease activity of ScathL; (3) the physiological damage caused by ScathL to insect tissues; and (4) the impact of AcMLF9.ScathL infection of H. virescens larvae on phenoloxidase (PO) and prophenoloxidase (pro-PO) titers. The potential use of ScathL in development of insect resistant transgenic plants is discussed.

2. Materials and methods

2.1. Insect cells, insects, and viruses

Spodoptera frugiperda Sf21 cells (Vaughn et al., 1977) were maintained in TC-100 medium (Sigma) supplemented with 10% fetal bovine serum (FBS; Intergen) and antibiotics (1 U/ml penicillin, 1 µg/ml streptomycin; Sigma). Trichoplusia ni BTI-TN-5B1-4 (High Five™) cells (Wickham et al., 1992) were maintained in Ex-Cell 405 medium (JRH Biosciences) supplemented with antibiotics only. Both cell lines were maintained at 28 °C. Larvae of H. virescens were reared individually from eggs (BioServ, Frenchtown, NJ) on a meridic diet in 1 oz. plastic cups (BioServ) at 28 °C with a 14:10 h light:dark cycle. An A. pisum colony was maintained on broad bean plants in a growth chamber at 24 °C and 24 h light.

The wild-type AcMNPV strain C6 and the recombinant viruses, AcMLF9.ScathL and AcMLF9.ScathLC146A were used for this study. AcMLF9.ScathL expresses a functional flesh fly cathepsin L protease (ScathL) (Harrison and Bonning, 2001), AcMLF9.ScathLC146A expresses a catalytic site mutant of ScathL (Li et al., 2007), and the expression of both proteins was directed by the AcMNPV p6.9 promoter (Harrison and Bonning, 2000; Hill-Perkins and Possee, 1990). Budded virus stocks were produced in Sf21 cells. The polyhedra were generated, purified as described previously (Harrison and Bonning, 2001), resuspended in glycerin/water (3:2, v/v), quantified using a hemocytometer, and stored at 4 °C. BV stocks were titered by end-point dilution.

2.2. ScathL activity in larval hemolymph

The expression patterns of ScathL were characterized by determination of specific activity and western blotting analysis for hemolymph plasma of fifth-instar H. virescens infected with the recombinant and control viruses. H. virescens larvae were inoculated with polyhedra within the first 1 h after ecdysis to fifth instar. Polyhedra of
AcMLF9.ScathL, AcMLF9.ScathL.C146A, or AcMNPyV-C6 were inoculated orally using a microapplicator (Burkard Scientific Ltd., UK) in a 1 µl mixture of water and glycerin (2:3, v/v) (Washburn et al., 1995). One µl of the mixture of water and glycerin was included as a control treatment. A dose of 5 × 10^5 polyhedra/larva, which caused 100% mortality of fifth instars, was used. Inoculated larvae were maintained at 28 °C.

After larvae were anaesthetized on ice and sterilized in 70% ethanol, 15–20 µl of hemolymph from infected larvae was prepared for protease assay at 12, 24, 30, 36, 42, and 48 h post-infection (hpi). Hemolymph was collected by cutting off a proleg tip, bleeding onto a piece of parafilm on ice, and transferring the hemolymph into a 1.5 ml tube containing 1 µl of 0.3% phenylthiourea (PTU) on ice. The hemolymph from three larvae in the same treatment was pooled and mixed as a replicate. Five such replicates were processed per treatment. The plasma was obtained by centrifuging the hemolymph at 500 g for 5 min at 4 °C to remove hemocytes. Protein content was quantified by Bradford assay. Ten µl of plasma was promptly transferred for each sample into a tube containing 2 × SDS loading buffer, immediately heated at 95 °C for 10 min, and stored at −20 °C for western blotting analysis as described previously (Li et al., 2007). The remaining plasma was transferred into clean tubes containing 100 µl of 0.1 M sodium acetate buffer (pH 5.0) for cysteine protease activity assay using azocoll as a substrate (Harrison and Bonning, 2001).

To examine the correlation between ScathL activity levels in the plasma and insect death, the melanization and mortality of insects were recorded throughout the experiment.

