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ORIGINAL CONTRIBUTION

Fragments of *Tenebrio molitor* cadherin enhance Cry3Aa toxicity for the red flour beetle, *Tribolium castaneum* (Herbst)

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Abstract

Bacillus thuringiensis crystalline (Cry) proteins are highly toxic to a wide range of insect pests, but some species resist their action. This is true for many economically important beetles, including stored product pests, such as *Tribolium castaneum*. In this article, we show that the susceptibility of *T. castaneum* larvae to natural as well as to a recombinant Cry3Aa-type toxin, applied in the diet, is enhanced by supplementing the diet with recombinant fragments of *Tenebrio molitor* cadherin; Cry toxin-binding sites occur in several cadherin repeats (CR). In our study, we used the toxin-binding region CRtb, which represents a substantial part of the repeat CR12-MPED (membrane-proximal extracellular domain). CRtb and CR12-MPED consistently increased Cry3Aa toxicity. This synergistic effect occured at diverse mass ratios between the toxin and the cadherin fragments, suggesting that optimal ratios can be found. In our 6-week-long assay with *T. castaneum,* we achieved mortality of up to 96.6% with toxin concentration 30 μg/g. Cadherin fragments CR11 and CR9-11 elicited small and diverse effects that require further analysis.

Introduction

The entomopathogenic bacterium *Bacillus thuringiensis* (Bt) has been used worldwide for more than 60 years to control agricultural and forestry pests and currently accounts for more than 90% of all bioagents (Gould 1998; Bel et al. 2009). *Bacillus thuringiensis* is a Gram-positive bacterium that harbours several kinds of insecticidal proteins. The crystalline (Cry) proteins produced during sporulation are of primary commercial importance against insects. There are more than an 100 kinds of Cry toxins that differ in both specificity and toxicity. Spore and crystals preparations containing diverse Cry proteins are most commonly used to control pests and disease vectors from the orders Lepidoptera, Coleoptera, Hymenoptera and Diptera (Bravo et al. 2007; Pigott and Ellar 2007). Moreover, modified genes encoding efficient Cry toxins have also been introduced into crops to render them resistant to the relevant pests (Betz et al. 2000; Whalon and Wingerd 2003).

The mode of action of insecticidal Cry toxins has been extensively studied in lepidopteran larvae (Bravo et al. 2007). Crystal solubilization and proteolytic activation of the Cry protoxin in the gut lumen belong to major factors that determine Cry toxicity (Fabrick et al. 2009). For example, the 130- to 140-kDa protoxin, Cry1A, is cleaved to form a 60- to 70-kDa active toxin (Schnepf et al. 1998), while the Cry3A protoxin (73 KDa) yields a 55-KDa protein (Carroll et al. 1997; Loseva et al. 2002). Additionally, it is known that activated toxins bind to specific receptor proteins in the midgut microvilli. Three different Cry1A toxin-binding proteins have been described in lepidopteran insects: a 120-kDa glycosylphosphatidylinositol (GPI)-anchored aminopeptidase-N (Knight et al. 1994), a cadherin-like protein (Vadlamudi et al. 1995) and a GPI-anchored alkaline phosphatase (Jurat-Fuentes and Adang 2004). Chen et al. (2009) have identified a putative cadherin-type receptor in the mosquito *Aedes aegypti* and Fabrick et al. (2009) in the beetle *Tenebrio molitor*.

Intensive research into Cry protein toxicity has shown that it is primarily due to the formation of pores in the plasma membrane of the midgut epithelial cells (Schnepf et al. 1998; Aronson and Shai 2001; De Maagd et al. 2001; Promdonkoy and Ellar 2003; Bravo et al. 2005). However, Zhang et al. (2006) have counter-suggested that the toxicity could be related to a G protein-mediated apoptosis of midgut cells that contain functional Cry receptors. Although, according to both models, the cadherin-like receptor is crucial for mediating the lethal action of Cry toxins.

