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Short communication

Activity of *Bacillus thuringiensis* δ-endotoxins against codling moth (*Cydia pomonella* L.) larvae

Rumyana Boncheva [a], Stefan Dukiandjiev [a], Ivan Minkov [a], Ruud A. de Maagd [b], Samir Naimov [a,*]

[a] *University of Plovdiv "Paisii Hilendarski", 4000 Plovdiv, Bulgaria*
[b] *Business Unit Bioscience, Plant Research International B.V., 6700 AA Wageningen, The Netherlands*

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Abstract

Solubilized protoxins of nine Cry1 and one hybrid Cry1 δ-endotoxin from *Bacillus thuringiensis* were tested for their activity against larvae of the codling moth (*Cydia pomonella* L). Cry1Da was the most toxic, followed by Cry1Ab, Cry1Ba, and Cry1Ac, while Cry1Aa, Cry1Fa, Cry1Ia, and SN19 were still less active. Cry1Ca and Cry1Cb showed no activity. In vitro trypsin activation increased activity of all eight active δ-endotoxins, and dramatically enhanced toxicity of hybrid SN19, Cry1Aa, Cry1Ac, and Cry1Fa. The differences between toxicity of proteins before and after trypsin digestion suggests that proteolytic activation in the *C. pomonella* digestive tract plays a critical role for the activity of Cry proteins against this insect.

Keywords: *Cydia pomonella* L. (Lepidoptera: Tortricidae); *Bacillus thuringiensis*; Cry; δ-Endotoxin; Hybrid protein; Trypsin; Bioassay

1. Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive bacterium which, during sporulation, produces crystalline inclusions consisting of one or more insecticidal proteins known as δ-endotoxins or Cry proteins. Insecticidal proteins produced by *B. thuringiensis* have been used for controlling many insect pest species from the orders Lepidoptera, Coleoptera, and Diptera, in sprayable spore/crystal-formulations or constitutively expressed in transgenic crops.

The codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) is one of the major pests of orchard crops throughout the world (Barnes, 1991; Putman, 1963). Fruit feeding by the codling moth can result in a high percentage of unmarketable fruit, since they often tunnel all the way to the core of the fruit. High quality standards of modern, conventional fruit production do not tolerate more than 1–2% of damaged apples or pear (Andermatt et al., 1988).

Bioinsecticides such as *B. thuringiensis* spore/crystal-formulations have been used and tested against *C. pomonella*, which is considered a low threshold pests because it causes damage at very low pest densities (Dent, 2002). There is considerable discrepancy between efficacy of Bt products under laboratory conditions and in field conditions, which is probably caused by the feeding behavior of the neonate larvae (Andermatt et al., 1988). Thus, an approach using expression of *cry* genes in transgenic plants may be the only effective option for *B. thuringiensis* toxin use against codling moth larvae (Dandekar et al., 1998).

In this study, we have tested nine different lepidopteran-active Cry1 proteins: Cry1Aa, Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca, Cry1Cb, Cry1Da, Cry1Fa, and Cry1Ia, as well as the hybrid protein SN19, previously proven to have dual activity against coleopteran and lepidopteran larvae (Naimov et al., 2001). Toxicity of solubilized protoxins was determined and compared with the activity of the same proteins after in vitro activation by trypsin.

* Corresponding author. Fax: +359 32 620944.
E-mail address: naimov0@pu.acad.bg (S. Naimov).

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2. Materials and methods

2.1. Bacillus thuringiensis toxin genes and preparation of proteins

Nine different Cry1 δ-endotoxins and one hybrid protein (SN19) were produced as inclusions in *Escherichia coli*. All used Cry protein expression vectors, with exception of pSB204, are based on pBD10, a derivative of pKK233-2 (Bosch et al., 1994). The vectors used were pPB08 (*cry1Aa*), pBD140 (*cry1Ab*), pB03 (*cry1Ac*), pMH19 (*cry1Ba*), pBD150 (*cry1Ca*), pMH15 (*cry1Da*), pMH21 (*cry1Fa*), pBD172 (*cry1Ia*), and SN19. SN19 encodes a hybrid protein with the domain composition (1Ba/1Ia/1Ba) (Naimov et al., 2001). 1Cb protein was produced from *E. coli* containing plasmid pSB204, with the *cry1Cb* gene from *B. thuringiensis* subsp. *galleriae* HD29. All other expression vectors have been described elsewhere (Bosch et al., 1994; de Maagd et al., 1999; de Maagd et al., 1996; de Maagd et al., 2000).

For large-scale production, all Cry1 and hybrid SN19 protoxins were expressed in *E. coli* strain XL-1, extracted, solubilized and, when necessary, activated with trypsin as described previously (Herrero et al., 2004). Protein concentrations were estimated in duplicate by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a calibration curve of bovine serum albumin.