2.3. Injection bioassays with yeast-expressed ScathL

Bioassays with purified yeast-expressed ScathL were performed to quantify the toxicity of ScathL to larvae of *H. virescens* by hemocoelic injection. Expression and purification of ScathL and ScathL.C146A from recombinant yeast cells were conducted as previously reported (Philip et al., 2007). Newly molted fifth-instar *H. virescens* (115–129 mg/larva) were injected via a proleg using a microapplicator (Burkard) with yeast-expressed ScathL at different doses. The injection was performed to the base of a hind leg using a pulled glass capillary needle (containing ScathL or ScathL.C146A in 0.1 µl 30 mM sodium acetate pH 5.5) held in place on a micromanipulator as previously described (Beernsten and Christensen, 1990). Mock treatments consisted of 30 mM sodium acetate, pH 5.5. For each dose, 20 adult aphids were injected. Injected aphids were placed in Petri dishes at room temperature and observed for any response to the injection. Mortality was recorded every 1–5 h until 16 h. At 16 h post-injection, aphids were dissected for examination of internal tissues.

2.4. ScathL-induced damage to larval tissues

Thirty newly molted fifth-instar larvae of *H. virescens* were orally inoculated with 5 × 10^5 polyhedra/larva with AcMLF9.ScathL, AcMLF9.ScathL.C146A or wild-type virus C6. The infected larvae were examined externally and internally for damage and melanization of the cuticle, gut, fat body, trachea, and malphigian tubules under a light microscope (Nikon SMZ1500) at 24, 36, 48, and 54 hpi. To observe the symptoms or tissue damage caused by ScathL alone, 90 newly molted fifth-instar *H. virescens* were injected using 20 µg ScathL/larva in 4 µl of sodium acetate (pH 5.5) supplemented with 1.0 mM glutathione. The injected larvae were observed every 3 h for mortality and melanization. Shortly after death, the dead larvae were dissected and examined for internal tissue damage. Larvae that survived for the 2 days were also dissected and examined.

In preliminary experiments, it was noted that the pericardial cells, dorsal aorta and alary muscles appeared to be melanized in some larvae killed by injection of ScathL. We examined these tissues in the larvae killed by ScathL injection using a light microscope (Nikon SMZ1500) and by scanning electron microscopy (SEM) as described previously (Tang et al., 2007).

2.5. Assay of phenoloxidase activity in hemolymph

To determine whether the widespread melanization of AcMLF9.ScathL virus-infected *H. virescens* larvae resulted from activation of the prophenoloxidase (pro-PO) cascade,
phenoloxidase (PO) activity was compared in the hemolymph of fifth-instar *H. virescens* larvae infected with AcMLF9.ScathL, AcMLF9.ScathL.C146A, or wild-type C6 viruses at 5 x 10^5 polyhedra/larva. At 24, 48, and 72 hpi, 5 μl of hemolymph was extracted from each virus or mock-infected larva as previously described. The extracted hemolymph was dispensed into a well containing 45 μl of 10 mM sodium phosphate buffer (pH 6.6) in a 96-well plate on dry ice. Six larvae were treated for each virus (six replicates). When all extractions were completed, the plate was maintained at room temperature to thaw the samples. After gentle mixing, 10 μl of each diluted hemolymph sample was transferred to individual wells containing 90 μl of 2 mM 3,4-DOPA (a substrate for PO) in 10 mM sodium phosphate buffer to assay PO activity in triplicate. Absorbance at 450 nm was read at 15 s intervals for 5 min at room temperature. The combined PO and pro-PO activities were also assayed using 2 mM 3,4-DOPA supplemented with 0.2% cetypyridinium chloride (CPC) that converts pro-PO to PO. Protein concentrations of samples were determined using the Bradford assay. Enzymatic activity data for PO, and PO plus pro-PO were tested for statistical significance using one-way analysis of variance (ANOVA).