Cadherins are modular proteins composed of three domains: an extracellular ectodomain formed by 5–12 (depending on insect species) cadherin repeats (CR), a transmembrane domain and an intracellular domain (Pacheco et al. 2009). The toxin-binding sites of cadherin receptors, that determine insect susceptibility to the Cry toxins, have been studied in several Lepidoptera (Pigott and Ellar 2007; Park et al. 2009), and most of these sites are localized within the extracellular CRs. The application of recombinant cadherin peptides containing such CRs is known to enhance Cry toxicity in several insect species. For example, in *Manduca sexta*, the recombinant peptide CR12-MPED (membrane-proximal extracellular domain), extracted from *Escherichia coli* inclusion bodies, elevated the activity of Cry toxin, Cry1Ab (Chen et al. 2007). Cadherin fragments corresponding to the CR7 and CR11 regions also augmented the activities of both the Cry1Ac and Cry1Ab toxins in *M. sexta* larvae, but not as efficiently as another CR12 fragment (Pacheco et al. 2009). Peng et al. (2010) have also reported that the toxin-binding regions of the *Helicoverpa armigera* cadherin (HaCad1) caused enhanced toxicity of Cry1Ac toxin against this serious lepidopteran pest. In Diptera, Hua et al. (2008) found that the CR11-MPED region of *Anopheles gambiae* cadherin (AgCad1) heightened Cry4Ba toxicity to the larvae of this mosquito. A fragment of the *T. molitor* cadherin (TmCad1) containing the predicted toxin-binding region has been shown to bind the Cry3Aa toxin and to promote its oligomerization (Fabrick et al. 2009). This cadherin fragment was also shown to increase Cry3Aa toxicity in *T. molitor* larvae (Oppert et al. 2008).

In our previous work, we attempted to increase Cry3Aa toxicity against *T. castaneum* larvae via structural modifications of the toxin (Mostafa et al. 2013). We confirmed a very low sensitivity of this species to Cry3Aa but found that the endogenous Cry3Aa was nevertheless more active than any of its recombinant derivatives. In this article, we explore the possible synergistic action of four fragments of the *T. molitor* cadherin with Cry3Aa against *T. castaneum* larvae. From a bioassay lasting 6 weeks, we show that 2–3 of the fragments consistently enhance Cry3Aa toxicity.

Material and Methods

Purification of the natural Cry3Aa toxin

A 100 ml sample of the commercial insecticide Novodor FC (Valent Biosciences Corp., Libertyville, IL, USA) was diluted with water to 1 l and centrifuged at 9000 ***g*** for 12 min. The sediment was washed again with 1 l water. Resulting Cry3Aa crystals were subsequently purified from the resuspended sediment (1 l) according to method of Murray and Spencer (1966) using chloroform extraction and Whatman No. 1 filtration. The resulting concentrated crystal suspension was centrifuged and resuspended in 11 ml of water containing 7 g CsCl. The crystals were purified by isopycnic CsCl centrifugation at 250 000 ***g*** for 18 h. The crystal zone was collected and dialysed against water, and the crystal concentration was adjusted to 2 mg protein/ml. Microscopic inspection of this preparation confirmed that it contained crystals, without any contaminating spores or cell debris.

The production of recombinant Cry3Aa and its modifications

The *Cry3Aa* gene of Bt var. *tenebrionis* (Sekar et al. 1987; GenBank J02978) was modified as described in Vlasák et al. (2012). Gene regions of very high (>80%) or very low (<30%) GC content, internal TATA-boxes, ribosomal entry sites and cryptic splice donor/acceptor sites were deleted. These changes increased the yield of recombinant proteins in our bacterial expression system. A *Nde*I cloning site, overlapping the Cry3Aa start codon, and a *Sac*I site localized after the stop codon were introduced into the gene design to facilitate cloning into the pET *E. coli* expression vector. The resulting gene was named *TOXst* and was synthesized commercially. Using the *TOXst* construct (GenBank JN989558) as a starting point, we prepared several related/modified constructs, staring from the Met_{48} codon of the original sequence. Modifications of the *TOX* gene yielded the following genes; *NdTOX*, *NdTOX-loop1FF-loop1YY* (abbreviated *NdTOX-loops*) and *TOX-Δloop*. The corresponding recombinant proteins TOXst (nearly identical with the natural Cry3Aa), NdTOX (TOX with N-terminal deletion of 112 amino acids), NdTOX-loops (TOX with N-terminal deletion of 112 amino acids and exchange of phenylalanines for tyrosines in positions 350 and 351 of loop 1) and TOX-Δloop (TOX

without the F_{346}–G_{361} region encoding loop 1 of domain II) were all used in the toxicity test to compare our previous assay, using 3rd instar *T. castaneum* larvae (Mostafa et al. 2013) with the current assay using the 2nd instar larvae. However, such modified Cry3Aa genes were not included in the assays with the cadherin fragments (see below).

