CHOLESTEROL OXIDASE: POTENT BOLL WEEVIL LARVICIDAL AND OöSTATIC AGENT SUITABLE FOR TRANSGENIC COTTON DEVELOPMENT

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Abstract

The enzyme cholesterol oxidase (E.C. 1.1.3.6), purified from a Streptomyces culture filtrate was found to have strong oral insecticidal activity on neonate larvae of the boll weevil (*Anthonomus grandis grandis* Boheman) from a laboratory population. The LC_{50} and LC_{95} against neonate boll weevil larvae in 14 day bioassays were 0.24 and 4.52 µg/mL, respectively. Cholesterol oxidase also showed moderate insecticidal activity against lepidopteran larvae. Larvae of several species were stunted severely by the enzyme at 100 µg/mL in 7 day assays. Extended feeding (27 days) by *Heliothis virescens* at this concentration resulted in 97% mortality with no survivors pupating. Cholesterol oxidase, cloned and expressed in tobacco plants, was bioactive as shown in bioassays with boll weevil larvae. These studies were conducted to further evaluate the utility of cholesterol oxidase in a program to establish boll weevil-resistant transgenic cotton.

Introduction

Pest control strategies involving the use of transgenic crops have been successfully demonstrated, specifically through the expression of genes for *Bacillus thuringiensis* proteins (Barton *et al.*, 1987; Fischhoff *et al.*, 1987; Perlak *et al.*, 1990; Vaeck *et al.*, 1987). Other orally active insecticidal proteins, such as lectins, amylase inhibitors, and proteinase inhibitors, will retard growth and increase development time, but afford little acute toxicity. Several recent studies reflect attempts to augment *B.t.* proteins in terms of pest spectrum and mode of action in transgenic crop development (Rahbé & Febvay, 1993; Purcell *et al.*, 1993; Purcell *et al.*, 1994). Previously, Purcell *et al.* (1993) described the larvicidal activity of cholesterol oxidase against *Anthonomus grandis* and suggested a mode of action that is direct and not mediated by diet alteration. Lethality appears to be effected by disruption of the midgut epithelial cell membrane (Purcell *et al.*, 1993). Larvae from field-collected adults were similarly susceptible to cholesterol oxidase in the diet (Greenplate *et al.*, 1995). When ingested by adult females during the mating/pre-oviposition period, cholesterol oxidase greatly reduced subsequent oviposition (83% reduction in eggs laid as compared to controls) and larval survival (97% reduction from controls). Dissection of treated adult females revealed poorly developed ovaries, few developing oöcytes, and compromised fat body development (Greenplate *et al.*, 1995). Corbin *et al* (1994) successfully cloned and expressed insect-active cholesterol oxidase in *E. coli*. Enzymatically active cholesterol oxidase was also expressed in tobacco protoplasts by Corbin *et al* (1994). The study described below further characterized cholesterol oxidase activity against the boll weevil by using a 14-day concentration-response diet bioassay to determine LC_{50} and LC_{95} values. The enzyme's activity upon lepidopteran larvae was also explored. Finally, the bioactivity of cloned and plant-expressed cholesterol oxidase was evaluated against boll weevil larvae. This work was designed to demonstrate the utility of the enzyme as a potential control agent if expressed in transgenic cotton.

Materials and methods

Cholesterol oxidase. Boll weevil concentration-response bioassays and lepidopteran diet assays utilized cholesterol oxidase purified, as described previously, from the *Streptomyces* enzyme purchased from SIGMA (Purcell *et al.*, 1993). Protein concentrations were estimated spectrophotometrically (Layne, 1957).

Expression of cholesterol oxidase in tobacco plants. Cholesterol oxidase-expressing transgenic tobacco plants were produced via inoculation of tobacco leaf disks with *Agrobacterium tumefasciens* strains containing plasmids bearing genes for cholesterol oxidase expression (Horsch *et al.*, 1985; Corbin, unpublished).

