Research Article

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Efficacy of *Bacillus thuringiensis* Cry3Aa protoxin and protease inhibitors against coleopteran storage pests

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Abstract

BACKGROUND: Environmental impacts and resistance to insecticides pose serious challenges to stored-product insect and other types of pest control. Insect-resistant transgenic grain is a potential alternative to fumigants, but candidate control proteins are needed, especially for coleopterans. Therefore, we evaluated the efficacy of a coleopteran-active toxin, *Bacillus thuringiensis* Cry3Aa, with or without protease inhibitors, in laboratory feeding assays against coleopteran storage pests.

RESULTS: In a comparison of the toxicity of Cry3Aa protoxin towards three species of coleopteran storage pests, *Tenebrio molitor* L. was found to be most sensitive, *Tribolium castaneum* (Herbst.) was most refractory and *Rhyzopertha dominica* F. displayed an intermediate response. For *R. dominica*, Cry3Aa combined with 3500 mg potato carboxypeptidase inhibitor or 5000 mg aprotinin kg^{-1} diet resulted in both delayed development and increased mortality. Potato carboxypeptidase inhibitor and bovine aprotinin reduced the LC_{50} of Cry3Aa for *R. dominica* two- and threefold respectively. Cry3Aa treatment resulted in fewer progeny from *R. dominica*, and progeny was further reduced when the protoxin was combined with potato carboxypeptidase inhibitor.

CONCLUSIONS: These data support the hypothesis that a combination of Cry3Aa protoxin and protease inhibitors, particularly a potato carboxypeptidase inhibitor, may have applications in control strategies for preventing damage to stored products and grains by coleopteran pests.
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Keywords: aprotinin; *Bacillus thuringiensis*; carboxypeptidase inhibitor; coleopteran; Cry3Aa; lesser grain borer; protease inhibitor; red flour beetle; *Rhyzopertha dominica*; *Tenebrio molitor*; *Tribolium castaneum*; yellow mealworm; stored-product insect

1 INTRODUCTION

The red flour beetle, *Tribolium castaneum* (Herbst.), and the lesser grain borer, *Rhyzopertha dominica* F., are major pests of stored products and grains worldwide.[1] Fumigants are often used to control these storage pests in stored products and grains, but loss of efficacy, regulatory restrictions and environmental impacts have provided the impetus to find alternative biologically targeted control solutions.[2] The latter species is particularly difficult to control in stored wheat because the young larvae bore directly into the seeds soon after hatching and feed internally, where they are sheltered from most control agents.

The yellow mealworm, *Tenebrio molitor* L., is considered to be a pest only in certain situations, such as poultry houses. Although *T. molitor* is generally not a target pest, this species serves as a model for studies of the efficacy of coleopteran-specific toxins from the bacterium *Bacillus thuringiensis* Berliner (*Bt*), and therefore may help to provide important information to promote the use of *Bt*-derived materials in the control of coleopteran pests. *Tenebrio molitor* is susceptible to the Cry3Aa toxin to a degree similar to that of Cry1A toxins in Lepidoptera.[3–6] Also similar to lepidopterans, a *Bt* toxin-binding protein in the gut of *T. molitor* larvae was identified as a cadherin-like protein.[7] However, other *Bt* entomocidal proteins, including Cry1A, Cry1I, Cry8, Cry34/35 and vip protoxins or toxins, have little or no effect on *T. molitor* growth and development.[8,9]

With regard to *T. castaneum*, *Bt* protoxins Cry3A, Cry23A/Cry37A and Cyt2C have been reported to have insecticidal activity.[8,10] However, *Bt* protoxins Cry1A, Cry8E/F/G and Cry34/35 were inactive.[8,9]

A previous report of *Bt* activity against *R. dominica* included isolates of *Bt* subsp. *darmstadiensis*, and, while there was little adult mortality, progeny was reduced by as much as 96% by spore/crystal preparations.[11] *Bt* subsp. *darmstadiensis* contains Cry1E, Cry5A and Cyt2A protoxins with activity in Lepidoptera, Diptera and Coleoptera.[8,12,13]

The potentiation of *Bt* toxicity by protease inhibitors has been proposed, but the experimental data are inconclusive. During the development of *Bt* transgenic plants, it was reported that inhibitors of trypsin and chymotrypsin potentiated the insecticidal activity of *Bt* toxins *in vivo* against several coleopteran and

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dipteran species.[14] In that study, transgenic tobacco expressing Cry1Ab and a squash trypsin protease inhibitor exhibited an approximately sixfold enhanced insecticidal activity towards the tobacco budworm, *Heliothis virescens* F. However, soybean trypsin inhibitors had no effect on the activity of a toxin formulation against the diamondback moth, *Plutella xylostella* L.[15]

To determine whether *Bt* protoxin Cry3Aa has a potential application in the control of coleopteran storage pests, feeding assays were conducted with *T. molitor, T. castaneum* and *R. dominica*. In addition, the effect of five protease inhibitors on Cry3Aa toxicity in *R. dominica* was also evaluated.