Preparation of the *T. molitor* cadherin fragment cDNAs

Total RNA was extracted from larval midgut tissue of *T. molitor* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA of *TmCad1* was synthesized from 5 μg of total RNA with the aid of oligo(dT) primers and the reverse transcriptase M-MuLV (Fermentas, Vilnius, Lithuania). The cDNA regions encoding cadherin repeats CR11, CR9-11, CR12-MPED and CRtb (toxin-binding domain within CR12-MPED) were amplified with specific primers shown in table 1. Sequencing of the amplified products confirmed that they corresponded to appropriate regions of the *TmCad1* gene (Fabrick et al. 2009, GeneBank No. DQ988044.2). We attempted, using degenerate primer, to prepare at least partial cadherin cDNAs based on the midgut mRNA of *T. castaneum,* but without success. The cadherin of *T. castaneum* was identified by Contreras et al. (2013) when our study has reached an advanced stage. The use of *T. molitor* cadherin allowed comparison with several similar studies.

Expression and purification of the TmCad1 fragments

cDNAs of 1050, 255, 681 and 582 bp encoding TmCad1 repeats CR9-11 (amino acid residues 947–1297), CR11 (residues 1212–1297), CR12-MPED (residues 1298–1525) and CRtb (residues 1322–1516), respectively, were amplified in the pGEM-T Easy vector (Promega) and then subcloned into the pET15b expression vector. Recombinant proteins were expressed in *E. coli* strain BL21 (DE3) grown at 37°C in Luria–Bertani broth. Protein production was induced with 1 M IPTG (isopropyl-beta-D-thiogalactopyranoside; AppliChem, Darmstadt, Germany). Proteins were dissolved and purified by affinity chromatography as described by Fabrick et al. (2009), and the recombinant fragments CR9-11, CR11, CR12-MPED and CRtb (of approximate sizes 55, 16, 30, and 27 kDa, respectively) were eluted from the columns with four portions of the binding buffer containing 250 mM imidazole. Aliquots of the eluates were TCA-precipitated and analysed by SDS-PAGE (10 μg protein per lane) to confirm the presence of proteins of expected size. Subsequent Western blotting employing a commercial antibody against the hexahistidine verified that the expressed proteins contained the hexahistidine tag (figure S1).

Insects rearing and bioassays

The stocks of the yellow mealworm *T. molitor* L. and the red flour beetle *T. castaneum* (Herbst) (both from Coleoptera: Tenebrionidae) have been inbred for about 20 years. All developmental stages were reared on wheat bran mixed with 5% yeast (S.I.Lesaffre, Marcq, France) at 25 ± 1°C, 60% humidity and in darkness. Insecticidal activities of the natural Cry3Aa and several recombinant toxins were tested on newly ecdysed 2nd instar larvae of *T. castaneum*. The toxin alone or in combination with a cadherin fragment was applied in 750 μl dH_2O mixed with 750 mg of the dry diet; the food of untreated control larvae was moistened with 750 μl dH_2O without any other additives. The freshly prepared diet was distributed in 25-mg portions per 1-ml well in a titre-plate kept in a humidity chamber. Larvae were individually added to the wells and their mortality was followed for 6 weeks, at which point survivors pupated. Each test was performed with 30 larvae divided into three

Table 1 Nucleotide primers used to obtain the cDNA of the TmCad1 fragments

Fragment	Position*	Sequences of the forward (F) and reverse (R) primers
CR11	3747–4004	F: 5′ GGCAGCCATATGGCAACGTATCCCGA 3′ R: 5′ CGAGGATCCTTAAAACACTGGAGATTC 3′
CR9-11	2952–4004	F: 5′ GGCAGCCATATGACGACCACTGAA 3′ R: 5′ CGAGGATCCTTAAAACACTGGAGATTC 3′
CR12-MPED	4005–4688	F: 5′ GGCAGCCATATGGACCAAACGGAGTATTTCACCAC 3′ R: 5′ CGAGGATCCTTATCCGAGAACTACCGAAACAC 3′
CRtb	4076–4661	F: 5′ GGCAGCCATATGACTGACAAGGATA 3′ R: 5′ CGAGGATCCTTACCACGCTTTCAAAAT 3′

*Nucleotide numbers correspond to the gene sequence of *Tenebrio molitor* cadherin published by Fabrick et al. (2009) and available in the GeneBank under accession no. DQ988044.2.

groups. Toxicity was expressed in toxin concentrations killing a half larvae contained (LC_{50}) or nearly all insects (LC_{90}).

Statistical analysis

Mortality was recorded in seven-day intervals for 6 weeks of exposure. The data were adjusted to the mortality of the untreated larvae using the Abbott's formula and then subjected to probit analysis (EPA Probit analysis program used for calculating LC/EC values, version 1.5; U.S. Environmental Protection Agency, Cincinnati, OH, USA) to estimate the effect of rising doses of the toxin alone or in combination with the cadherin peptides. The results were also subjected to regression analysis. Synergistic activity of the cadherin fragments was analysed using Duncan's multiple range test.

