Bacillus thuringiensis δ-endotoxin Cry1Ac domain III enhances activity against Heliothis virescens in some, but not all Cry1-Cry1Ac hybrids

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Abstract

We investigated the role of domain III of Bacillus thuringiensis δ-endotoxin Cry1Ac in determining toxicity against Heliothis virescens. Hybrid toxins, containing domain III of Cry1Ac with domains I and II of Cry1Ba, Cry1Ca, Cry1Da, Cry1Ea, and Cry1Fb, respectively, were created. In this way Cry1Ca, Cry1Fb, and to a lesser extent Cry1Ba were made considerably more toxic.

Keywords: Bacillus thuringiensis; δ-Endotoxin; Cry1Ac; Hybrid protein; Heliothis virescens

1. Introduction

Bacillus thuringiensis is a gram-positive soil bacterium that forms during sporulation a parasporal crystal containing insecticidal proteins (called Cry proteins or δ-endotoxins). Cry proteins are formed as protoxins, which are normally activated by proteases of the insect gut. The elucidation of the three-dimensional structure has shown that several, and probably most, activated Cry toxins share a common three-domain structure [for review, see (de Maagd et al., 2001)]. The N-terminal domain I is thought to insert into the target membrane and form part of a membrane pore. Domain II is involved in receptor binding and thereby in co-determining the insect specificity. The C-terminal domain III is also involved, among other proposed functions, in determining specificity through receptor binding. Several studies have demonstrated that the creation of hybrid Cry proteins can result in substantially improved toxins, in terms of toxicity or target spectrum [reviewed in (de Maagd et al., 2001)].

A serious pest of cotton and tobacco is Tobacco budworm, Heliothis virescens (Lepidoptera: Noctuidae). It is susceptible to Cry1Aa, Cry1Ab, Cry1Ac, Cry1Fa, Cry1Ja, Cry2A, and Cry9Ca, with Cry1Ac being the most toxic (K. Van Frankenhuyzen and C. Nystrom, Bacillus thuringiensis specificity database, http://www.glfcc.cfs.nrcan.gc.ca/Bacillus/btsearch.cfm). Several groups have suggested a role for domain III of Cry1Ac in specificity by showing that hybrids containing domains I and II of Cry1Aa and domain III of Cry1Ac have increased activity against H. virescens (Ge et al., 1991; Schnepf et al., 1990). In this paper, we have studied the extent to which Cry1Ac domain III is a determinant for specificity by combining it with domains I and II of various other Cry1 proteins.
1.1. Production and selection of hybrid toxins

All used Cry protein expression vectors were based on pBD10, a derivative of pKK233-2 (Bosch et al., 1994). Expression plasmids pB03 (cry1Ac), pMH19 (cry1Ba), pBD150 (cry1Ca), pMH15 (cry1Da), pBD160 (cry1 Ea), and pMH21 (cry1Fa), have been described before (Bosch et al., 1994; de Maagd et al., 2000). The cry1Fb gene from strain BTS00349A was obtained from Aventis CropScience NV. For production of Cry1Fb, an EcoNI–MunI (nucleotides 35–3494) fragment of cry1Da in pMH15 was replaced with the corresponding fragment of cry1Fb (nucleotides 36–3514), resulting in cry1Fb expression vector pMH35.

cry1Ca-cry1Ac tandem plasmid pHK12 (Fig. 1) has been described before (de Maagd et al., 1996). Tandem plasmids pRK1 (cry1Ba-cry1Ac), pRK2 (cry1Da-cry1Ac), and pRK3 (cry1Ea-cry1Ac) were produced by replacing the cry1Ca containing NcoI–NotI fragment of pHK12 by that of the corresponding cry1-fragments of previously described cry1-cry1Ca tandem plasmids pMH22 (cry1Ba-cry1Ca) (de Maagd et al., 2000), pMH18 (cry1Da-cry1Ca) (de Maagd et al., 2000), and pBD650 (cry1Ea-cry1Ca) (Bosch et al., 1994), respectively. For cry1Fb-cry1Ac tandem plasmid pRK5, the cry1Ca NcoI–SacII fragment from pHK12 was replaced by the cry1Fb NcoI–FspI fragment of pMH35 using a synthetic FspI–SacII linker. The common arrangement of cry1 5’ fragments, followed by a polylinker and an identical 3’ fragment of cry1Ac for the 5 tandem plasmids is shown in Fig. 1A.

After allowing intramolecular recombination of tandem plasmids in Escherichia coli JM101 (recA+), plasmid DNA was isolated and digested with SacI and NotI to linearize non-recombinant plasmids. NotI has a unique recognition site in the polylinker of all tandem plasmids and SacI has a unique recognition site at position 1350.