2 MATERIALS AND METHODS

2.1 Insects

Insects used in this study were from laboratory colonies. *Rhyzopertha dominica* was reared on whole wheat kernels, *T. castaneum* on 95% flour/5% yeast mixture and *T. molitor* on a mixture of 50% rolled oats and 50% flour/yeast at 28 °C, 16 : 8 h light : dark photoperiod and 50% RH. Adults of *T. molitor* or *T. castaneum* were placed on flour/yeast, and *R. dominica* was placed on 99% wheat and 1% flour/yeast for 16–24 h to obtain eggs.

2.2 Inhibitors and toxins

The protease inhibitors aprotinin, amastatin [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valyl-L-valyl-L-aspartic acid], ethylenediaminetetraacetic acid (EDTA) and carboxypeptidase inhibitor (CPI, labeled as 35% protein) were obtained from Sigma Chemical Co. (St Louis, MO). Bestatin [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine] was purchased from Boehringer Mannheim (Indianapolis, IN). Amastatin and bestatin are microbial, slow-binding, competitive inhibitors of aminopeptidase M;[16] aprotinin is a peptide from bovine lung and targets serine peptidases;[17] EDTA is a metal chelator that inhibits enzymes such as metallopeptidases or disrupts structural proteins such as cadherin; CPI was prepared from potato tuber.[18]

M-one, containing 105 mg Cry3Aa protoxin g^{-1} powder, was obtained from Mycogen (now Dow AgroSciences, Indianapolis, IN). The amount of M-one that was added in all experiments corresponded to the reported doses of Cry3A that were incorporated into the wheat germ or wheat kernel diet (mg Cry3A kg^{-1} powdered or solid diet). Mycogen also provided a formulation blank containing fermentation solids, sugars and a silicate bulking agent.

2.3 Bioassay of immature stages

For bioassays, raw wheat germ was ground through a 20 mesh screen in a Wiley mill and stored at −20 °C. Test compounds were mixed with wheat germ (mg kg^{-1}) and water was added (1 : 1.5 w/v), lyophilized and ground together in an alumina mortar with a pestle.

The assay container for *R. dominica* was a 0.2 mL microcapillary pipet tip with the capillary portion removed by twisting to form a seal. For *T. molitor* and *T. castaneum*, standard 1.5 mL microcentrifuge tubes without lids were used. Assays were performed using individuals placed on 4 mg of untreated diet or diet treated with a formulation blank for M-one (controls) or treated diet as indicated, each dispensed into a pipet tip for *R. dominica*, or 30 mg dispensed into microcentrifuge tubes for *T. molitor* or *T. castaneum*. After equilibration at 28 °C and 75% RH, an egg was added to each tube and monitored to determine the average date of hatch. Larvae of *T. molitor* or *T. castaneum* were weighed individually on a Cahn microbalance. Mortalities were recorded from egg hatch to adult eclosion for *R. dominica* and *T. castaneum* and during the first 50 days of the larval stage of *T. molitor*. Probit analysis (POLO-PC; LeOra Software, Berkeley, CA) was used to estimate LC_{50} and lethal dose ratio (LDR)[19] for Cry3Aa with *R. dominica*, using mortality data from control treatments and seven doses of Cry3Aa only (11–2100 mg kg^{-1}), five doses of Cry3Aa (11–525 mg kg^{-1}) with 3500 mg kg^{-1} CPI and three doses of Cry3Aa (105–525 mg kg^{-1}) with 5000 mg kg^{-1} aprotinin.

2.4 Bioassay of Cry3Aa and protease inhibitors on intact wheat kernels

M-one and/or CPI were ground with hard red winter wheat flour (160 mg) in a mortar with a pestle prior to mixing with 40 g of wheat kernels, and in some experiments M-one alone was mixed with wheat kernels. Doses of Cry3Aa, CPI and flour were expressed as mg kg^{-1} wheat kernels. Wheat kernels (with or without addition of M-one, CPI and flour) were infested with adults of *R. dominica* at 25 °C and 60% RH. Specifically, samples of 40 g of wheat in 120 mL glass jars, capped with filter paper, were infested with 20 adults for 7 days, and adult progeny were counted after an additional 9 week period.