To further determine if ScathL activity was able to convert pro-PO to PO in vitro, PO activity was assayed in larval hemolymph after addition of purified yeast-expressed ScathL. Hemolymph was prepared from healthy early fifth-instar larvae of *H. virescens* as above described. Two μl of 5 μg/μl ScathL was added to 100 μl of diluted hemolymph extracted from two larvae in 10 mM sodium phosphate buffer and mixed. PO activity in the mixture was immediately assayed as described above using 2 mM 3,4-DOPA as substrate. Two μl of water or 10% (0.2% in diluted hemolymph) CPC served as negative and positive controls, respectively. The experiment was repeated six times.

3. Results and analyses

3.1. Relationship between larval death and ScathL titer in fifth-instar hemolymph

The protease activity detected in hemolymph plasma from *H. virescens* larvae infected with AcMLF9.ScathL was shown to result from cysteine protease activity by use of the cysteine protease-specific inhibitor E64 in preliminary experiments (data not shown). The accumulation of ScathL and ScathL.C146A in hemolymph plasma of baculovirus-infected larvae was analyzed at 12, 24, 30, 36, 42, and 48 hpi using activity assays and western blot analyses (Fig. 1). Prior to 24 hpi, the cysteine protease activity in the plasma of larvae infected with AcMLF9. ScathL was at a similarly low level to that of larvae infected with the control virus AcMLF9.ScathL.C146A or mock (Fig. 1A). From 24 to 36 hpi, the cysteine protease activity was significantly elevated in the plasma of AcMLF9. ScathL-infected larvae compared to the control virus- and mock-infected larvae, but remained at a relatively low level. However, cysteine protease activity drastically increased after 36 hpi. At 48 hpi, more than half of the larvae infected with AcMLF9.ScathL died shortly after melanization. The increase in cysteine protease activity in plasma harvested from melanized and non-melanized larvae infected with AcMLF9.ScathL at 48 hpi. (B) Correlation of larval cuticular melanization and mortality. Error bars represent standard errors. Larvae infected with AcMLF9.ScathL died shortly after melanization.

ScathL-infected larvae to the control virus- and mock-infected larvae, but remained at a relatively low level. However, cysteine protease activity drastically increased after 36 hpi. At 48 hpi, more than half of the larvae infected with AcMLF9.ScathL had melanized. Melanized larvae exhibited much higher cysteine protease activity in the plasma than non-melanized larvae (inset in Fig. 1A). The increase in cysteine protease activity in the plasma was consistent with results of the western blot analysis (data not shown).

In larvae infected with AcMLF9.ScathL, elevated cysteine protease activity in the plasma closely correlated with larval melanization and subsequent death. Prior to 42 hpi, the specific activity of cysteine proteases was relatively low and there were no melanized larvae and no larval mortality (Fig. 1B). However, after 42 hpi, cysteine protease activity was drastically increased in the plasma of AcMLF9.ScathL-infected larvae. Melanization occurred, followed by increased mortality of larvae infected with
AcMLF9.ScathL. By 48 hpi, approximately 60% of larvae melanized, and by 54 hpi more than 90% of larvae melanized and about 50% died. By 66 hpi almost all larvae died that were infected with AcMLF9.ScathL. No melanization occurred for the control virus- or mock-infected larvae, and mortality was observed for the control virus-infected larvae only after 96 hpi. Because ScathL-C146A, a mutant lacking protease activity, was expressed at levels similar to ScathL, these data indicate that elevated cysteine protease activity was responsible for the observed melanization and rapid larval death.

