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PII: S0022-2011(16)30068-4
DOI: http://dx.doi.org/10.1016/j.jip.2016.06.004
Reference: YJIPA 6823

To appear in: *Journal of Invertebrate Pathology*

Received Date: 22 March 2016
Revised Date: 10 June 2016
Accepted Date: 13 June 2016

Please cite this article as: Radosavljevic, J., Naimov, S., Toxicity of *Bacillus thuringiensis* (L.) Cry proteins against summer fruit tortrix (*Adoxophyes orana* - Fischer von Rösslerstamm), *Journal of Invertebrate Pathology* (2016), doi: http://dx.doi.org/10.1016/j.jip.2016.06.004

Toxicity of *Bacillus thuringiensis* (L.) Cry proteins against summer fruit tortrix (*Adoxophyes orana* - Fischer von Rösslerstamm)

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Abstract

The activity of seven Cry1, one Cry9 and one hybrid Cry1 protoxins against neonate larvae of summer fruit tortrix (*Adoxophyes orana* - Fischer von Rösslerstamm) has been investigated. Cry1Ia is identified as the most toxic protein, followed by Cry1Aa and Cry1Ac. Cry1Ca, Cry1Cb, Cry1Da and Cry1Fa were less active, while SN19 (Cry1 hybrid protein with domain composition 1Ba/1Ia/1Ba) and Cry9Aa exhibited negligible toxicity against *A. orana*. *In vitro* trypsin-activated Cry1Ac is still less active than Cry1Ia protoxin, suggesting that toxicity of Cry1Ia is most probably due to more complex differences in further downstream processing, toxin-receptor interactions and pore formation in *A. orana*'s midgut epithelium.

Keywords: *Adoxophyes orana* (Fischer von Rösslerstamm); summer fruit tortrix moth; *Bacillus thuringiensis*; Cry proteins; Cry1Ia.

Abbreviations: PBS - phosphate-buffered saline, Bt - *Bacillus thuringiensis*

1. Introduction

The summer fruit tortrix moth, *Adoxophyes orana* (Fischer von Rösslerstamm) is the most important leaf-rolling pest in Europe (Kocourek and Stará, 2005). Since it is a polyphagous species, it damages all types of pome and stone fuits and many ornamental trees. This species is recognized as a key pest in apple orchards in many countries (EPPO, 2015).

Depending on climate conditions, summer fruit tortrix has two to three generations per year. It overwinters at second or third instar larvae and emerges in spring, feeding on leaves, shoots and later on fruits. In early summer first-generation females fly and usually lay eggs on leaves. Upon hatching, second-generation larvae migrate to shoots or fruits. The major economic loss is produced by summer larvae since they are making large feeding patches on the fruits. By the end of summer second-generation moths are flying and frequently lay eggs directly on the fruit (Cross, 1997; Stamenković and Stamenković, 1985). Since decrease of photoperiod to less than 12 h induces diapause in *A. orana* larvae, the autumn fruit damage is minor. (EPPO, 2015)

Bacillus thuringiensis is a Gram-positive bacterium widely used as a biocontrol agent active against different Lepidopteran, Coleopteran and Dipteran pest insects. Its toxicity mainly associates with delta-endotoxins accumulated as crystalline inclusions during sporulation.

Field studies investigating efficacy of sprayable preparations of Bt spores/crystals against *Adoxophyes orana* showed variable results (Cross et al., 1999). To the best of our knowledge the toxicity of different Cry proteins towards summer fruit tortrix moth in laboratory conditions has not been reported so far. Hence, we have tested the activity against summer fruit tortrix moth (*Adoxophyes orana* (Fischer von Rösslerstamm)) larvae of eight different lepidopteran-active Cry proteins (Cry1Aa, Cry1Ac, Cry1Ca, Cry1Cb, Cry1Da, Cry1Fa, Cry1Ia and Cry9Aa) and the hybrid protein SN19 (active against coleopteran and lepidopteran larvae (Naimov et al., 2001).