3 RESULTS

3.1 Comparison of the effect of Cry3Aa on the mortality of coleopteran storage pests

The efficacy of Cry3Aa against *T. castaneum, T. molitor* and *R. dominica* was compared using a wheat germ diet. *Tribolium castaneum* tolerated doses of Cry3A as high as 2100 mg kg^{-1} without significant mortality (Table 1). Cry3Aa was most effective against *T. molitor*, resulting in 100% mortality at a concentration of 42 mg kg^{-1} and suggesting that the LC_{50} was between 11 and 42 mg kg^{-1} (Table 2). In contrast to the two extreme responses of *T. castaneum* and *T. molitor, R. dominica* larvae were intermediate in their response to Cry3Aa, with a LC_{50} of 1177 mg kg^{-1} (851–1828 mg kg^{-1} 95% CI) (Table 3). These data demonstrate that coleopteran storage pests exhibit a range of responses to Cry3Aa protoxin.

3.2 Efficacy of Cry3Aa and/or protease inhibitors in bioassays with *Tribolium castaneum* and *Tenebrio molitor*

Although *T. castaneum* mortality was not observed at high doses of the protoxin, the duration of development, from egg hatch to adult eclosion, was nevertheless extended substantially (Table 1). In larvae fed the highest dose of 2100 mg kg^{-1} Cry3Aa, the developmental time was approximately 50% longer for Cry3Aa-treated larvae. At this dose, the growth of larvae was significantly reduced by 81%. These data suggest that, although *T. castaneum* larvae survive the Cry3Aa toxin treatments, high doses impact upon this pest's population by decreasing larval weight and increasing developmental time. The addition of CPI to the diet, with or without Cry3Aa, did not affect larval growth or development.

For *T. molitor*, 100% mortality was observed at day 33 when larvae were fed 42 mg kg^{-1} Cry3Aa (Table 2). The mortality of *T. molitor* was similar to previous results using a *Bt* subsp. *tenebrionis* preparation, with LC_{50} values of 19 and 8 mg kg^{-1} at days 14 and 35 respectively.[6] The addition of CPI to Cry3Aa had no effect on larval weight or mortality, according to Tukey's test.

Table 1. Effect of Cry3Aa and/or CPI on the growth, development and mortality of Tribolium castaneum on a raw wheat germ diet[a]

Treatment	Cry3Aa dose[b] (mg kg^{-1})	Larval weight[c] (± SEM) (mg)	Mortality[c] (± SEM) (%)	Developmental time[d] (± SEM) (days)
Control	–	2.1 (±0.3)ab	0 (±0)a	24.9 (±0.7)ab
Formulation	–	2.2 (±0.1)a	6 (±6)a	24.3 (±0.3)ab
CPI	–	2.3 (±0.2)a	0 (±0)a	24.2 (±0.5)a
Cry3Aa	525	1.1 (±0.2)bc	5 (±5)a	27.7 (±0.7)abc
	1050	0.8 (±0.0)c	5 (±5)a	30.2 (±0.1)bc
	2100	0.4 (±0.1)c	6 (±6)a	37.3 (±1.2)d
Cry3Aa + CPI	525	1.3 (±0.3)abc	0 (±0)a	27.2 (±1.1)abc
	1050	0.8 (±0.2)c	5 (±5)a	31.1 (±2.4)c

[a] Means ± SEM of two replicates (7–11 larvae per replicate per treatment). Means followed by the same letter are not significantly different, $P < 0.05$ according to Tukey's test. For ANOVA of larval weight: $F = 14.8$; df = 7, 8; $P < 0.01$; mortality: $F = 0.4$; df = 7, 8; $P = 0.9$; developmental time: $F = 17.2$; df = 7, 8; $P < 0.001$.
[b] Formulation blank was 2100 mg kg^{-1}; CPI dose was 3500 mg kg^{-1}.
[c] Recorded on day 14.
[d] From egg hatch to adult eclosion.