Boll weevil larval bioassay. Boll weevil larval bioassays were performed essentially as described (MacIntosh *et al.*, 1990; Purcell *et al.*, 1992; Greenplate *et al.*, 1995) using an agar based artificial diet modified slightly from that described by Marrone *et al.* (1985). The published diet was altered by replacing formalin with 3 mL of an aqueous solution of phosphoric acid (3.8%) and propionic acid (42%) for each liter of final diet volume. An additional 10.5 mL of 10% KOH was also added for each liter of final diet volume. The diet was prepared as described (Marrone et al., 1985) except that 20% of the required water was held back. The water was replaced when the aqueous samples to be assayed were thoroughly incorporated into the warm (40-45° C) liquid diet. Hepes buffer (25 mM, pH 7.5) served as sample diluent and was used in untreated controls. Treated diet was immediately poured into 1 ml wells of 96-well insect diet trays (Jarold Mfg., St. Louis, MO) and allowed to cool and gel. Boll weevil eggs (4-8 in 50 µL of 0.15% agar), within 12-24 hours of hatch, were added to each well. Eggs from lab-reared weevils were secured through GAST laboratory, Mississippi State University. The 96 well trays were covered with a mylar film (Clear Lam Pkg., Elk Grove Village, IL) and sealed with a tacking iron (Seal® Products Inc., Naugatuck, CT). An insect pin (#2) was used to punch a small aeration hole in the mylar over each well. The trays were incubated at 27° C for 14 days at which time mortality was noted. LC_{50} and LC_{95} values were calculated by probit analysis (Statistical Analysis System, SAS Institute, Cary, NC).

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Lepidopteran diet bioassays. Lepidopteran bioassay diet trays were prepared as described above. After diet was prepared and treated, either eggs (4-8 in 50 µL of 0.15% agar), within 12-24 hours of hatch, or neonate larvae were added to each well. Tobacco budworm *(Heliothis virescens*), beet armyworm (*Spodoptera exigua*), cotton bollworm (*Helicoverpa zea*), and black cutworm (*Agrotis ipsilon*) were delivered as eggs; European cornborer (*Ostrinia nubilalis*), tobacco hornworm (*Manduca sexta*), and pink bollworm (*Pectinophora gossypiella*) were delivered as neonates. In most cases, assays were evaluated after 7 days at 27° C. An extended bioassay was performed in which *H. virescens* survivors were transferred to fresh diet (appropriately to either treated or control diet) at periodic intervals over a 27 day span.

Bioassay of transgenic plant material. Leaves from transgenic cotton plants were lyophilized and ground into a fine powder. A 2% slurry (w/v) of tobacco powder was made in a 0.15% agar solution. The slurry was overlaid upon clean insect diet (prepared as described above) at a ratio of 200µL per 1mL diet well. The slurry was allowed to surface dry under a laminar flow hood, then boll weevil eggs were added and the wells were again allowed to surface dry. Trays were covered and incubated as described above and after 7 days mortality and larval weights were recorded.

Results

Neonate larvae of *A. grandis* were highly susceptible to cholesterol oxidase in the diet, showing LC_{50} and LC_{95} values of 0.24 and 4.52 µg/mL, respectively in 14 day bioassays (Table 1).

Cholesterol oxidase had a significant effect upon the development of every lepidopteran species tested. When neonates were challenged for 7 days with cholesterol oxidase at 100µg/mL, stunting ranged from 27% for pink bollworm to 86% for tobacco budworm (Figure 1). In a 7 day concentration response study, the most sensitive lepidopteran (*H. virescens*) showed an approximate 50% reduction in weight in response to a cholesterol oxidase concentration as low as 5 µg/mL (Figure 2).

Chronic exposure to cholesterol oxidase at 100 µg/mL led to significant mortality in *H. virescens* larvae (Figure 3). After reaching 30% at 7 days, mortality rose dramatically to 67% at 10 days and reached 90% at 21 days. By 27 days, at which time all control larvae had pupated, mortality reached 97% among the treated larvae with no likelihood of survivors completing development.

Cholesterol oxidase was effectively expressed in phenotypically normal tobacco plants and showed powerful insecticidal activity against boll weevil larvae. When lyophilized powders were overlaid upon synthetic diet, cholesterol oxidase-expressing plants resulted in high levels of mortality and survivor stunting while non-expressing tobacco powder had no effect (Figure 4).

Discussion

The boll weevil, *Anthonomus grandis* has been regarded as the single most destructive cotton insect pest in the United States. The boll weevil utilizes cotton almost exclusively as a food source. Adults feed upon and oviposit into the buds and bolls; each female can lay 100-300 eggs during a 3-6 week oviposition period. Within the protected environment of the bud or boll, larvae develop through three larval instars and the pupal stage; accordingly traditional chemical control measures have targeted adults.

