Rapid Bioassay to Screen Potential Biopesticides in *Tenebrio molitor* Larvae

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ABSTRACT A simplified assay was devised to evaluate the response of *Tenebrio molitor* larvae to potential insect control products. The assay incorporates punched disks of flattened whole-grain bread placed in 96-well plates, with treatments applied topically, and neonate larvae added to each well. To evaluate the method, increasing doses of the *Bacillus thuringiensis* Cry3Aa protoxin were added as a suspension to diet disks. Larval mortality was recorded, and LD_{50} values were obtained at different intoxication time intervals. The LD_{50} decreased as the interval of toxin exposure increased. At day 35, the LD_{50} for Cry3Aa protoxin was 8.06 mg/disk, approximately an order of magnitude higher than values obtained for lepidopteran larvae exposed to Cry1A toxins in diet overlay assays at much shorter time intervals. The lower efficacy and slower response probably account for the lack of incorporation of Cry3Aa into integrated pest management of coleopteran storage pests. The slower response of *T. molitor* to Cry3Aa may be behavioral, or it may be an indication of differences in the mode of action of Cry3A and Cry1A toxins. The diet-disk assay offers improvements over previous assays, including shorter preparation time, relatively simple and inexpensive, and larger numbers of larvae screened.

KEY WORDS : *Tenebrio molitor*, mealworm larvae, insect bioassay, Cry3Aa

INTRODUCTION

Coleopteran insects from the family Tenebrionidae are among the most damaging to agriculture worldwide. As solubility and digestion by midgut enzymes is often a factor in the efficacy of oral insect control products, knowledge of the gut physiology greatly aides in interpretation of the insect response. The yellow mealworm, *Tenebrio molitor*, has been the focus of many biochemical studies because of the relatively larger size of the larval stage as compared to other Coleoptera (reviewed in Vinokurov *et al.*, 2006a). *T. molitor* larvae have a complex of midgut proteases, and digestion is compartmentalized to an anterior that is more acidic with active cysteine proteases, and a basic posterior with mostly serine protease activities (Vinokurov *et al.*, 2006a,b; Prabhakar *et al.*, 2007).

The earliest report of a coleopteran-specific *Bacillus thuringiensis* (Bt) toxin, Cry 3Aa, was from Bt subsp. *tenebrionis* and was found in *T. molitor* larvae (Herrnstadt *et al*., 1986). The first characterized receptor for Cry3Aa toxin was a midgut cadherin, also from *T. molitor* larvae (Fabrick *et al.*, 2009). There are many reports that cadherins in the lepidopteran midgut bind to Cry1A toxins and are involved in toxin mode of action (Bravo *et al.*, 2007). The finding that Cry3Aa toxins bind to cadherin in coleopteran larvae suggests similarities in the mode of actions of Cry3Aa and Cry1A toxins.

While Cry3Aa was isolated from *T. molitor* lar-

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Fig. 1. Components of the bread diet-disk assay. A) Rolling pin, pizza cutter, 96-well microplate, and 4 mm cork borer on wax paper, used in the preparation of the bioassay. B) Flattened and trimmed bread, demonstrating disks punched with the 4 mm cork borer and placed into each well of a 96-well microplate.

vae, it has not been integrated into pest control programs for storage pests. *T. molitor* are particularly problematic in bioassays because of the slower generation time compared to other tenebrionids. Extremely adaptable to environmental conditions, *T. molitor* displays plasticity in all of the developmental stages (Cotton and St. George, 1929). Under optimal environmental rearing and dietary conditions, complete larval development requires approximately nine months, but under suboptimal conditions such as their natural environment, larvae can undergo as many as 20 instars, and development from egg to adults can last more than 20 months. Typical laboratory bioassays that measure developmental times and progeny are not practical for *T. molitor*, even reared under optimal conditions.