Table 2. Effect of Cry3Aa and/or CPI on growth and mortality of *Tenebrio molitor* on raw wheat germ[a]

Treatment	Cry3Aa dose[b] (mg kg^{-1})	Larval weight[c] (± SEM) (mg)	Mortality[c] (± SEM) (%)	Time to death[d]
Control	–	3.6 (±0.1)a	0 (±0)a	–
Formulation	–	4.1 (±0.4)a	6 (±6)a	–
CPI	–	3.0 (±1.5)a	5 (±5)a	–
Cry3Aa	11	0.8 (±0.1)a	25 (±3)a	40 (±2)a
	42	–	100 (±0)b	18 (±2)b
Cry3Aa + CPI	11	0.9 (±0.1)a	6 (±6)a	–
	42	–	89 (±11)b	18 (±2)b

[a] Means (± SEM) of two biological replicates (6–10 larvae per replicate for each treatment). For ANOVA of larval weights: $F = 4.8$; df = 6, 7; $P = 0.06$; mortality: $F = 56.3$; df = 6, 7; $P < 0.001$; time to death: $F = 57.0$; df = 2, 3; $P < 0.01$.
[b] Formulation blank was 263 mg kg^{-1}; CPI dose was 3500 mg kg^{-1}.
[c] Recorded on day 30.
[d] Day mortality was recorded.

3.3 Effect of Cry3Aa and/or peptidase inhibitors on *Rhyzopertha dominica*

To further explore the use of Cry3Aa for the control of *R. dominica*, bioassays were conducted with M-one and various protease inhibitors. Cry3Aa at 105 mg kg^{-1} resulted in a longer developmental time for *R. dominica*. However, when Cry3Aa was combined with 3500 mg kg^{-1} CPI, mortality was observed in addition to increased developmental time (Table 4). Aprotinin or amastatin at 2000 mg kg^{-1} combined with Cry3Aa resulted in an increase in developmental times but no mortality. Bestatin or EDTA, with or without Cry3Aa, did not impact *R. dominica* growth or survival.

The effect of increasing doses of Cry3Aa and a fixed concentration of inhibitor was also evaluated (Fig. 1). Carboxypeptidase inhibitor at 3500 mg kg^{-1} or aprotinin at 5000 mg kg^{-1} combined with 263 and 525 mg kg^{-1} Cry3Aa increased the developmental time of *R. dominica* (Fig. 1a). However, either inhibitor alone or the combination of CPI and 105 mg kg^{-1} Cry3Aa had no significant effect on developmental time. The combination of CPI or aprotinin with Cry3Aa increased mortality at all Cry3Aa doses, but the letter rankings of mean mortalities from Tukey's test indicated a significant effect only when CPI or aprotinin was combined with 1050 mg kg^{-1} Cry3Aa (Fig. 1b).

We used doses of 5000 mg kg^{-1} aprotinin, 3500 mg kg^{-1} CPI and multiple concentrations of Cry3Aa to estimate the LC_{50} in *R. dominica* larvae (Table 3, Fig. 1). For Cry3Aa alone the LC_{50} was 1177 mg kg^{-1} (851–1828 mg kg^{-1} 95% CI), 384 mg kg^{-1} (268–778 mg kg^{-1}95% CI) for aprotinin combined with Cry3Aa and 606 mg kg^{-1} (403–1502 mg kg^{-1}95% CI) for CPI combined with Cry3Aa. To determine significance, LDR was used to compare the effect of Cry3Aa alone or with inhibitors. With LDR, the LC_{50} values do not differ if the 95% confidence interval (CI) overlaps the value of 1.[19] In the present study, the LDR indicated that the efficacy of Cry3Aa was increased significantly with either CPI (LDR = 1.9, 1.1–3.5 95% CI) or aprotinin (LDR = 3.1, 1.9–5.0 95% CI).

3.4 Efficacy of Cry3Aa and CPI mixed in flour and applied to seed

To test the applicability of using Cry3Aa and CPI as a seed treatment, the two materials were mixed with wheat flour and the mixture was applied to wheat kernels. In wheat kernels mixed with 11 mg kg^{-1} Cry3Aa and 4000 mg kg^{-1} flour and infested with adult *R. dominica*, there was no adult mortality after 1 week (data not shown), but there was a 54% reduction in progeny after an additional 9 weeks (Table 5). With the combination of 11 mg kg^{-1} protoxin and 35 mg kg^{-1} carboxypeptidase inhibitor,

Table 3. Effect of Cry3Aa on the development and mortality of *Rhyzopertha dominica* on raw wheat germ