In artificial diet assays, the activity of cholesterol oxidase was extremely potent against neonate boll weevil larvae; LC_{50} and LC_{95} values were 0.24 and 4.52 µg/mL, respectively (Table 1). These values are consistent with levels of activity exhibited by B.t. proteins against the most susceptible lepidopteran targets (MacIntosh *et al*., 1990). The potential utility of cholesterol oxidase as a transgenically expressed control agent in cotton is obvious, since commercially effective levels of B.t. proteins have been expressed in cotton for the control of lepidopteran species. A transgenic approach for the control of *A. grandis* larvae would remedy a missing link in boll weevil IPM by exposing heretofore protected larvae to an effective control agent. In addition to its larvicidal activity, cholesterol oxidase has exhibited significant oöstatic effects on adult females, reducing fecundity by over 80% and subsequent larval viability by 97% (Greenplate *et al*., 1995).

The putative target in *A. grandis* is the midgut epithelial cell layer (Purcell *et al*., 1993). In mammalian systems, the ability of cholesterol oxidase to lyse erythrocytes is associated with a hypercholesteremic or hyperlipidemic erythrocyte membrane (Lange *et al.*, 1984; Bernheimer *et al.*, 1987). If midgut epithelia in wild *A. grandis* populations differ in membrane composition from those in lab populations due to variances in cholesterol and lipid content in artificial diet and cotton tissue, then it is possible that cholesterol oxidase would affect populations unequally. Neonate larvae from one wild population were susceptible to cholesterol oxidase (Greenplate *et al*., 1995) suggesting that laboratory colony susceptibility to the enzyme is not artifactual.

Dissection of cholesterol oxidase-fed females showed poorly developed ovaries with few developing oöcytes (Greenplate *et al*., 1995). *Bacillus thuringiensis* proteins, which disrupt susceptible larval midguts (Gill *et al.*, 1992), are able to reduce fecundity when fed to adult *Heliothis virescens* (Ali and Watson, 1982). Purcell *et al.*, (1993) demonstrated by light microscopy studies that cholesterol oxidase leads to extreme disruption of the boll weevil larval midgut epithelial cells. Midgut disruption of like nature, but less severe, in female adult boll weevils could effectively reduce the ability to assimilate nutrients and thereby greatly compromise

oögenesis. The observed atrophy of fat body in treated females is consistent with this hypothesis. The exact nature of the cholesterol oxidase interaction with larval and adult midgut epithelial membranes will be the subject of future work.

The activity of cholesterol oxidase against lepidopteran larvae is interesting in terms of its relative uniformity (All species tested were effected.) and its lack of acute mortality (Figure 1; Figure 3). Despite the lack of acute activity, concentrations as low as 5 µg/mL reduced growth in *H. virescens* as much as 50% (Figure 2) and prolonged exposure effectively prevented development to adulthood (Figure 3). Microscopy studies with *H. virescens* show marked pathology of the midgut epithelium when exposed to cholesterol oxidase (unpublished observations). These data suggest potential utility of cholesterol oxidase to augment the activity of B.t. proteins against lepidopterans in a transgenic plant.

Corbin *et al.* (1994) successfully cloned and expressed the cholesterol oxidase gene in *E. coli* and in tobacco protoplasts; subsequent *Agrobacterium* transformation of tobacco resulted in phenotypically normal cholesterol oxidase-expressing plants (Horsch *et al*., 1985; Corbin, unpublished). Tissue from cholesterol oxidase-positive plants exhibited potent insecticidal activity against boll weevil larvae even when fed in tandem with optimal synthetic insect diet (Figure 4).

We have shown, through these data and previous studies, that cholesterol oxidase is an effective larvicidal and oöstatic agent against *A. grandis* with significant bioactivity against larvae of several lepidopteran species. We have also successfully demonstrated the production of bioactive cholesterol oxidase in a transgenic plant.

If expressed in cotton buds, squares, and bolls, cholesterol oxidase could effectively reduce the incidence of bolls dropped by reducing the number of *A. grandis* eggs laid. Larvae resulting from any viable eggs would be effectively controlled by the expressed cholesterol oxidase. The potential for cholesterol oxidase to augment plant-expressed B.t. proteins for improved lepidopteran control, and/or as a component of resistance management, also appears to be viable. For these reasons we are currently pursuing the evaluation of this enzyme as a control agent in a transgenic cotton program.