2. Material and methods

2.1. Preparation of Bt Cry protein variants

Seven different Cry1 delta-endotoxins (Cry1Aa, Cry1Ac, Cry1Ca, Cry1Cb, Cry1Da, Cry1Fa and Cry1Ia), Cry9Aa and SN19 hybrid protein have been produced in *E. coli* XL-1 Blue. Expression vector for the

Cry1Cb was designated as pSB204 and obtained by cloning *cry1Cb* gene from *B. thuringiensis* subsp. *galleriae* HD29 in pUC19 as described earlier (Kalman et al., 1993). All other expression vectors are based on pBD10, a derivative of pKKK233-2 (Bosch et al., 1994). Vectors pPB08 (*cry1Aa*), pB03 (*cry1Ac*), pBD150 (*cry1Ca*), pMH15 (*cry1Da*), pMH21 (*cry1Fa*), pBD172 (*cry1Ia*), pCIz1 (*cry9Aa5*) and pSN19 (hybrid protein SN19 with domain composition 1Ba/1Ia/1Ba) were used in this study. All vectors are thoroughly described elsewhere (Bosch et al., 1994; de Maagd et al., 1999; de Maagd et al., 1996; de Maagd et al., 2000; Naimov et al., 2014; Naimov et al., 2001).

E. coli XL1-Blue cells were transformed by Mix & Go! *E.coli* Transformation Buffer Set (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's instructions. Transformants carrying desired expression vector were grown on terrific broth for 48 h at 28 ˚C for large-scale production. Protein was partially purified from inclusion bodies according to (Herrero et al., 2004). Briefly, 1 g of bacterial pellet was frozen and resuspended in 3 mL of lysis buffer (50 mM Tris pH=8.00, 5 mM EDTA, 100 mM NaCl, 0.13 mM PMSF, 0.25 mg/mL lysozyme, 3.3 mM sodium deoxycholate). Inclusion bodies' pellets obtained upon centrifugation were washed initially three times with washing buffer (20 mM Tris pH = 7.50, 1 M NaCl, 1% Triton X-100) and subsequently three times with PBS. Bt delta-endotoxins were solubilized in buffer containing 50 mM sodium-bicarbonate pH=10.50, 100 mM NaCl and 10 mM DTT for two hours at 37 ˚C. Solubilized proteins were analyzed on 7.5% SDS-PAGE gel (Laemmli, 1970). Cry1Ac has been activated by trypsin as previously described (Herrero et al., 2004).

Protein concentration was assessed by densitometry using different concentrations of bovine serum albumin as a standard with Gel-Pro Analyzer (Media Cybernetics, Inc., Rockville, USA).

2.2 Insect bioassay

For insect bioassay modified artificial diet from (Damos et al., 2009) was used. Briefly, 100 g of alfa-alfa meal, 100 g of yeast extract, 1.7 g of potassium sorbate, 1.7 g of methyl-*p*-hydroxybenzoate, 300 mL of tomato juice, 5 g of Wesson's salt mixture, 20 g of Vanderzant vitamin mixture were suspended in 300 mL of distilled water and mixed with 200 mL of 10%(w/v) agar solution in distilled water. Toxin solutions were incorporated in fresh diet before solidification to obtain desired concentrations.

Adoxophyes orana eggs were purchased from Andermatt Biocontrol AG (Grossdietwil, Switzerland). Neonate larvae were transferred to 24-well plates containing 1 g of artificial diet per well (one larva per each well) and mortality was scored after 4 days at 25 °C.

2.3 Data analysis

All data were analyzed using PoloPC program (Russel et al., 1977). Probit analysis was performed for determination of LC_{50} and LC_{90} and their respective 95% fiducial limits. Relative toxicity was calculated as LC_{90} ratio (Lethal Dose Ratio in PoloPC) against value obtained for Cry9Aa. Also, pairwise calculation of LC_{90} ratio (Table 1) or LC_{50} ratio (Table 2) and their 95% fiducity limit ranges was done for all proteins. Protein pairs with fiducity limit ranges including the ratio 1 are not considered to have significantly different toxicities ($P<0.05$) and are assigned to the same toxicity class.