Treatment	Cry3Aa dose[a] (mg kg^{-1})	Mortality[b] (± SEM) (%)	Developmental time[b] (± SEM) (days)
Control	–	3 (±3)a	20.8 (±0.2)a
Formulation	–	3 (±3)a	20.8 (±0.3)a
Cry3Aa	105	3 (±3)a	25.9 (±0.5)b
	263	13 (±8)ab	27.8 (±0.4)bc
	525	23 (±6)ab	31.2 (±0.4)cd
	1050	37 (±9)b	33.3 (±1.5)d
	2100	79 (±5)c	39.5 (±0.2)e

[a] Formulation blank was for the 2100 mg kg^{-1} concentration.
[b] Means (± SEM) of two replicates (15–20 larvae per replicate for each treatment) followed by the same letter are not significantly different, $P < 0.05$ according to Tukey's test. For ANOVA of mortality: $F = 25.3$; df = 6, 7; $P < 0.001$; development: $F = 112.1$; df = 6, 7; $P < 0.001$.

Table 4. The effect of Cry3Aa and/or protease inhibitors on the development and mortality of *Rhyzopertha dominica* on raw wheat germ

Treatment (mg kg^{-1})	Developmental time[a] (± SEM) (days)	Mortality (%)
Control	22.8 (±0.3)[25]a	7
105 Cry3Aa	27.2 (±0.5)[12]bc	0
3500 CPI	23.4 (±0.4)[14]a	0
2000 aprotinin	23.2 (±0.4)[11]a	0
2000 amastatin	24.4 (±0.5)[13]ab	0
2000 bestatin	24.2 (±0.4)[13]ab	0
1000 EDTA	23.7 (±1.0)[14]a	0
105 Cry3Aa + 3500 CPI	33.3 (±2.2)[8]d	43*
105 Cry3Aa + 2000 aprotinin	31.8 (±1.1)[13]d	7
105 Cry3Aa + 2000 amastatin	30.8 (±0.9)[12]d	14
105 Cry3Aa + 2000 bestatin	30.3 (±0.6)[12]cd	0
105 Cry3Aa + 1000 EDTA	27.0 (±0.7)[11]bc	8

[a] Means (± SEM) for the developmental period from egg hatch to adult eclosion, with the number of adults in square brackets. ANOVA results were $F = 25.5$; df = 11, 146; $P < 0.001$. Means followed by the same letter are not significantly different, $P < 0.05$ according to Tukey's test.
* Significantly different from the control according to the Fisher exact test ($P < 0.05$).

Table 5. Effect of Cry3Aa and/or CPI on the number of progeny from adult *Rhyzopertha dominica* on wheat kernels

Treatment (mg kg^{-1})	Number of progeny[a] (± SEM)
Control	156 (±16)a
4000 wheat flour	169 (±2)a
35 CPI + 4000 wheat flour	165 (±17)a
11 Cry3Aa	82 (±8)b
11 Cry3Aa + 4000 wheat flour	71 (±2)b
11 Cry3Aa + 35 CPI + 4000 wheat flour	42 (±5)b

[a] Means (± SEM), $n = 3$. Wheat kernels were infested with 20 adults for 1 week, and progeny were counted 9 weeks after removal of adults. ANOVA results were $F = 28.1$; df = 5, 12; $P < 0.001$. Means followed by the same letter are not significantly different, $P < 0.05$ according to Tukey's test.

the number of progeny was approximately one-fourth of the number present in control samples, although the combination was not statistically different from that of wheat treated with only the M-one formulation. These data indicate that Cry3Aa has a detrimental effect on the number of *R. dominica* progeny, and that the addition of CPI may further increase the potency of Cry3Aa.

4 DISCUSSION

The data in this study are the first to demonstrate the efficacy of Cry3Aa against *R. dominica*, and add information about Cry3Aa toxicity to *T. molitor* and *T. castaneum*. Furthermore, the activity of Cry3Aa in *R. dominica* is enhanced when combined with aprotinin or CPI. For pest management of *R. dominica*, applications of Cry3Aa and protease inhibitors may be possible through the development of transgenic cereals expressing both *Bt* toxin and protease inhibitors, or in commercial preparations where the proteins can be mixed with a carrier such as diatomaceous earth. The use of CPI in a transgenic application may be more favorable than one employing aprotinin, because the former is a smaller protein and of plant (not animal) origin.