A major goal of our research is to identify new insecticidal proteins to use as a tool in integrated pest management of coleopteran storage pests. One limiting factor in screening midgut proteins and genetic transcripts as potential targets for tenebrionid pests is a simple, rapid-screen bioassay. While several assays have been used previously to screen potential control agents in storage pests (Johnson *et al.*, 1991; Herrero *et al.*, 2001; Epsky, 2001), improvements were needed in delivery of the agents, preparation time, and assay method. To address these problems, a simple assay was devised based on punched disks of flattened multi-grain bread. The bread-disk bioassay is inexpensive and relatively fast compared to previous methods. The bioassay procedure was demonstrated by evaluating the mortality of *T. molitor* larvae over time with increasing doses of Cry3Aa applied to the diet disks.

MATERIALS AND METHODS

T. molitor are maintained as a laboratory colony on a standard diet of 50% rolled oats mixed with 47.5% wheat flour and 2.5% brewer's yeast, under a controlled environment of 60% RH and 28°C. Adults are supplemented with drinking water on bulk cotton in a Petri dish on the standard diet. To collect eggs, adults were allowed to mate and oviposit on standard diet, and eggs and oat flake fragments were removed using a series of 8, 15, and 25 mesh/in screens.

To prepare the bioassay diet, items were assimi-

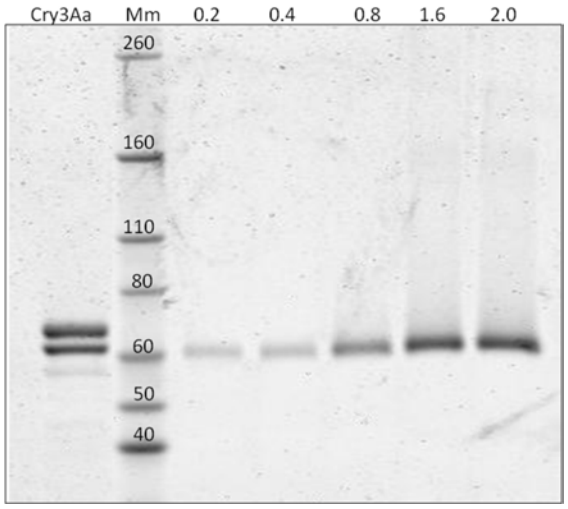

Fig. 2. Coomassie-stained sodium dodecyl sulfate polyacrylamide electrophoresis gel of Cry3Aa suspension (left), molecular mass markers (Mm, Sharp, Invitrogen), and bovine serum albumin (right) in increasing amounts, as indicated at the top in µg.

lated from the kitchen: rolling pin, pizza cutter, and wax paper; and from the laboratory, a 4 mm cork borer and standard polystyrene 96-well microplates (Evergreen Scientific, Los Angeles, CA; Fig. 1A). A slice of multi-grain bread was placed on the wax paper overlaid on cardboard, and the crust was removed using the pizza cutter. The bread was flattened with the rolling pin, and the cork borer was used to remove 4 mm disks of diet and place each in a microplate well (Fig. 1B). Microplates with diet disks were equilibrated in a salt box with saturated NaCl (approximately 75% RH), 28°C, for 24 h. To each row, either suspensions of Cry3Aa (dilutions containing a range of 0.015 to 60 µg, 11–12 doses) or water as a control were added to the diet disks in no more than 4 µl total volume. The treated diet disks were again incubated in the salt box for 24 h, and neonate larvae were added to each well using a fine no. 2 artist's paintbrush. Bioassay trays were covered with an air permeable membrane (Breathe-EASIER, Diversified Biotech, Boston, MA). Bioassays were incubated in the salt box, and membranes were temporarily removed to evaluate mortality at weekly intervals.

Cry3Aa protoxin was purified from sporulated cultures of Bt var. *tenebrionis* as previously described (Herrnstadt *et al.*, 1986). The amount of Cry3Aa protoxin was estimated by densitometry in a Coomassie-stained 3–8% tris-acetate sodium dodecyl sulfate polyacrylamide electrophoresis gel (Invitrogen, Carlsbad, CA) using bovine serum albumin (Pierce Chemical Co., Rockford, IL) as a standard (Fig. 2). Gels were scanned and protein estimated by the Odyssey Imager and related software (LiCor, Lincoln, NE). Cry3Aa protoxin was estimated as mostly a doublet of approximately 67 and 73 kDa peptides, as has been previously described for the purified crystalline protoxin (Carroll *et al.*, 1989).