2.2. Insect bioassays and toxicity

The toxin solutions were mixed with freshly prepared artificial diet (kept at 35–40 °C) to obtain the desired concentrations. The artificial diet consisted of 780 g water, 20 g agar, 50 g maize grit, 50 g wheat germ, 50 g baker's yeast, 4.5 g ascorbic acid, 1.8 g benzoic acid, and 1.8 g 4-hydroxybenzoic acid methylester per liter.

Cydia pomonella eggs were delivered by Andermatt Biocontrol AG, Switzerland. Neonate larvae were transferred to 5 ml tubes (one larvae per tube) with 2.5 g of artificial diet and mortality was scored after 4 days at 28 °C. The concentrations causing 50% mortality (LC_{50}) or 95% mortality and their respective 95% fiducial limits were determined by Probit analysis of results from three or more independent experiments using PoloPC program (Russel et al., 1977). Ranking of LC_{50}'s of protoxins or toxins was achieved by pairwise calculation of the Lethal Dose Ratio and its 95% fiducity limits for each pair of toxin or protoxin, using the PoloPC program. Toxin or protoxin pairs with fiducity limit ranges on either side (but not including) the ratio 1, are considered to have significantly different toxicities ($P < 0.05$). Likewise, the Lethal Dose Ratios and 95% fiducity limits were determined for LC_{50}'s of each of the protoxin/toxin-pair.

3. Results and discussion

We produced and partially purified 10 Cry1 protoxins from *E. coli* inclustion bodies. SDS–PAGE profiles of protoxins are shown in Fig. 1A. All protoxins, with the exception of Cry1Ia, which is C-terminally truncated to an 81 kDa protein, show a major band at the expected size of approximately 130 kDa.

Results of the Probit analysis of our *C. pomonella* bioassays are shown in Table 1, presented as LC_{50} and LC_{95}. The toxicity of proteins was tested by using a diet incorporation bioassay. All δ-endotoxins tested as solubilized protoxins, with the exception of Cry1Ca and Cry1Cb, showed significant toxicity for *C. pomonella* larvae. A ranking was made of the toxicities based on the ratio of the LC_{50} with that of the most active of the protoxins, Cry1Da, including the significance of any observed differences by determining the 95% fiducity limits of the ratio (Table 2, first column). Cry1Da is significantly more toxic then all other protoxins, and is followed by Cry1Ab, Cry1Ba, and Cry1Ac, which in turn are significantly more active then Cry1Aa, Cry1Fa, Cry1Ia, and SN19. The latter four have the lowest, yet still measurable activity with LC_{50}'s approximately 12–17 times higher than that of Cry1Da. Comparison with the only other report of toxicity of individual Cry proteins against *C. pomonella* (Rang et al., 2000), in that case with purified unsolubilized inclusions from recombinant *B. thuringiensis* strains, shows different relative toxicities of Cry1Aa and Cry1Ab. As opposed to in the earlier study, solubilized Cry1Ab protoxin was found by us to be 3 times more toxic then Cry1Aa. The difference in relative activities is possibly due to the use of solubilized inclusions instead of intact inclusions and differences in the assay method (diet incorporation in stead of diet surface contamination). For all other tested protoxins, this is the first report describing their activity against this insect.

In the insect midgut, protoxins are converted to an active core protein by proteases from the gut, as well as possibly by crystal-associated proteases from *B. thuringiensis*, a process which may be mimicked in vitro by trypsin

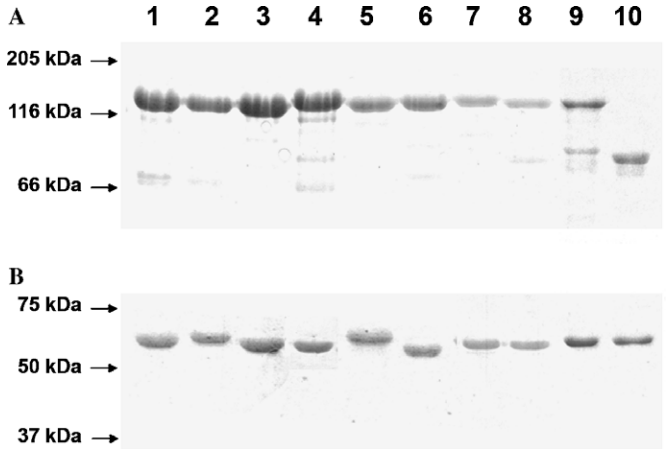

Fig. 1. SDS–PAGE profiles of proteins isolated from *E. coli* XL1-Blue. (A) Protoxins (7.5% polyacrylamide). (B) Trypsin-activated toxins (10% polyacrylamide). Lanes: 1, Cry1Aa; 2, Cry1Ab; 3, Cry1Ac; 4, Cry1Ba; 5, Cry1Da; 6, Cry1Fa; 7, Cry1Ca; 8, Cry1Cb; 9, SN19; 10, Cry1Ia.