3. Results and discussion

Nine Cry protoxins were produced in *E. coli* and partially purified from inclusion bodies. Electrophoretic profiles of Cry protoxins used in this study are shown in Fig. 1. All proteins, except the C-terminally truncated Cry1Ia are present as major bands of approximately 135 kDa. Cry1Ia is present as a protein of 81 kDa. Trypsin-activated Cry1Ac was detected as single band of approximate weight of 60 kDa with no obvious signs of further degradation (data not shown).

We used diet incorporation bioassays to test the toxicity of Cry protoxins against *A. orana* larvae. Data obtained by Probit analysis of bioassay results are shown in Table 1 as LC_{50} and LC_{90}. Among tested proteins, Cry1Ia showed significant toxicity for *A. orana* larvae. All tested Cry1 toxins, except hybrid SN19 protein, had lower LC_{50} and LC_{90} in comparison to Cry9Aa. Toxicity ranking of proteins was based on the ratio of the LC_{90} of the Cry1 protoxins with LC_{90} of Cry9Aa proteins. Cry1Ia is significantly more toxic than all other tested proteins. Toxicity of Cry1Ia is followed by toxicity of Cry1Aa, Cry1Ac and Cry1Ca, which are more toxic than Cry1Cb, Cry1Fa and Cry1Da, respectively. Toxicity of hybrid SN19 is comparable to the toxicity of Cry9Aa.

Cry proteins exhibit their toxic effects upon proteolytic activation in insects' midgut. Sometimes, toxic activity of Cry proteins towards insects is hidden until proteins have been activated *in vitro* by trypsin (Lambert et al., 1992). In order to investigate the effect of trypsin activation on toxicity towards *A. orana* , we used protein with the lowest LC_{50} after Cry1Ia, ie. Cry1Ac. Upon activation, Cry1Ac toxicity has not

been significantly improved (LC_{50} Cry1Ac/LC_{50} activated Cry1Ac=1.85, 95% fiducity limits: 0.73 - 4.68 at $P<0.05$)(Boncheva et al., 2006). Moreover, LC_{50} calculated for Cry1Ia is significantly lower than LC_{50} of both Cry1Ac protoxin and toxin (Table 2). These findings suggest that Cry proteins are activated by proteases in *A. orana* larvae midgut.

Being a widely distributed pest in Europe and Asia, *A. orana* represents a serious economical threat to agriculture. Its hosts include species from different plant families, with major hosts belonging to the families *Grossulariaceae* (*Ribes* sp.) and *Rosaceae* (*Malus* sp., *Prunus* sp., *Pyrus* sp., *Rubus* sp., *Cydonia oblonga* Mill.). Previous reports on exploitation of *B. thuringiensins* for controlling *A. orana* in fruit orchards have been reviewed by (Cross et al., 1999). Unfortunately, the use of various, at that time available, preparations containing Bt spores showed variable efficacy against *A. orana*. The only report of purified Bt crystal inclusions against *A. orana* revealed significant toxicity (van der Geest, 1981). Probably those inconsistent results neglected further investigation of Cry proteins for control of summer fruit tortrix. Here we showed that Cry1Ia protein is a promissing candidate for controlling *A. orana*. Since this toxin is produced by *B. thuringiensis* early during sporulation (Kostichka et al., 1996), our finding could explain variable results obtained by field studies (Cross, 1997) using different preparations containing Bt spores. Probably, different amounts of Cry1Ia were present in media, hence affecting controlling efficacy toward *A. orana*. Also, in some Bt strains *cryI* gene is silenced since it is located downstream of strong transcriptional terminator of *cry1* genes (Gleave et al., 1993; Shin et al., 1995; Song et al., 2003; Tailor et al., 1992; Tounsi et al., 2003), that probably produced discrepancy among field studies using Bt formulations containing different strains. We showed that toxicity of Cry1Aa and Cry1Ac against summer fruit tortrix should not be neglected, and it is possible that previously reported toxicity of purified Bt crystal inclusions (van der Geest, 1981) is caused by the presence of proteins with similar toxicity in the purified crystals.

In conclusion, *A. orana* larvae are susceptible to action of Cry1Aa, Cry1Ac and, in the highest extent, to Cry1Ia. Results of this study revealed the need for re-evaluation of Cry proteins' use in biocontrol of *A. orana*. Generally, sprayable preparations containing Bt spores or crystals are of very short persistence and are avoided for biocontrol applications. Hence, our results might direct further development of transgenic plants resistant to this important pest.