Data was analyzed by probit (POLO, Robertson *et al.*, 1980). Each data set included 2–3 biological replicates from different generations of progeny (n = 11–27), and in most cases, lower doses were not used in calculations. POLO produces a heterogeneity factor, which is χ^2 divided by the degrees of freedom and is a measure of how well the data fit the model; a heterogeneity factor greater than 1 indicates that the data fit the model poorly.

RESULTS

T. molitor larvae on control or Cry3Aa-treated diet disks were monitored at seven day intervals for

Table 1. Effect of Cry3Aa from *Bacillus thuringiensis* subsp. *tenebrionis* on *Tenebrio molitor* larvae (n = 11–27, 2–3 biological replicates, d.f. = 9; POLO analysis)

Day	LD_{50} (95% CI)[1]	χ^2	Heterogeneity
7	n/a	-	-
14	18.5 (11.8–37.7)	4.60	0.51
21	21.4 (12.2–53.2)	6.85	0.76
28	8.81 (5.61–11.6)	6.03	0.82
35	8.06 (5.61–11.6)	5.68	0.63

[1]Data are in µg, applied to each diet disk

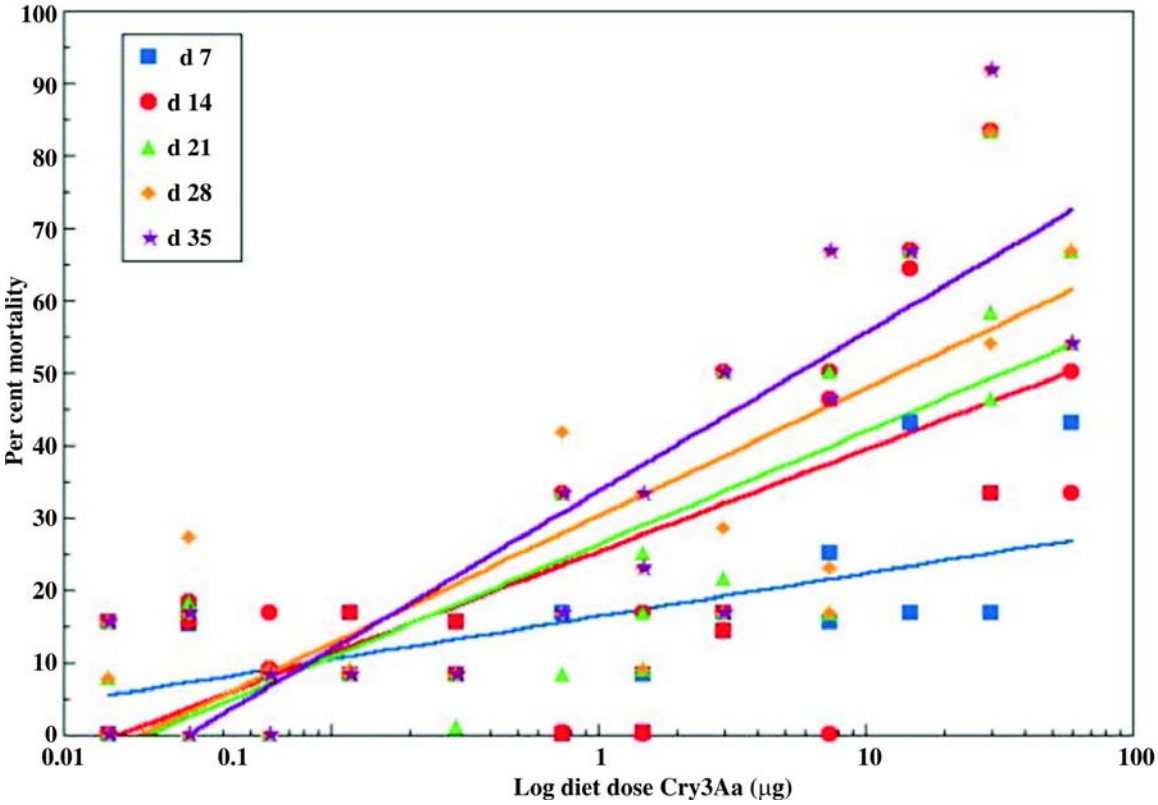

Fig. 3. Per cent mortality as a function of increasing doses of Cry3Aa protoxin, each data point representing one row of the bioassay in replicate treatments, recorded at day 7, 14, 21, 28, or 35, as indicated. Regression lines were given for all data points at each time interval.

mortality (Table 1 and Fig. 3). Control mortalities ranged from 3.9 to 7.7% in all replicates. At day 7 post treatment, per cent mortality was less than 50%, even at the higher doses, and probit analysis was unable to determine a lethal dose for 50% mortality (LD_{50}). Incomplete mortality was likely due to the fact that surviving larvae did not ingest a lethal dose, even at the highest dose of 60 μg.

As incubation times were extended on Cry3Aa-treated diets, *T. molitor* larvae suffered increasing mortality. However, because mortalities with the highest dose, 60 μg, were lower than with 30 μg, the highest dose was excluded from POLO analysis. At each time interval, the heterogeneity scores were less than 1, providing confidence in linearity of the data. In comparing days 14–35, the hypothesis of equality was rejected, an indication that the regression lines for each day are not equal, while the hypothesis of parallelism was not rejected, indicating that the slopes for the regression lines are not different. On days 14 to 21, the LD_{50} values were similar, as