The efficacy of Cry3Aa against *R. dominica* applied to wheat kernels in a flour dust carrier contrasted with the lack of mortality observed with Cry3Aa in wheat germ. The efficacy of Cry3Aa applied to wheat kernels may be due to ingestion of the protoxin by the adults and/or young larvae prior to their penetration into the kernel. Therefore, the insects may have been exposed to a higher level of protoxin when flour dust was the carrier.

The data in this study are in general agreement with results from previous bioassays of *T. molitor*, in which the range of toxicity reported for Cry3Aa was from about 5 to 21 mg kg^{-1} (LC_{50}), depending on when the assays were evaluated, from 5 days to 5 weeks.[3–6] There were also differences in the assay methods and diets, which may account for some of the variability in mortality. However, the response of *T. molitor* is much slower than that of most lepidopterans to Cry1A toxins. Lepidopteran larvae stop feeding within hours after ingesting Cry1A toxins and die soon after, whereas *T. molitor* often suffer mortality only after weeks of Cry3Aa exposure.

CPI potentiated Cry3Aa toxicity in *R. dominica* but not in *T. molitor* and *T. castaneum* under the experimental conditions of this study. *T. molitor* and *T. castaneum* larvae rely on cysteine proteolytic activity in an acidic anterior midgut for initial digestion of protein.[20–23] Therefore, the lack of potentiation of Cry3Aa may be due to the instability of CPI at acidic pH and/or hydrolysis by digestive proteases. In fact, it has been proposed that the evolution