3.2. Toxicity of purified ScathL to fifth-instar *H. virescens* and adult *A. pism*

Fifth-instar larvae of *H. virescens* were injected with different doses of purified yeast-expressed ScathL and the larval mortality was recorded daily for the next 5 days. The mortality of larvae injected with catalytically active ScathL occurred in a dose-dependent manner (Fig. 2). Injection with 20 μg ScathL/larva resulted in 100% cumulative mortality at 72 hpi and injection with 10 μg/larva resulted in approximately 50% cumulative mortality at 96 hpi. At lower doses tested viz., 5.0 and 2.5 μg/larva, the mortalities were less than 20% at the end of experiment. Probit analysis with cumulative mortality data at 48 hpi was used to determine that the median lethal dose (LD$_{50}$) of yeast-expressed wild-type ScathL was 11.0 (8.31–13.8) μg per fifth-instar *H. virescens*. For the mock-injected control, only one larva died throughout the experiment within 24 hpi. This death likely resulted from injury caused by injection. As expected, injection with 20 μg ScathL.C146A/larva did not result in any mortality, and all larvae grew and pupated normally. These results are comparable with those for another lepidopteran insect pest, *L. oleracea* injected with ScathL (Philip et al., 2007), indicating similar toxicity of ScathL to lepidopteran insects. Interestingly, injection with 20 μg/larva of bovine trypsin did not cause any mortality. When 40 μg of bovine trypsin was injected, 40% of the larvae died within 2–3 hpi and thereafter no further mortality occurred. The mortality may result from internal tissue fragmentation with symptoms similar to type one symptoms as described below. All survivors pupated normally (data not shown).

Injection of *A. pism* adults with purified yeast-expressed ScathL also resulted in dose-dependent mortality (Table 1). The LD$_{50}$ value was between 40 and 50 ng of ScathL per aphid. Injection with 300 ng ScathL.C146A per aphid or injection of buffer alone had no effect on the aphids.

3.3. ScathL damage to insect tissues

Infection of fifth-instar *H. virescens* with AcMLF9.ScathL always caused cuticular melanization at around 48 hpi (Fig. 3A). Melanized larvae showed a gradual increase in black pigment deposition on the cuticle, and when probed, the cuticle or integument was tougher than that of either healthy larvae or wild-type virus-infected larvae. However, the deposition of black pigment was not significantly visible on the cuticle of larvae injected with purified ScathL enzyme (Fig. 3B).

The effects of AcMLF9.ScathL on the insect gut, fat body, malpighian tubules and trachea were examined under a light microscope (Fig. 4). After 42 hpi melanin appeared at the tips of tracheae that were attached to the surfaces of other tissues such as the gut and fat body (Fig. 4C and D). This symptom occurred prior to the appearance of external melanization on the cuticle. In larvae where cuticular melanization was not prominent, we did not observe any abnormality of the gut, fat body, or malpighian tubules. However, fragmentation followed by complete disappearance or digestion of all the internal tissues was observed in larvae with melanized cuticles and in dying larvae (Fig. 4E). The fragmented tissues were floating in darkened hemolymph and when the larvae were dissected, all tissues were digested except the integument and the tracheae which appeared to be more resistant to proteolysis (Fig. 4F). None of these symptoms were seen in larvae in the two control treatments (Fig. 4A and B).

Injection of purified ScathL resulted in two types of symptoms with each type observed in approximately 50% of the injected larvae (Fig. 3B). Larvae with type one symptoms usually died within a few hours. Internal tissues were fragmented and the body cavity was filled with blackened hemolymph, similar to that of larvae killed by AcMLF9.ScathL. Externally, the larvae appeared black but there was no pigment on the cuticle. The appearance of external darkening resulted from damage to the internal tissues and blackened hemolymph. Larvae with the second type of symptoms did not die until 12–24 h post-injection. The dead larvae showed slight pigmentation of the cuticle. Most of the internal tissues were not damaged and remained intact. However, dark particles were present in the hemolymph. Dark spots were also seen at the base of larger tracheal trunks (Fig. 5A and B) similar to type one symptoms but clearly different from the darkened tracheal tips observed in larvae infected with AcMLF9.ScathL (Fig. 4C and D).

Another typical characteristic of the type two responses to ScathL injection was that the pericardial cells, dorsal aorta and alary muscles were dark or brown, and that the alary muscles appeared to be broken on both sides in dead larvae (Fig. 6A–C). This was not observed in larvae infected with AcMLF9.ScathL (Fig. 6D). Examination of tissues by SEM indicated that the dark pericardial cells, dorsal aorta and alary muscles of the larvae killed by ScathL injection were fragmented (Fig. 7A), in contrast to the non-melanized pericardial tissues of either control larvae or ScathL injected but surviving larvae (Fig. 7B and C). These observations demonstrate that darkening of pericardial tissues of fifth-instar *H. virescens* injected with pure ScathL is a consequence of damage to these tissues that may be responsible for insect death. This damage may result from digestion of the BM overlying these tissues.
The gut and other internal tissues of aphids injected with lethal doses of ScathL lysed. When ScathL-injected aphids were dissected, unborn progeny were easily released into the dissection solution (water) and did not show obvious external abnormalities, indicating that the BM surrounding the embryos within the mother had lysed. The cuticle of both the injected mother and the unborn progeny appeared to be intact with no apparent external or internal melanization (Fig. 8C and D). The gut and other internal tissues of mock-treated aphids or aphids injected with ScathL-C146A were intact (Fig. 8A and B).