Results

Toxicity of Cry3Aa and its derivatives to the larvae of *Tribolium castaneum*

Newly ecdysed 2nd instar larvae of *T. castaneum* were transferred to diets supplemented with toxins at concentrations of 200, 100, 50, 10 and 5 μg per 1 g of diet, respectively. Larvae grown on a diet treated with water alone were used as controls. No pupation occurred during the first 5 weeks of the test. About 10% of the control larvae perished and the remainder pupated, mostly in the 6th week. The death rates observed were higher in larvae that consumed food containing a toxin (table 2). Larvae seemed to be more susceptible to the natural Cry3Aa (LC_{50} = 30.5 μg/g) than to any of its derivatives. This result is consistent with the low activities of 18 Cry3A-related recombinant toxins tested on 3rd instar larvae of *T. castaneum* (Mostafa et al. 2013). The recombinant TOXst was the second best toxin in the assay using the 3rd instar larvae but ranked last in the LC_{90} comparisons in the mortality tests against 2nd instar larvae (table 2). In this study, we refrained from employing our assay using 3rd instar larvae because of its low sensitivity. Some larvae are obviously killed only after a long exposure to the toxin and are scored as unaffected in the short assay using 3rd instar larvae.

Table 2 Lethality of *Tribolium castaneum* larvae receiving diets containing natural Cry3Aa or its recombinant derivatives in doses 5, 10, 50, 100 and 200 μg/g diet. The assay began with newly ecdysed 2nd instar larvae and was terminated after 6 weeks

Toxins	LC_{50} (μg/g), 95% confidence limits	LC_{90} (μg/g), 95% confidence limits	Slope ± SE
Natural Cry3Aa	30.5 (2.17–58.90)	334.8 (188.5–2795.1)	1.13 ± 0.22
TOXst	43.7 (18.77–66.63)	769.7 (431.9–2733.5)	1.02 ± 0.21
NdTOX	41.6 (20.03–61.39)	513.5 (327.4–1241.9)	1.17 ± 0.21
NdTOX-loops	45.6 (23.38–65.94)	546 (346.4–1321.5)	1.18 ± 0.21
TOX-Λloop	53 (32.19–71.95)	461.9 (315.2–907.2)	1.36 ± 0.22

Effectiveness of toxins applied with cadherin fragments in 1 : 1 mass ratio

A number of studies have shown that peroral application of cadherin fragments have no direct effect, but in some cases, potentiate the toxicity of Cry proteins (Chen et al. 2007; Hua et al. 2008; Liu et al. 2009; Park et al. 2009; Oppert et al. 2013). Using degenerate primers, we attempted to prepare at least partial cadherin cDNAs based on the midgut mRNA of *T. castaneum* but without success. We therefore decided to use in our study the *T. molitor* cadherin that has previously been tested as a Cry synergist in a diverse spectrum of insects. It is noteworthy that the cadherin gene of *T. castaneum* has now been identified by Contreras et al. (2013), but only when our study had reached an advanced stage.

We supplied the diet of *T. castaneum* with equal amounts (200, 100, 50, 10 and 5 μg per 1 g of diet) of Cry3Aa and of a particular cadherin fragment. The fragments CR11, CR12-MPED and CRtb reduced the LC_{50} value of Cry3Aa 2.02 times, 5.21 times and 3.14 times, respectively, while the large CR9-11 fragment (353 amino acid residues) appeared inactive in our assay. Low immunoreactivity of this fragment (figure S1) indicated that it might be present in lower molar concentration than the other fragments (antibody was directed against a His tag present in one copy per protein molecule, small or large). Poor immunoreactivity could also be caused by constrained antibody access to the poly(His) binding site. Partial loss of the tag also cannot be excluded. The fragments CR12-MPED and CRtb were subsequently assayed with the recombinant TOXst and caused a 1.20- and 1.17-fold LC_{50} decrease, respectively (table 3). This was a small effect by comparison with the natural Cry3Aa but nevertheless indicative of the potential of some cadherin fragments to be used as synergists in the applications of Cry toxins that have low inherent activity. Both CR-11 and CR9-11, applied with TOXst in 1 : 1 ratio, showed inhibitory effects on the toxicity (table 3). Plots of the cumulative mortality on dose dependence (data not

Table 3 Effects of cadherin fragments on the LC_{50} activities of the natural toxin Cry3Aa and the structurally slightly different recombinant TOXst. Toxins and cadherin fragments were used in 1 : 1 ratio in concentrations 5, 10, 50, 100 and 200 μg per gram of diet. Tests were initiated with the newly ecdysed 2nd instar larvae of *Tribolium castaneum* and evaluated after 6 weeks when the surviving insects pupated