Acknowledgements

Jelena Radosavljevic acknowledges support from The Ministry of Education, Science and Technological Development of Republic of Serbia by Postdoctoral Fellowship awarded through the Project Grant No 172024.

References

Boncheva, R., Dukiandjiev, S., Minkov, I., de Maagd, R. A., Naimov, S., 2006. Activity of *Bacillus thuringiensis* delta-endotoxins against codling moth (*Cydia pomonella* L.) larvae. J. Invertebr. Pathol. 92**,** 96-99.

Bosch, D., Schipper, B., van der Kleij, H., de Maagd, R. A., Stiekema, W. J., 1994. Recombinant *Bacillus thuringiensis* crystal proteins with new properties: possibilities for resistance management. Nat. Biotechnol. 12**,** 915-918.

Cross, J. V., 1997. Susceptibility of the summer fruit tortrix moth, *Adoxophyes orana* (Lepidoptera: Tortricidae), to chlorpyrifos and strategies for insecticidal control in orchards. Ann. Appl. Biol. 131**,** 197-212.

Cross, J. V., Solomon, M. G., Chandler, D., Jarret, P., Richardson, P. N., Winstanley, D., Bathon, H., Huber, J., Keller, B., Langenbruch, G. A., Zimmermann, G., 1999. Biocontrol of pests of apples and pears in northern and central Europe: 1. Microbial agents and nematodes. Biocontrol Sci. Technol. 9**,** 125-149.

Damos, P., Spanoudis, C. G., Savopoulou-Soultani, M., 2009. Artificial diets for larvae of *Anarsia lineatella* Zeller (Lepidoptera: Gelechiidae). Comm. Agric. Appl. Biol. Sci. 74**,** 321-330.

de Maagd, R. A., Bakker, P. L., Masson, L., Adang, M. J., Sangadala, S., Stiekema, W., Bosch, D., 1999. Domain III of the *Bacillus thuringiensis* delta-endotoxin Cry1Ac is involved in binding to *Manduca sexta* brush border membranes and to its purified aminopeptidase N. Mol. Microbiol. 31**,** 463-471.

de Maagd, R. A., Kwa, M. S. G., van der Klei, H., Yamamoto, T., Schipper, B., Vlak, J. M., Stiekema, W. J., Bosch, D., 1996. Domain III substitution in *Bacillus thuringiensis* delta-endotoxin CryIA(b) results in superior toxicity for *Spodoptera exigua* and altered membrane protein recognition. Appl. Environ. Microbiol. 62**,** 1537-1543.

de Maagd, R. A., Weemen-Hendriks, M., Stiekema, W., Bosch, D., 2000. *Bacillus thuringiensis* delta-endotoxin Cry1C domain III can function as a specificity determinant for *Spodoptera exigua* in different, but not all, Cry1-Cry1C hybrids. Appl. Environ. Microbiol. 66**,** 1559-1563.

EPPO, 2015. PQR - EPPO database on quarantine pests (available online). http://www.eppo.int. 28/09/2015.

Gleave, A. P., Williams, R., Hedges, R. J., 1993. Screening by polymerase chain reaction of *Bacillus thuringiensis* serotypes for the presence of *cryV*-like insecticidal protein genes and characterization of a *cryV* gene cloned from *B. thuringiensis* subsp. *kurstaki*. Appl. Environ. Microbiol. 59**,** 1683-1687.

Herrero, S., González-Cabrera, J., Ferré, J., Bakker, Petra L., de Maagd, Ruud A., 2004. Mutations in the *Bacillus thuringiensis* Cry1Ca toxin demonstrate the role of domains II and III in specificity towards *Spodoptera exigua* larvae. Biochem. J. 384**,** 507-513.

Kalman, S., Kiehne, K. L., Libs, J. L., Yamamoto, T., 1993. Cloning of a novel cryIC-type gene from a strain of *Bacillus thuringiensis* subsp. *galleriae*. Appl. Environ. Microbiol. 59**,** 1131-1137.