### 3.4. Phenoloxidase activity in hemolymph plasma

Specific activities of PO, and PO+pro-PO were compared in the hemolymph of fifth-instar *H. virescens* larvae infected with AcMLF9.ScathL, AcMLF9.ScathL.C146A, and wild-type C6 viruses at 24, 48, and 72 hpi. There were no significant differences in PO activity in the hemolymph extracted from either virus or mock-infected larvae at either 24, 48, or 72 hpi (Fig. 9A). Interestingly, at 72 hpi, the overall activity of PO+pro-PO was significantly reduced in wild-type C6 and control virus AcMLF9.ScathL.C146A-infected larvae relative to mock-infected larvae, and it was further reduced in AcMLF9.ScathL-infected larvae (Fig. 9B). There was no significant difference in either PO or PO+pro-PO activity in hemolymph between melanized and non-melanized larvae (Fig. 9C). These data suggest that the pool of pro-PO was reduced by virus infection and further reduced by ScathL expression.

The PO activity was elevated in the positive control by addition of the pro-PO activator CPC. These data suggest that ScathL activity alone was not able to convert pro-PO to PO in vitro.
4. Discussion

A cathepsin L-like cysteine protease (ScathL) was isolated from the culture medium of NIH-Sape-4 cells, an embryonic cell line of *S. peregrina* (Homma et al., 1994). The proenzyme (50 kDa) automatically activated to the mature enzyme (35 kDa). This was confirmed by our observation of 35-kDa ScathL in both the medium of AcMLF9.ScathL-infected High Five cells (data not shown) and the hemolymph of *H. virescens* larvae infected with the virus. Our previous western blot analysis revealed that AcMLF9.ScathL-infected tissues (hemocytes, gut, and fat body) had only the mature form of ScathL (Li et al., 2007), but in this study both mature and proenzyme forms were observed in the hemolymph plasma (data not shown). This is in agreement with the observation of Homma et al. (1994) that the proenzyme was secreted into the medium and the activated form remained in the cells. This differential targeting of ScathL was also observed for imaginal discs cultured in the presence of ecdysone.

A high level of ScathL activity in hemolymph was consistently associated with widespread melanization of the cuticle and tracheae, tissue damage including ruptured guts and fragmented fat body, and ultimately larval death. Melanization, tissue damage and mortality were not observed in larvae infected with AcMLF.ScathL.C146A. Bioassays with purified ScathL that was injected into the hemocoel further confirmed that the cysteine protease activity of ScathL was able to kill insects in the absence of baculovirus infection. It is also conceivable that ScathL activates zymogens of endogenous proteases that contribute to BM and tissue damage. The widespread melanization of *H. virescens* larvae associated with AcMLF9.ScathL infection could indicate that the pro-PO cascade pathway is activated by ScathL, resulting in melanin production. However, our data demonstrated that the cysteine protease activity of purified ScathL was not able to activate pro-PO to PO \textit{in vitro}. PO activity in the hemolymph of larvae was not altered by AcMLF9.ScathL, although the pool of pro-PO was significantly reduced. This suggests that the PO level in the hemolymph is regulated and that virus infection may inhibit production of pro-PO.