Treatment	LC_{50} (μg/g), 95% confidence limits	Slope ± SE	Reduction factor of LC_{50}*
Natural Cry3Aa			
Natural Cry3Aa alone	30.5 (2.17–58.90)	1.13 ± 0.22	–
Cry3Aa + CR11	15.21 (3.15–29.80)	1.03 ± 0.27	2.02
Cry3Aa + CR9-11	27.75 (1.64–114.9)	0.47 ± 0.23	1.1
Cry3Aa + CR12-MPED	5.83 (1.27–11.42)	0.96 ± 0.25	5.24
Cry3Aa + CRtb	9.72 (1.19–20.84)	0.98 ± 0.27	3.14
Recombinant TOXst			
TOXst alone	43.7 (18.77–66.63)	1.02 ± 0.21	–
TOXst + CR11	60.48 (30.47–279.54)	0.80 ± 0.23	0.72
TOXst + CR9-11	73.99 (38.01–336.57)	0.86 ± 0.24	0.59
TOXst + CR12-MPED	36.39 (14.73–221.35)	0.62 ± 0.22	1.20
TOXst + CRtb	37.27 (17.16–147.97)	0.70 ± 0.22	1.17

*Reduction factor = LC_{50} of toxin alone/LC_{50} of toxin applied with a cadherin fragment.

shown), and a probit analysis, presented in table 3, revealed a high mortality increase with increasing toxin dose (values close to 1 in column 'Slope' in table 3) and lesser increases in case of toxins combined with cadherin fragments (low slope values in table 3). This difference in the dose/response relationship possibly indicates the complexity of toxin interactions with the provided cadherin fragment and the endogenous cadherin receptor. The fragments CR12-MPED and CRtb seemed to increase toxicity, probably by mimicking or enhancing the interaction of endogenous receptor with the toxin. The apparent reduction of TOXst toxicity observed with cadherin fragments CR9-11 and CR11 (table 3) may have different causes. For example, the fragments may slow down toxin degradation and bind toxin with high affinity but be inferior to the endogenous cadherin receptor in inducing toxin oligomerization. The low impact of CR12-MPED and CRtb on the activity of TOXst suggests that the match of TOXst with CR12-MPED and CRtb binding sites is not perfect.

Impact of the ratio between toxin doses and cadherin fragments doses

Potential effects of the toxin: cadherin fragment ratio were examined in larvae treated with the LC_{50} dose of a toxin, while cadherin fragments were applied in mass doses ten times lower or higher. Hence, 30.6 μg/g of Cry3Aa was used with 3 μg/g (1 : 0.1) or 306 μg/g (1 : 10) of cadherin fragments. Similarly, 43.7 μg/g of TOXst was combined with 4.37 μg/g or 437 μg/g of cadherin fragments. Fragment concentration in the 1 : 0.1 ratio was apparently too low, because mortality was similar as with the LC_{50} doses of the toxins alone (table 4). By contrast, cadherin fragments CR9-11, CR12-MPED and CRtb applied in 10 times higher doses than the LC_{50} of Cry3Aa, enhanced mortality 1.27, 1.6 and 1.6 times (table 4). Similar increase (1.35, 1.58 and 1.5, respectively) was elicited with cadherin fragments applied together with the recombinant TOXst. These relatively small differences in the synergistic activities of the three fragments on insect mortality contrasted with the effects of fragment applications in a 1 : 1 ratio with the toxin amount on the LC_{50} value (table 3). In combination with CR11, CR12-MPED and CRtb, the LC_{50} value of Cry3Aa decreased 2.02, 5.24 and 3.14 times, respectively. In similar combinations of TOXst with cadherin fragments, the LC_{50} values of CR12-MPED and CRtb decreased 1.20 and 1.17 times, respectively (table 3). As such, these two fragments were efficient synergists and their addition nearly eliminated the activity differences between Cry3Aa and TOXst. The effects of CR11 and CR9-11 were marginal and in some cases contradictory. More data are thus needed to comprehend these contradictions.