Kocourek, F., Stará, J., 2005. Predictive value of a model of the flight activity of Adoxophyes orana (Lep.: Tortricidae). J. Pest Sci. 78**,** 205-211.

Kostichka, K., Warren, G. W., Mullins, M., Mullins, A. D., Craig, J. A., Koziel, M. G., Estruch, J. J., 1996. Cloning of a *cryV*-type insecticidal protein gene from *Bacillus thuringiensis*: the *cryV*-encoded protein is expressed early in stationary phase. J. Bacteriol. 178**,** 2141-2144.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227**,** 680-685.

Lambert, B., Höfte, H., Annys, K., Jansens, S., Soetaert, P., Peferoen, M., 1992. Novel *Bacillus thuringiensis* insecticidal crystal protein with a silent activity against coleopteran larvae. Appl. Environ. Microbiol. 58**,** 2536-2542.

Naimov, S., Nedyalkova, R., Staykov, N., Weemen-Hendriks, M., Minkov, I., de Maagd, R. A., 2014. A novel Cry9Aa with increased toxicity for *Spodoptera exigua* (Hubner). J. Invertebr. Pathol. 115**,** 99-101.

Naimov, S., Weemen-Hendriks, M., Dukiandjiev, S., de Maagd, R. A., 2001. *Bacillus thuringiensis* delta-endotoxin Cry1 hybrid proteins with increased activity against the Colorado potato beetle. Appl. Environ. Microbiol. 67**,** 5328-5330.

Russel, R. M., Robertson, J. L., Savin, N. E., 1977. POLO: a new computer program for probit analysis. Bull. Entomol. Soc. Am. 23**,** 209-213.

Shin, B. S., Park, S. H., Choi, S. K., Koo, B. T., Lee, S. T., Kim, J. I., 1995. Distribution of *cryV*-type insecticidal protein genes in *Bacillus thuringiensis* and cloning of *cryV*-type genes from *Bacillus thuringiensis* subsp. *kurstaki* and *Bacillus thuringiensis* subsp. *entomocidus*. Appl. Environ. Microbiol. 61**,** 2402-2407.

Song, F., Zhang, J., Gu, A., Wu, Y., Han, L., He, K., Chen, Z., Yao, J., Hu, Y., Li, G., Huang, D., 2003. Identification of *cry1I*-type genes from *Bacillus thuringiensis* strains and characterization of a novel *cry1I*-type gene. Appl. Environ. Microbiol. 69**,** 5207-5211.

Stamenković, S., Stamenković, T., 1985. The life cycle of the summer fruit tortrix moth, *Adoxophyes orana* F. v. R. (*Lepidoptera, Tortricidae*) in western Serbia. Plant Protection. 36**,** 65-80.

Tailor, R., Tippett, J., Gibb, G., Pells, S., Pike, D., Jordan, L., Ely, S., 1992. Identification and characterization of a novel *Bacillus thuringiensis* delta-endotoxin entomocidal to coleopteran and lepidopteran larvae. Mol. Microbiol. 6**,** 1211-1217.

Tounsi, S., Zouari, N., Jaoua, S., 2003. Cloning and study of the expression of a novel cry1Ia-type gene from *Bacillus thuringiensis* subsp. *kurstaki*. J. Appl. Microbiol. 95**,** 23-28.

van der Geest, L. P. S., 1981. Mode of action of *Bacillus thuringiensis* on the summer fruit tree leafroller *Adoxophyes orana* (F. v. R.). Zeitschrift für Angewandte Entomologie. 91**,** 84-86.

Figure and Table legends:

Figure 1: Purified Cry protoxins. MM-molecular weight markers.

Table 1: Toxicity and relative toxicity of Cry proteins against *A. orana* larvae.

Table 2: Effect of trypsin activation on toxicity of Cry1Ac.