the Lethal Dose Ratio (LDR) was 0.87 (95% C.I. 0.38–1.98) and indicates that they are not different; this also was true in comparing days 28 and 35 (LDR = 1.09, C.I. 0.65–1.84). The decrease in the LD_{50} for Cry3Aa, from 18.5 and 21.4 μg/disk on days 14 and 21, respectively, to 8.81 and 8.06 μg/disk on days 28 and 35, respectively, suggests that significant events may be occurring in the 3–4 week time interval post intoxication.

After 35 days of incubation, mortalities greater than 90% were achieved with 30 μg of Cry3Aa protoxin (Fig. 3). However, when the dose was increase to 60 μg, mortality decreased, suggesting that larvae may have been able to detect the toxin and avoid ingestion.

Table 2. Results of previous bioassays of Cry toxins from different *Bacillus thuringiensis* (Bt) isolates in *Tenebrio molitor* larvae

Cry Toxin	Isolate	Expression Host	Delivery	Instar	Response	Reference
Cry01Ia2	*kurstaki* DSIR32	*E. coli*	diet incorporation	1	not active, 2 mg/ml lysate	Gleave *et al.*, 1993
Cry03Aa	*tenebrionis* 1911	Bt *tenebrionis*	forced feeding	n/a[1]	some activity, 5 µg/d, 5 d	Carroll *et al.*, 1989
Cry03Aa1	*san diego*	Bt *san diego*	diet incorporation	1–3	some activity, no dose given, 30 d	Hernstadt *et al.*, 1986
Cry03Aa2	*tenebrionis*	Bt *tenebrionis*	forced feeding	7	LD_{50} 10.5 (7.50–15.1) µg/larva	Wu *et al.*, 1996
Cry08D,E	Bt 185	Bt 185	diet incorporation	1	not active	Yu *et al.*, 2006

[1]Data not available

DISCUSSION

Previously, our laboratory has employed several different methods to screen individual storage pests. In one assay developed to screen moths for the effects of Bt toxins, small cubes (2 mm) of diet were cut with a razor blade from semi-dehydrated apple, placed into individual compartments of an insect bioassay tray, and dosed with 2 µl of toxin suspension (Johnson *et al.*, 1991). In that assay, individual 2nd and 3rd instars were added to each dosed apple cube, covered with clear perforated plastic, and mortality was recorded every 3–4 days until all insects were either dead or pupae. The main limitation of this assay was that larvae did not always readily consume the apple cube, and often yeast extract was added to make the diet more palatable. Also, the manual preparation of diet was imprecise and time-consuming, and the bioassay trays and covers were an additional expense.