The impact of cadherin fragments on Cry3Aa toxicity was plotted in graphs detailing the cumulative mortality at six time points, during the course of the bioassay (figs 1 and 2). With some approximation, it was possible to construct straight lines fitting the points throughout the developmental period of 6 weeks. We do not claim that there is a linear correlation between mortality and time, but rather wish to emphasize that the mortality was evenly distributed during the assay of 6 weeks. After this time, it would obviously rapidly increase. The graphs also show that the mortality rates depended on the kind of treatment. Overall mortality of the controls reached 10%, while the mortality of toxin-treated insects was distinctly higher. The addition of cadherin fragments to the toxin-treated diet shifted the trend lines towards higher mortality values without changing the slopes, indicating that the initial toxin consumption killed up to a half of insects. The course of lethality in the larvae treated with the toxin alone or with a combination of the toxin and cadherin fragment CR11, followed a track starting with near zero mortality in the first week and ending with ca. 50% mortality in the 6th

Table 4 Per cent mortality (average ± SD) of *Tribolium castaneum* larvae grown since the 2nd instar on diets containing LC_{50} doses of the natural Cry3Aa or of the recombinant TOXst toxins in combination with 10 times higher (1 : 10 by weight) or 10 times lower (1 : 0.1 by weight) doses of cadherin fragments

	Natural Cry3Aa				Recombinant TOXst			
Treatment*	1 : 0.1 Cry:Cad ratio, % death	Increment factor	1 : 10 Cry:Cad ratio, % death	Increment factor	1 : 0.1 Cry:Cad ratio, % death	Increment factor	1 : 10 Cry:Cad ratio, % death	Increment factor
Toxin alone	60 ± 10.0^{b} (% mortality)				56.6 ± 14.4^{b} (% mortality)			
Toxin + CR11	50.0 ± 3.3^{a}	0.83	56.6 ± 22.2^{b}	0.94	50.0 ± 5.7^{b}	0.88	53.3 ± 8.8^{b}	0.94
Toxin + CR9-11	56.6 ± 13.3^{a}	0.94	76.6 ± 20.8^{ba}	1.27	50.0 ± 5.7^{b}	0.88	76.6 ± 3.3^{a}	1.35
Toxin + CR12-MPED	63.3 ± 11.9^{a}	1.05	96.6 ± 3.3^{a}	1.60	66.6 ± 5.7^{a}	1.17	90.0 ± 6.6^{a}	1.58
Toxin + CRtb	66.6 ± 6.6^{a}	1.10	96.6 ± 3.3^{a}	1.60	60.0 ± 6.6^{ba}	1.05	90.0 ± 6.6^{a}	1.50

*Control larvae were grown on pure diet and their mortality reached 10% (not shown). Cry3Aa was applied at 30 μg/g and in some cases supplemented with 3 μg/g (1 : 0.1 ratio) or 300 μg/g (1 : 10) of a cadherin fragment. TOXst was used in 43.7 μg/g concentration either alone or with cadherin fragments present at 4.37 μg/g (1 : 0.1) and 437 μg/g (1 : 10). Values marked with the same letters are not significantly different ($P > 0.05$: Duncan's multiple range test). Increment factor = mortality% of Cry3Aa with CR peptides/mortality% of Cry3Aa alone.

week. Mortality was about twice as high in larvae that were treated with the natural or recombinant toxin along with the cadherin fragments CR12-MPED or CRtb. As many as 96.6% of these larvae perished during the 6 weeks of the test. Cadherin fragments applied in 10 times lower doses than the LC_{50} of Cry3Aa exerted only slight synergistic effect.

Lethal activity of recombinant TOXst rose in the presence of cadherin fragments CR12-MPED or CRtb to 90%, whereas the recorded mortality of larvae receiving the recombinant toxin alone was only about 50%. This was higher than the mortality of larvae fed the recombinant toxin with the cadherin fragment CR11. The low doses of fragments CR11 and CR9-11 reduced lethal activity of both Cry3Aa and TOXst toxins.

Discussion

Chen et al. (2007) and other authors have demonstrated the binding of Cry toxins to cadherin proteins anchored in the plasma membrane of microvilli of midgut cells. This cadherin has been identified as a Cry receptor (Vadlamudi et al. 1995) with multiple binding sites (Pigott and Ellar 2007; Park et al. 2009) that apparently differ in their affinities to a diversity of Cry toxins. The concurrent application of cadherin or cadherin fragments with Cry toxins, usually augments but in some cases can also reduce Cry toxicity (Liu et al. 2009; Hua et al. 2013). Our results contribute to the recognition of cadherin regions with great impact on the toxicity of Cry3Aa. Tested regions were chosen from the cadherin gene of *T. molitor* (Fabrick et al. 2009). We show that the extracellular repeat CR12-MPED (ca. 30 kDa) exerted a synergistic effect when administered with natural Cry3Aa, as well as with its slightly modified recombinant version, the TOXst toxin. Similar synergy was obtained with the CRtb fragment (27 kDa, internal portion of CR12-MPED). This observation is consistent with other reports that similar fragments of *T. molitor* cadherin promote the formation of Cry3Aa oligomers and thereby increase their toxicity against *T. molitor* (Oppert et al. 2008; Fabrick et al. 2009). Another independently prepared fragment of *T. molitor* cadherin has also been shown to enhance Cry3Aa mediated toxicity in three beetles from the Chrysomelidae family (Gao et al. 2011). Cultured S2 and Hi5 cells expressing CRtb have been shown to synergistically respond to a Cry treatment with 40% and 25% mortality, respectively (Oppert et al. 2013). Based on these data, and our results, we conclude that the synergistic effect of CR12-MPED is largely confined to