Figure 1

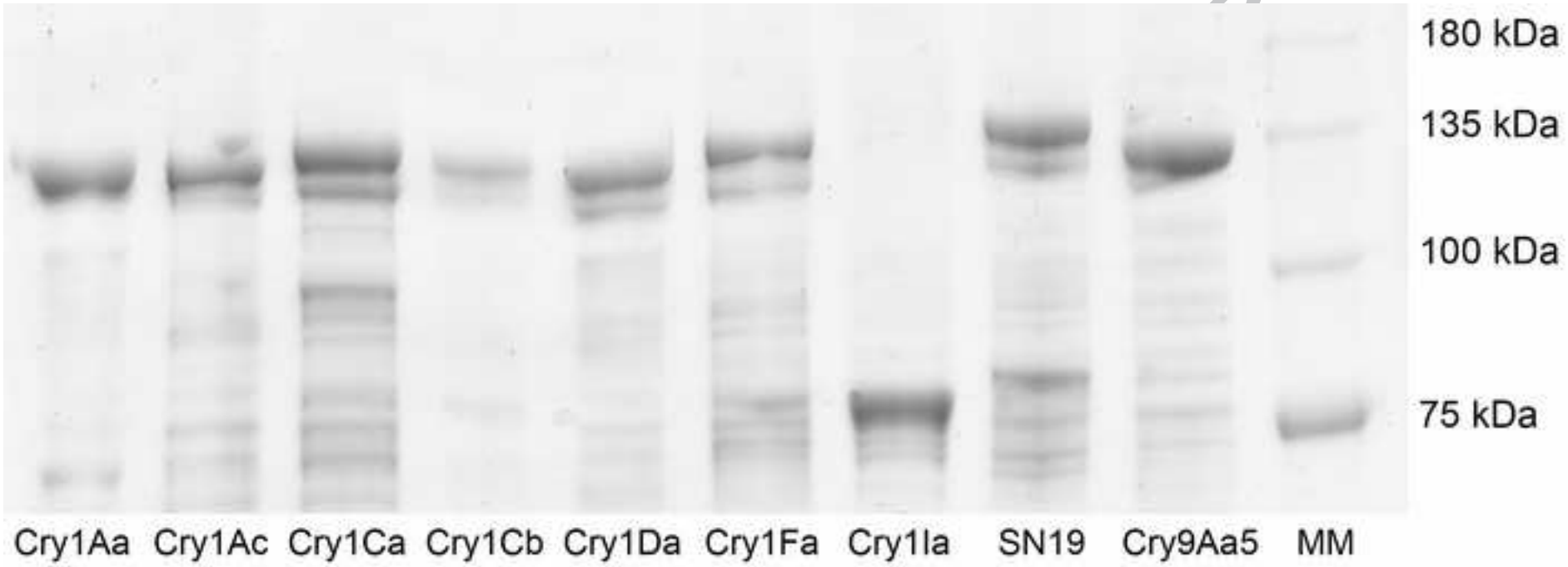


Table 1: Toxicity and relative toxicity of Cry proteins against *A. orana* larvae.

	LC_{50} (95% fiducial limits)[A]	LC_{90} (95% fiducial limits) [A]	Relative toxicity[B]
Cry1Aa	378 (196-495)	987 (774-1,673)	24[b, c]
Cry1Ac	209 (27-372)	1,448 (1,039-3,525)	17[b, c, d]
Cry1Ca	272 (13-501)	4,460 (2,141-21,3860)	5[c, d, e]
Cry1Cb	562 (213-800)	6,868 (3,127-142,380)	3[d, e]
Cry1Da	1,370 (1,046-2,133)	10,222 (4,683-91,450)	2[d, e]
Cry1Fa	671 (408-882)	6,858 (3,505-43,182)	3[d, e]
SN19	911 (494-1,438)	26,218 (6,757-27,704,000)	1[d, e]
Cry9Aa	1,911 (1,261-9,574)	23,931 (6,327-43,799,000)	1[d, e]
Cry1Ia	11 (4-18)	50 (38-69)	479[a]

[a-e] Assignment of toxins to significantly different toxicity classes. Same letter indicates no significant difference at P=0.05 or lower.

[A] LC_{50} and LC_{90} are given in nanograms per gram of diet.

[B] Relative to Cry9Aa. Ratio LC_{90} of Cry9Aa/LC_{90} of protoxin.