Another bioassay was developed using a flattened "pie-crust" of semi-hydrated cereal, wheat germ, yeast, and water, with sorbic acid and methylparaben added to prevent mold (Herrero *et al.*, 2001). This assay was superior to the apple cube assay because the insects more readily consumed the diet, and neonate larvae were used to evaluate the response in earlier larval stages. In addition, a 4 mm cork borer was more efficient in extracting diet disks and provided disks that were more uniform. While this bioassay was an improvement, making the flattened cereal diet was time consuming.

A bioassay for storage pests also was described that used dog food encapsulated in parafilm (Epsky *et al.*, 2001). In the parafilm bioassay, food scatter was one of the parameters used to evaluate insect feeding, but *T. castaneum* responded slowly, while *T. molitor* was not tested.

The present bioassay provided a reasonable evaluation of the effects of Bt Cry3Aa protoxin on *T. molitor* larvae. Although Bt *tenebrionis* Cry3Aa was first isolated from *T. molitor* larvae, it is ironic that scarce bioassay data are available on larval toxicity (http://www.glfc.forestry.ca/bacillus; Table 2). Cry01Ia and Cry08D,E had no activity in *T. molitor* (Gleave *et al.*, 1993; Yu *et al.*, 2006). The first report of Cry3Aa activity in *T. molitor* was with an isolate from Bt *san diego* (identical to Bt *tenebrionis*), in which a spore/crystal mixture was incorporated into the diet of 1st, 2nd, or 3rd instars and the effects were evaluated after 30 days (Herrnstadt *et al.*, 1986). However, no details on the amount of Cry3Aa or evaluation criteria were provided in this study, with activity judged simply as "++" on a scale of "-" to "++++". *T. molitor* larvae that were force-fed 5 µg of purified Bt *tenebrionis* Cry3Aa protoxin or toxin each day for five days suffered some effects from

the toxin, but it wasn't clear how activity was determined (again, a "+" scale was used) or what larval stage was used in the experiment (Carroll *et al.*, 1989).

Force feeding is of course the most precise dosing mechanism for *T. molitor* larvae, but it is time-consuming and necessitates using older larvae, which may be less sensitive to the potential biopesticide, or may require increased amounts of test agent due to increased body mass. Early instar larvae can be difficult to force feed, even when starved. Therefore, the diet-disk bioassay described in this paper was developed to use as a large-scale screening tool for potential control proteins or dsRNA in *T. molitor*. In this overlay bioassay, the LD_{50} for Cry3Aa in neonate larvae at day 28 was 8.81 µg/disk, most similar to the only previously reported LD_{50} for Cry3Aa in *T. molitor* larvae, 10.5 µg, which was force-fed to 7th instar and evaluated 5 days post feeding (Wu and Dean, 1996).

After a week of feeding on Cry3Aa-treated diet, less than 50% mortality was observed at even the highest treatment dose. *T. molitor* larvae consume diet intermittently, which contributes to a large variation in larval instar duration (Cotton and St. George, 1929). Alternatively, although Cry3Aa and Cry1A toxins interact with cadherin as part of their mode of action there may be downstream, post-binding event with the two toxins in the two insect orders. Comparing these data to Cry1A protoxins in lepidopteran larvae in similar diet overlay assays, Cry1A-intoxicated Lepidoptera usually die within hours instead of days or weeks, and the LC_{50} values are generally an order of magnitude lower (van Frankenhuyzen, 2009).

In summary, the diet-disk assay was an improvement to previous bioassays because of a relatively shorter preparation time and the ability to screen larger numbers of larvae. The assay used simple and relatively economical supplies from the kitchen and laboratory. Perhaps more importantly, the diet-disk bioassay more realistically represents larvae feeding *in vivo*, providing a more accurate assessment of the performance of the biological agent in the field.

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