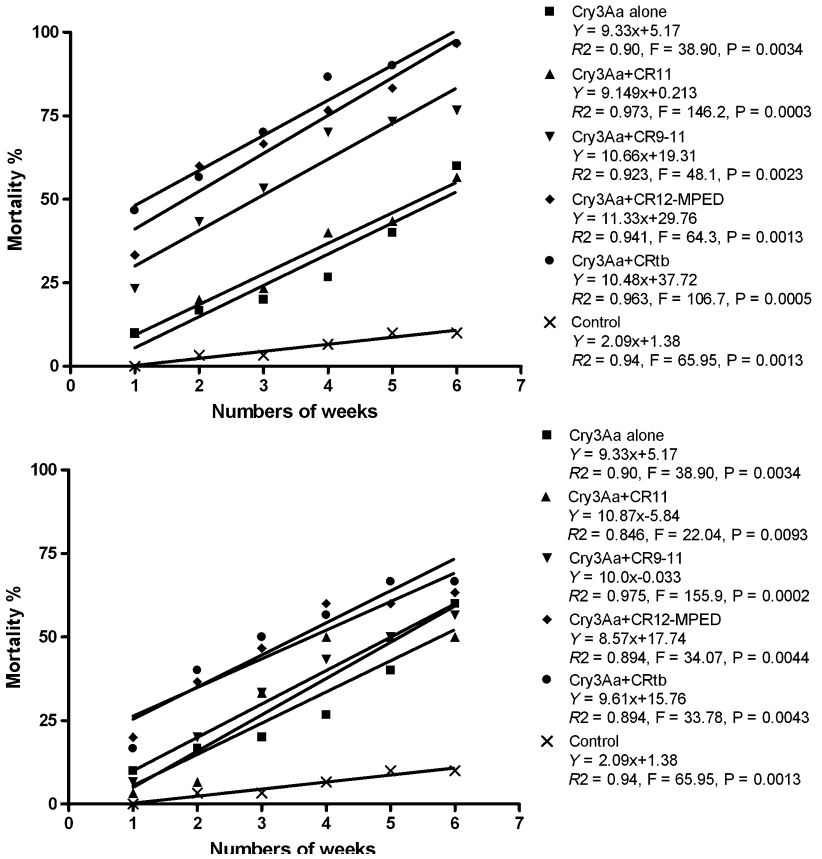

Fig. 1 Cumulative mortality of larvae taken to the assay at the start of the 2nd instar and inspected in weekly intervals. Control larvae received untreated diet; the diet of other larvae contained LC_{50} concentration (30 μg/g) of Cry3Aa applied alone or with a ten times higher (top) or ten times lower (bottom) concentrations of cadherin fragments CR11, CR9-11, CR12 and CRtb, respectively.

its CRtb region. Activity of the remaining part of CR12-MPED remains to be determined. The synergistic action of CR12-MPED has also been repeatedly demonstrated in representatives of order Lepidoptera (Chen et al. 2007; Pacheco et al. 2009; Peng et al. 2010). It is therefore justified to propose that this cadherin fragment could be used as a synergist in the efforts to expand Cry toxin use in the pest control (Pardo-Lopez et al. 2009).