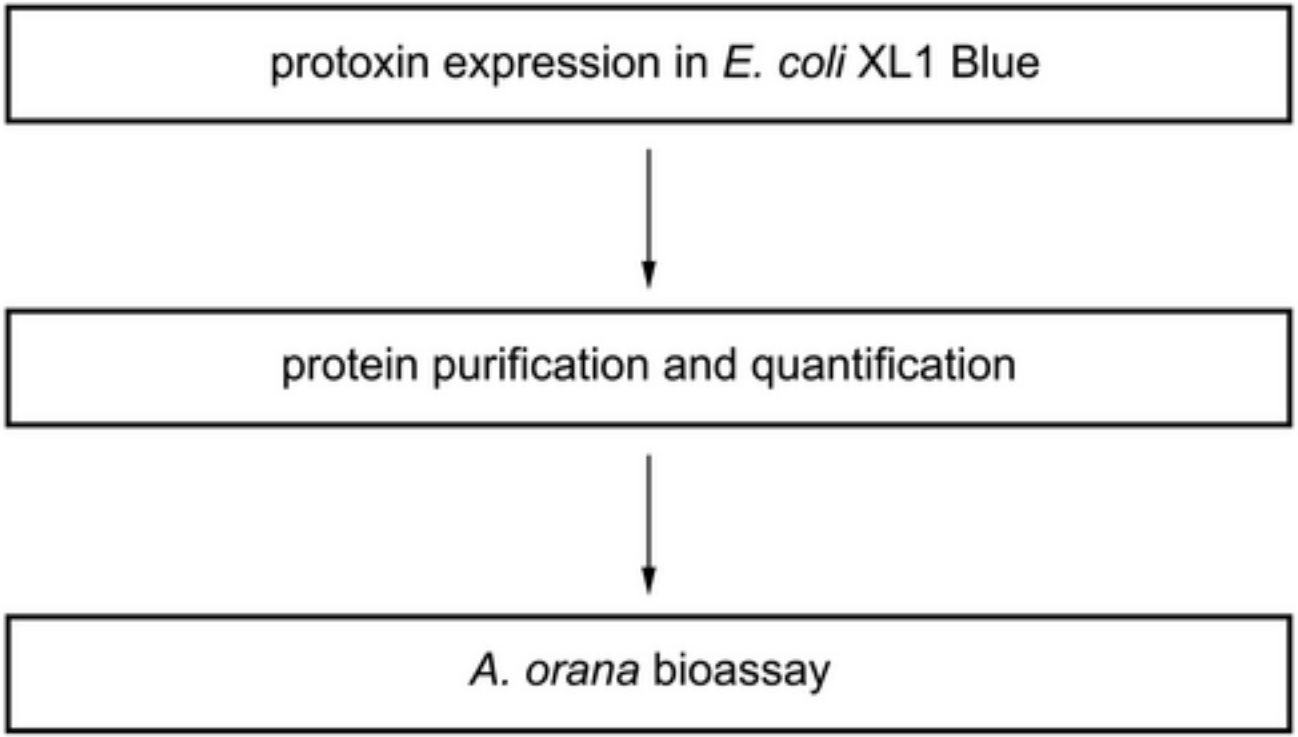
Table 2: Effect of trypsin activation on toxicity of Cry1Ac.

	LC_{50} (95% fiducial limits) [A]	(LC_{50} of Cry1Ia/LC_{50} of protein) ×100
Cry1Ac	1.55 (0.20-2.75)	9[b]
activated Cry1Ac	0.83 (0.55-1.02)	17[b]
Cry1Ia	0.14 (0.05-0.22)	100[a]

[a,b] Assignment of toxins to significantly different toxicity classes. Same letter indicates no significant difference at P=0.05 or lower.

[A] LC_{50} is expressed in pmol per gram of diet.

Graphical Abstract



Research highlights

- Eight different Cry delta-endotoxins (Cry1Aa, Cry1Ac, Cry1Ca, Cry1Cb, Cry1Da, Cry1Fa, Cry1Ia, Cry9Aa5) and SN19 hybrid protein (1Ba/1Ia/1Ba) have been expressed in *E. coli* XL-1 Blue.
- Cry1Ia is identified as the most toxic protein for *Adoxophyes orana* (Fischer von Rösslerstamm).
- Compared to trypsin-activated Cry1Ac, higher toxicity of Cry1Ia for *A. orana* was found.