The melanization of tracheal tips observed in larvae infected with AcMLF9.ScathL is similar to melanization of midgut-associated tracheae in *Helicoverpa zea* and *Manduca sexta* larvae infected with AcMNPV (Washburn et al., 1996, 2000). These two host species are semipermisive for AcMNPV infection. In those cases, infection of the host species with AcMNPV elicits a melanotic encapsulation immune response targeted at infected tracheae closely
associated with the midgut on the hemocoel side. The junction of tracheae and midgut appears to be a site at which the NPV infection process can be halted by encapsulation of infected tracheal epidermal cells. In the case of AcMLF9.ScathL, infection is not halted by melanization at this site, because the LC50s for AcMNPV-C6 and AcMLF9.ScathL are not significantly different (Harrison and Bonning, 2001; Li et al., 2007). How is it that melanotic encapsulation of tracheae can block the spread of infection in one case, but not in the other? The melanization of tracheal tips was not observed until 42 hpi in H. virescens larvae infected with AcMLF9.ScathL. At this stage, viral systemic infection is well established. The delayed encapsulation of tracheal tips...
Fig. 6. Melanized pericardial cells, dorsal aorta and alary muscles of fifth-instar *H. virescens* injected with 20 μg of purified yeast-expressed ScathL at 24 hpi (A, B, C) and non-melanized pericardial tissues of larvae infected with AcMLF9.ScathL at 48 hpi (D). d, dorsal aorta; p, pericardial cells; a, alary muscle; f, fat body. Scale bar = 1 mm.

Fig. 7. SEM of pericardial tissues of fifth-instar *H. virescens* injected with 20 μg of purified yeast-expressed ScathL at 24 hpi (A, B) or uninjected control larvae (C). A’, B’, and C’ show the boxed areas at left at higher magnification. d, dorsal aorta; a, alary muscle; p, pericardial cells. Scale bar = 60 μm.
Fig. 8. ScathL-induced lysis of gut and other internal tissues in pea aphids. Control aphid injected with: (A) 0.1 μl of 30 mM sodium acetate, pH 5.5; (B) 300 ng of ScathL.C146A; (C, D) aphids injected with 50 ng of purified yeast-expressed ScathL. Aphids were dissected 12 h after injection. Note the absence of the gut and reduced fat body in (C) and (D).

Fig. 9. Impact of ScathL on phenoloxidase (PO) and prophenoloxidase (pro-PO). (A) PO activity of hemolymph extracted from fifth-instar H. virescens larvae infected with AcMLF9.ScathL, AcMLF9.ScathL.C146A, or wild-type C6 viruses at 5 × 10⁵ polyhedra/larva at the indicated time points. (B) PO activity after addition of water (mock) and the combined activity of PO and pro-PO after addition of the pro-PO activator CPC in the hemolymph of H. virescens larvae infected with viruses at 72 hpi. (C) Activity of PO after addition of water (mock) or CPC to hemolymph extracted from melanized and non-melanized H. virescens larvae. (D) PO activity in the hemolymph extracted from healthy early fifth-instar H. virescens larvae after addition of water (mock), ScathL, or CPC. Means with the same letter are not significantly different at P = 0.05, and error bars represent standard errors.
resulting from ScathL action may be why we failed to see an increased LC$_{50}$ of AcMLF9.ScathL for *H. virescens*.

Abnormality of the BM is considered an important means by which the immune system distinguishes self from non-self in insects. In some strains of *Drosophila melanogaster* Meigen that produce melanotically encapsulated tumors, the formation of melanotic tumors was elicited by disruption of the BM of the fat body, and followed by melanotic encapsulation of the affected area (Rizki and Rizki, 1974, 1980a, b). Tissue grafts in *D. melanogaster* bearing mechanically or enzymatically damaged BM also underwent melanotic encapsulation, while tissue grafts with undamaged BM were not encapsulated (Rizki and Rizki, 1980a, b). Hemocytes normally spread rapidly on foreign surfaces, but did not spread on a dish surface coated with BM (Pech et al., 1995). This study suggests that BM can make a surface appear to be self. In addition, antibodies raised against surface proteins of the insect pathogenic fungus *Nomuraea rileyi* were cross-reactive with antigens on the surface of the fat body of the host, *Spodoptera exigua* Hübner (Pendland and Boucias, 1998, 2000). Hyphae of *N. rileyi* are capable of evading the immune response of *S. exigua*. This suggests that *N. rileyi* utilizes a form of molecular mimicry in which the hyphae present a surface that resembles the BM of the host insect. These studies provide an explanation for the melanization caused by ScathL: ScathL degrades the insect BM, which causes the host’s immune defenses to recognize its own BM as foreign. We did not see melanization of any internal or external tissues in aphids or in mosquitoes that were killed by injection with ScathL (Li et al. unpublished observation). The reason for the lack of melanization in these insects is unknown. ScathL-induced damage may not trigger the same immune response in all types of insects. Variation in susceptibility to melanization may also explain why only some tissues (such as tracheae, cuticle, pericardial cells, dorsal aorta and alary muscles) were melanized in *H. virescens* larvae killed by ScathL.