The impact of other CRs on Cry toxicity has been less tested. Recombinant CR7 and CR11 enhanced activity of Cry1Ab and Cry1Ac in *Manduca sexta,* but not as efficiently as CR12 (Pacheco et al. 2009). CR8-10 of a cadherin from *Diabrotica virgifera* enhanced the toxicity of Cry3 in two coleopteran pests (Park et al. 2009). Park et al. (2014) also reported that the CR8-10 fragment enhanced toxicity of Cry3Aa, Cry3Bb and Cry8Ca against the lesser mealworm (*Alphitobius diaperinus*). In our assays, with both Cry3Aa and TOXst applied in the same masses as the cadherin fragments (from 5 to 200 μg/g), both CR11 and CR9-11 were inferior to CRtb and CR12-MPED. CR11 nevertheless doubled the activity of Cry3Aa, while CR9-11 had no effect on Cry3Aa and curbed the TOXst activity. In experiments with the Cry3Aa concentration 30.6 μg/g and the TOXst concentration 43.7 μg/g, ten times lower doses of any cadherin fragments elicited no effect. At the 306 μg/g (for combination with Cry3Aa) and 437 μg/g (for the trial with TOXst), the CR11 was inactive while CR9-11 enhanced toxicity of Cry3Aa about 1.3 times (the CRtb and CR12-MPED fragments 1.6 times). No data are available on the effects of other CRs, but their roles are probably negligible by comparison with CR12-MPED and its component CRtb.

Our results and the data of other authors demonstrate that CR12-MPED and CRtb can be administered in different ratios, according to the Cry concentration. This has two important implications: (i) As cadherins appear to be less species specific than the Cry toxins, it might be possible to enhance specific toxic activity with the aid of non-specific cadherin fragments; and (ii) Some species, including *T. castaneum*, are inherently little susceptible to the natural Cry toxins. It will be easier to find combination of a standard and modestly active toxin with a suitable cadherin fragment than to seek potentially more active structural Cry modifications. Develop-

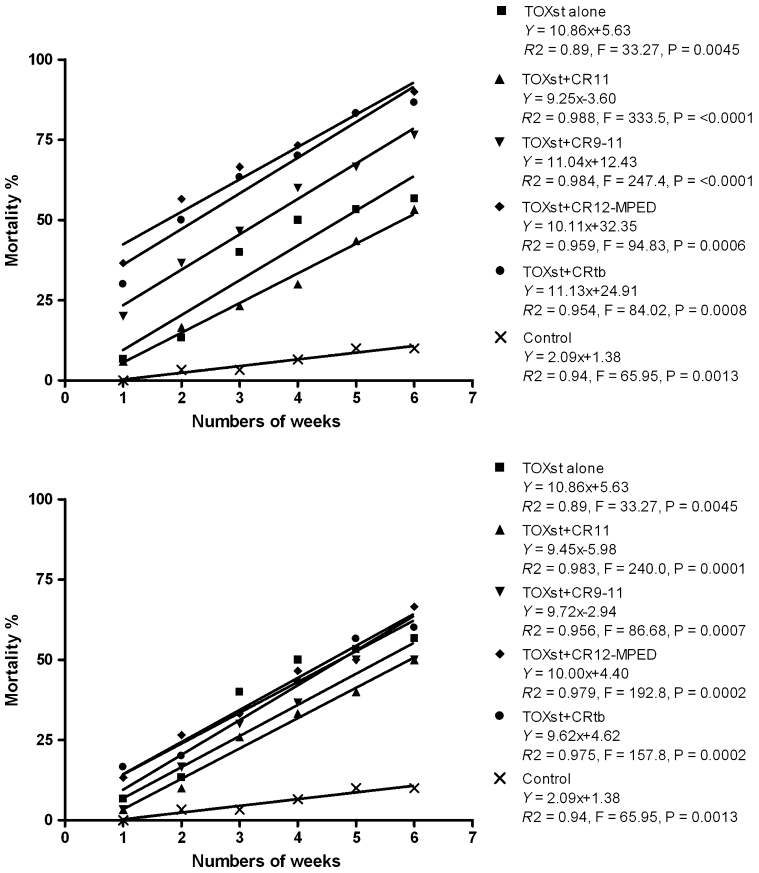

Fig. 2 Cumulative mortality of larvae taken to the assay at the start of the 2nd instar and inspected in weekly intervals. Control larvae received untreated diet; the diet of other larvae contained LC_{50} concentration (43.7 μg/g) of TOXst applied alone or with a ten times higher (top) or ten times lower (bottom) concentrations of cadherin fragments CR11, CR9-11, CR12 and CRtb, respectively.

ment of new pest control methods based on the synergy of cadherin and Cry proteins is feasible but requires the ascertaining of the optimal ratios of cadherin fragments and the Cry toxins to be used. More attention should also be paid to the time course of insect poisoning, enhanced by the addition of cadherin fragments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Western blotting analysis of recombinant TmCad1 fragments. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was treated with 5% skim milk in TBST and probed with mouse Anti - polyHistidine primary antibody (Sigma) (1 : 1000) and Anti-Mouse IgG Peroxidase conjugate (1 : 5000) as secondary antibody. Antigens were detected by chemoluminescence using Amersham ECL Direct Nucleic Acid Labelling and Detection System.