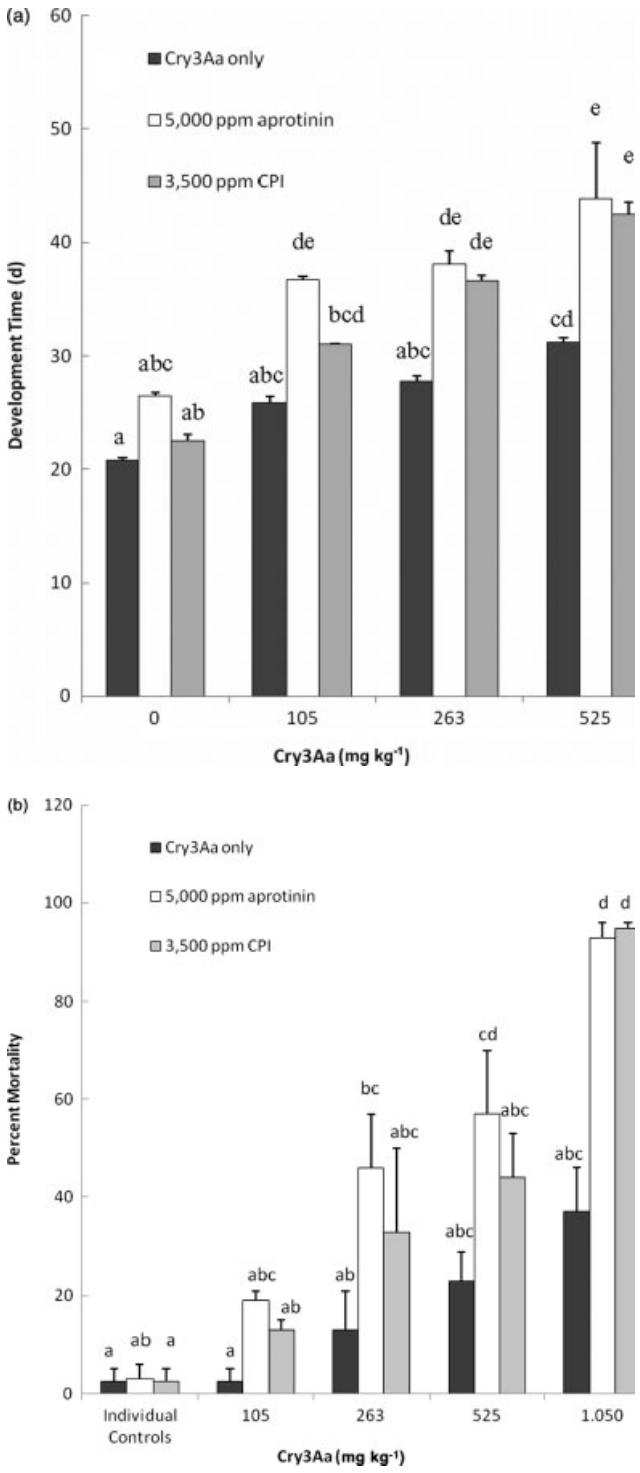

Figure 1. The effect of increasing doses of Cry3Aa and either 5000 mg kg^{-1} aprotinin or 3500 mg kg^{-1} CPI on (a) development or (b) mortality of *Rhyzopertha dominica* using a raw wheat germ diet. Controls (0) were with wheat germ only (no toxin or inhibitor), 5000 mg kg^{-1} aprotinin or 3500 mg kg^{-1} CPI. Data are the means ± SEM of two replicates (17–20 larvae per replicate for each treatment). For the developmental period from egg hatch to adult eclosion, ANOVA results were: $F = 24.7$; df = 11, 12; $P < 0.001$; mortality: $F = 16.1$; df = 14, 15; $P < 0.001$. Means followed by the same letter are not significantly different, $P < 0.05$ according to Tukey's test.

of an acidic anterior midgut containing cysteine proteases in tenebrionid larvae may be a compensatory response to the presence of serine protease inhibitors in coadapting seeds and cereals.

Genes encoding both trypsin and chymotrypsin-like enzymes have been reported in the midgut of *R. dominica* larvae.[24,25] In these previous studies, aprotinin was found to partially inhibit the activity of crude extracts of *R. dominica* midgut proteases towards trypsin and chymotrypsin substrates. Therefore, the effects of aprotinin in the present study are most likely due to the inhibition of serine protease activity in the midgut.

Since the first report of *Bt* toxin potentiation by protease inhibitors,[14] there is now a better understanding of the response of insects to dietary protease inhibitors.[26,27] *Tribolium castaneum* larvae mount a complex genetic response to cysteine protease inhibitors,[28] in agreement with bioassay and biochemical studies supporting the hypothesis that larvae shift from cysteine cathpesin-like proteases to elevated chymotrypsin-like activity when fed a cysteine protease inhibitor.[29–31] In designing an effective combined *Bt* toxin/protease inhibitor strategy for pest control, understanding the genetic and physiological responses of a target pest to insecticidal proteins is necessary to increase potency and to nullify the potential for adaptive responses by the host.

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REFERENCES

1 Phillips TW and Throne JE, Biorational approaches to managing stored-product insects. *Annu Rev Entomol* **55**:375–397 (2010).
2 Thoms E and Phillips TW, Fumigation, in *Mallis Handbook of Pest Control*, 9th edition, ed. by Hedges S. Mallis Handbook and Technical Training Company, Cleveland, OH, pp. 1165–1261 (2004).
3 Carroll J, Li J and Ellar DJ, Proteolytic processing of a coleopteran-specific delta-endotoxin produced by *Bacillus thuringiensis* var. *tenebrionis*. *Biochem J* **261**:99–105 (1989).
4 Herrnstadt C, Soares GG, Wilcox ER and Edwards DL, A new strain of *Bacillus thuringiensis* with activity against coleopteran insects. *BioTechnol* **4**:305–308 (1986).
5 Wu SJ and Dean DH, Functional significance of loops in the receptor binding domain of *Bacillus thuringiensis* CrylIIA δ-endotoxin. *J Mol Biol* **255**:628–640 (1996).
6 Oppert B, Rapid bioassay to screen potential biopesticides in *Tenebrio molitor* larvae. *Biopestic Internat* **6**:67–73 (2010).
7 Fabrick J, Oppert C, Lorenzen MD, Morris K, Oppert B and Jurat-Fuentes JL, A novel *Tenebrio molitor* cadherin is a functional receptor for *Bacillus thuringiensis* Cry3Aa toxin. *J Biol Chem* **284**:18401–18410 (2009).
8 van Frankenhuyzen K and Nystrom C, *The Bacillus thuringiensis Toxin Specificity Database*. [Online]. (2009). Available: http://www.glfc.cfs.nrcan.gc.ca/bacillus [17 May 2010].
9 Oppert B, Ellis RT and Babcock J, Effects of Cry1F and Cry34Ab1/35Ab1 on storage pests. *J Stored Prod Res* **46**:143–148 (2010).
10 Beeman RW, Stuart JJ, Denell RE, McGaughey WH and Dover BA, *Tribolium* as a model insect for study of resistance mechanism, in *Molecular Mechanisms of Insecticide Resistance, Diversity Among Insects*, ed. by Mullin CA and Scott JG. *ACS Symposium Series* 505, American Chemical Society, Washington, DC, pp. 202–208 (1992).