A recent study indicated that a cathepsin L (Har-CL) from cotton bollworm, *Helicoverpa armigera*, plays an important role in molting and may function in cuticle degradation (Liu et al., 2006a). In our experiments with fifth-instar *H. virescens*, we did not see cuticle damage following either oral infection with AcMLF9.ScathL or injection with purified ScathL. In contrast, the cuticle of larvae infected with AcMLF9.ScathL was much harder than those of uninfected control larvae, likely due to widespread melanin deposition on the cuticle. In addition to the results presented here, ScathL-mediated damage to the BM overlying the gut and fat body in larvae infected with AcMLF9.ScathL was confirmed by electron microscope analyses (Tang et al., 2007). Whether or not the cuticular melanization is associated with cuticle damage requires further investigation.

Injection with purified ScathL did not cause significant cuticular melanization, but resulted in tracheal melanization in both types of responses of *H. virescens* larvae to ScathL. Tracheae appear to be the most sensitive for melanization but tracheal melanization may not be lethal to *H. virescens* larvae. Type one responses are characterized by rapid death due to tissue lost to ScathL, protease activity and type two responses are characterized by slow mortality, without apparent tissue damage or change in coloration except for pericardial cells, dorsal aorta and alary muscles. The difference in responses to ScathL injection may be associated with a difference in dispersal of ScathL in the hemocoel following injection and/or with differences in concentration of a cysteine protease inhibitor in the hemocoel (Miyaji et al., 2007).

The current use of toxins for development of insect resistant transgenic plants has been largely restricted to agents that act within the gut of the insect pest, such as Bt toxins, enhancin, protease inhibitors, and avidin (Liu et al., 2006b). However, as expected from the mode of action of ScathL within the body cavity, bioassays indicated that ScathL had no insecticidal effect when orally ingested (unpublished data). The high-level toxicity of ScathL when delivered into the hemocoel suggests that ScathL would serve as an excellent transgene to confer plant resistance to insect pests when combined with an appropriate delivery system. Some plant lectins are able to cross from the gut into the hemocoel of Lepidoptera and aphids (Down et al., 2006). Lectins such as *Galanthus nivalis* agglutinin (GNA) are resistant to proteolysis in the guts of herbivores (Fitches et al., 2004). Upon ingestion by Lepidoptera and Hemiptera, GNA binds to the gut epithelium and passes into the hemocoel most likely by endocytosis (Fitches et al., 2001). The ability of GNA to cross the gut epithelium gives this protein the potential to act as a carrier to deliver fused peptides into the hemocoel of targeted insect pests. This concept has been demonstrated with a variety of intrahemocoelic effectors (Down et al., 2006; Fitches et al., 2002). Hence, ScathL fused to GNA may have potential for use in development of transgenic plants for insect management.

**Acknowledgments**

The authors thank Drs. Jan O. Washburn and Loy E. Volkman at University of California, Berkeley, CA, for technical assistance; Dr. Sijun Liu for assistance with antibody production. This material is based upon work supported by USDA NRI 2003-35302-13558 as well as Hatch Act and State of Iowa funds.

**References**


Homma, K., Jurata, S., Natori, S., 1994. Purification, characterization, and cDNA cloning of procathepsin L from the culture medium of NIH-Sape-4, an embryonic cell line of Saccharomyces cerevisiae (flesh fly), and its involvement in the differentiation of imaginal discs. Journal of Biological Chemistry 269, 15258–15264.