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Table 1. Effect of cholesterol oxidase on neonate larvae of *A. grandis* when presented in artificial diet.

	µg/mL diet	95% confidence limits
LC_{50}	0.24	0.09 - 0.41
LC_{95}	4.52	2.71 - 13.09

Neonate larvae of *A. grandis* were exposed to a range of concentrations of cholesterol oxidase in artificial diet. At 14 days LC_{50} and LC_{95} values were determined by probit analysis (Statistical Analysis System, SAS Institute, Cary, NC).

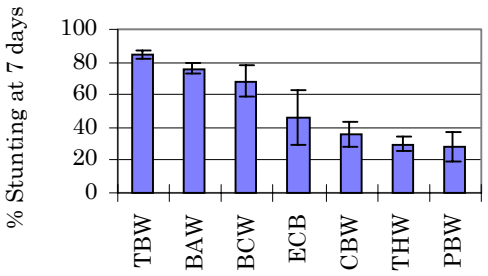

Figure 1. Effect of cholesterol oxidase on lepidopteran larvae. Neonate larvae were fed artificial diet containing cholesterol oxidase at 100 µg/mL. After seven days larval weights were measured and stunting relative to the average untreated control weight was calculated for each treated larva. Values represent means ± SEM. In all cases treated larvae were significantly smaller ($P < 0.05$) than conspecific controls. TBW - tobacco budworm; BAW - beet armyworm; BCW - black cutworm; ECB - European cornborer; CBW - cotton bollworm; THW - tobacco hornworm; PBW - pink bollworm.

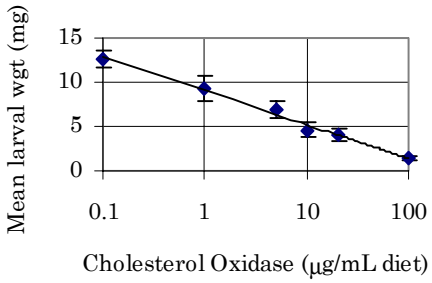

Figure 2. Concentration response curve of cholesterol oxidase against *H. virescens*. Eggs were added to treated diet; after 7 days at 27◦C, larvae were weighed. Values represent means ± SEM.

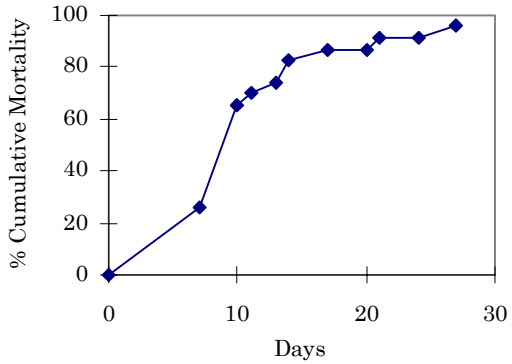

Figure 3. Effect of exposure of *H. virescens* larvae to cholesterol oxidase over time. Eggs of *H. virescens* were added to diet containing cholesterol oxidase at 100 µg/mL. At intervals, surviving larvae were transferred to fresh diet (either control- or cholesterol oxidase-treated) until 27 days when all controls had pupated.

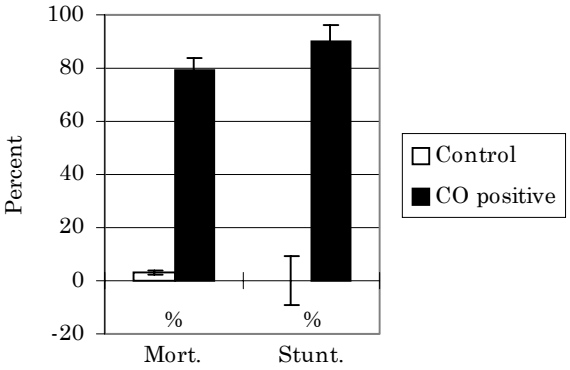

Figure 4. Effects of tobacco-expressed cholesterol oxidase upon *A. grandis* larvae. A 2% slurry of lyophilized plant material was overlaid upon synthetic insect diet. *A. grandis* eggs were added; mortality and relative stunting (as compared to diet treated with control tobacco powder) was recorded after 7 days. The figure compares non-transformed control tobacco plants with plants from a single cholesterol oxidase-expressing line. Values represent means ± SEM.