11 Beegle CC, Efficacy of *Bacillus thuringiensis* against lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Biocont Sci Technol* **6**:15–21 (1996).
12 Crickmore N, Zeigler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, *et al*., Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol R* **62**:807–813 (1998).
13 Crickmore N, Zeigler DR, Schnepf E, Van Rie J, Lereclus D, Baum J, *et al*., *Bacillus thuringiensis* Toxin Nomenclature. [Online]. Available: http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/ (2010).
14 MacIntosh SD, Kishore GM, Perlak FJ, Marrone PG, Stone TB, Sims SR, *et al*., Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. *J Agric Food Chem* **38**:1145–1152 (1990).
15 Tabashnik BE, Finson N and Johnson MW, Two protease inhibitors fail to synergize *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J Econ Entomol* **85**:2082–2087 (1992).
16 Rich DH, Moon BJ and Harbeson S, Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding processes. *J Med Chem* **27**:417–422 (1984).
17 Kraut H and Bhargava N, Versuche zur Isolierung des Kallikrein-Inaktivators aus Rinderlunge and seine Identifizierung mit dem Inaktivator aus Rinderparotis. *Hoppe-Seyler's Z Physiol Chem* **338**:231–237 (1964).
18 Ryan CA, Hass CM and Kuhn RW, Purification and properties of a carboxypeptidase inhibitor from potatoes. *J Biol Chem* **249**:5495–5499 (1974).
19 Robertson JL, Russell RM, Preisler HK and Savin NE, *Bioassays with Arthropods*, 2nd edition. CRC Press, Boca Raton, FL (2007).
20 Vinokurov KS, Elpidina EN, Oppert B, Prabhakar S, Zhuzhikov DP, Dunaevsky YE, *et al*., Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. *Comp Biochem Physiol* **145B**:126–137 (2006).
21 Vinokurov KS, Elpidina EN, Oppert B, Prabhakar S, Zhuzhikov DP, Dunaevsky YE, *et al*., Fractionation of digestive proteinases from *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae and role in protein digestion. *Comp Biochem Physiol* **145B**:138–146 (2006).
22 Vinokurov KS, Elpidina EN, Zhuzhikov DP, Oppert B, Kodrik D and Sehnal F, Digestive proteolysis organization in two closely related tenebrionid beetles: red flour beetle (*Tribolium castaneum*) and confused flour beetle (*Tribolium confusum*). *Arch Insect Biochem Physiol* **70**:254–279 (2009).
23 Prabhakar S, Chen M-S, Elpidina EN, Vinokurov KS, Smith CM and Oppert B, Molecular characterization of digestive proteinases and sequence analysis of midgut cDNA transcripts of the yellow mealworm, *Tenebrio molitor* L. *Insect Mol Biol* **16**:455–468 (2007).
24 Zhu YC and Baker JE, Molecular cloning and characterization of a midgut chymotrypsin-like enzyme from the lesser grain borer, *Rhyzopertha dominica*. *Arch Insect Biochem Physiol* **43**:173–184 (2000).
25 Zhu YC and Baker JE, Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Insect Biochem Mol Biol* **29**:1053–1063 (1999).
26 Oppert B, Transgenic plants expressing enzyme inhibitors and the prospects for biopesticide development, in *Phytochemical Biopesticides*, ed. by Koul O and Dhaliwal GS. Harwood Academic, Amsterdam, The Netherlands, pp. 83–95 (2000).
27 Agrawal AA, Phenotypic plasticity in the interaction and evolution of species. *Science* **294**:321–326 (2001).
28 Oppert B, Elpidina EN, Toutges M and Mazumdar-Leighton S, Microarray analysis reveals strategies of *Tribolium castaneum* larvae to compensate for cysteine and serine protease inhibitors. *Comp Biochem Physiol* Part D, **5D**:280–287 (2010).
29 Oppert B, Morgan TD, Culbertson C and Kramer KJ, Dietary mixtures of cysteine proteinase and serine proteinase inhibitors exhibit increased toxicity toward the red flour beetle, *Tribolium castaneum*. *Comp Biochem Physiol* **105C**:379–385 (1993).
30 Oppert B, Morgan TD, Hartzer K, Lenarcic B, Galesa K, Brzin J, *et al*., Effects of proteinase inhibitors on growth and digestive proteolysis of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Comp Biochem Physiol* **134C**:481–490 (2003).
31 Oppert B, Morgan TD, Hartzer K and Kramer KJ, Compensatory proteolytic responses to dietary proteinase inhibitors in the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *Comp Biochem Physiol* **140C**:53–58 (2005).