Fig. 1. (A) Schematic representation of tandem plasmids and in vivo recombination strategy. Locations of domain III borders are indicated by a dotted vertical line. The overlapping regions of the involved genes are aligned vertically, and the polylinker between the two genes is shown as cut by NotI. Since recombinants were selected with NotI and SacI, recombination within the dotted area of cry1Ac was not found. The hatched area indicates the protoxin-specific encoding part of the gene. (B) Amino acid alignment of Cry1Ac and other parental toxins in the area of border between domains II and III. Amino acid identity to Cry1Ac is represented by dots. The homologous area that contains the cross-over is underlined for each hybrid. For the hybrids, the parental toxin sequence is shown in lowercase beyond the crossover site.
of cylAc. After digestion, the products were used to transform E. coli XL-1 Blue. This strategy selected for recombinants with a cross-over in or near the domain III-encoding sequences, since only recombination events 3’ of the SacI-site will lead to the loss of this site and hence no linearization. From a total of 100 recombinants (20 from each plasmid) initially screened, 21 produced a soluble protoxin. DNA sequencing subsequently showed that these represented seven unique recombinants at the protein level. The locations of the crossover sites in these hybrid proteins, designated RK6 (Cry1Ba), RK15 (Cry1Ca), RK7 (Cry1Da), RK8 (Cry1Ea), RK9 (Cry1Ea), RK10 (Cry1Ea), and RK12 (Cry1Fb) are shown in Fig. 1B. For large-scale production, all parental and hybrid protoxins were expressed in E. coli strain XL-1 Blue, extracted, solubilized, and activated with trypsin as described earlier (Herrero et al., 2004). Solubilized protoxins and activated toxins were dialyzed overnight against 25 mM NaHCO3–100 mM NaCl, pH 10. Protein concentrations were estimated in duplicate by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a calibration curve of bovine serum albumin.

1.2. Insect bioassays and toxicity

The toxicity of proteins was tested by spreading protoxin and activated toxin dilutions on artificial diet. Neonate larvae of H. virescens (Benzon Research, Carlisle, PA) were used, and mortality was scored after 6 days at 28°C. The concentrations causing 50% mortality (LC50) and their 95% fiducial limits were determined by Probit analysis of results from three or more independent experiments using the PoloPC program (Russel et al., 1977). Results are shown in Table 1.

<table>
<thead>
<tr>
<th>Toxin or hybrid</th>
<th>LC50 (95% fiducial limits) for Protoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cry1Ac</td>
<td>22 (14–31)</td>
</tr>
<tr>
<td>Tmut</td>
<td>151 (117–193)</td>
</tr>
<tr>
<td>Cry1Ba</td>
<td>&gt;8000</td>
</tr>
<tr>
<td>RK6</td>
<td>&gt;3200</td>
</tr>
<tr>
<td>Cry1Ca</td>
<td>&gt;16,000</td>
</tr>
<tr>
<td>RK15</td>
<td>93 (62–134)</td>
</tr>
<tr>
<td>RK15mut</td>
<td>&gt;5000</td>
</tr>
<tr>
<td>Cry1Da</td>
<td>&gt;6400</td>
</tr>
<tr>
<td>RK7</td>
<td>&gt;6400</td>
</tr>
<tr>
<td>Cry1Ea</td>
<td>&gt;16,000</td>
</tr>
<tr>
<td>RK8</td>
<td>&gt;16,000</td>
</tr>
<tr>
<td>RK9</td>
<td>&gt;16,000</td>
</tr>
<tr>
<td>RK10</td>
<td>&gt;16,000</td>
</tr>
<tr>
<td>Cry1Fb</td>
<td>&gt;8000</td>
</tr>
<tr>
<td>RK12</td>
<td>115 (79–154)</td>
</tr>
</tbody>
</table>

ND, not determined.

* Toxicity is indicated as LC50 (in nanograms per square centimeter).

Cry1Ba protoxin or activated toxin had no significant activity against H. virescens. Hybrid pRK6 (Cry1Ba-Cry1Ac) showed considerable activity only as a protoxin (at least a 16-fold improvement compared to Cry1Ba). Lack of activity of trypsin-activated toxin of Cry1Ba and its hybrids could be due to trypsin processing in domain I, leading to a less active 55 kDa protein as was seen before in our studies on Spodoptera exigua (de Maagd et al., 2000) and by Bradley et al. (1995) for Manduca sexta. We noticed that during trypsin-activation the 65 kDa intact toxin appeared to be a relatively stable intermediate, while the prolonged trypsin treatment required to convert all protoxin leads to increasing amounts of inactive 55 kDa protein (results not shown). The relative stability of the 65 kDa intermediate may explain why protoxin was active, if the 65 kDa intermediate in vivo was present long enough to bind and form pores.

Cry1Ca shows only very weak activity as a protoxin, and even less as activated toxin. Cry1Ca-Cry1Ac hybrid RK15 was tested as activated toxin. Also here replacement of domain III with that of Cry1Ac increased activity, in this case at least 115 times compared to parental toxin Cry1Ca. In contrast, like its parental toxin Cry1Da, Cry1Da-Cry1Ac hybrid RK7 showed neither detectable toxicity as activated toxin or as protoxin, at the maximum concentration used here. The same was observed for Cry1Ea-Cry1Ac hybrids RK8, RK9, and RK10, which have different cross-over sites and no measurable activity, either as protoxin or as activated toxin.

Domain III of Cry1Ac is involved in N-acetylgalactosamine (GalNAc)-mediated binding to a putative receptor in M. sexta, aminopeptidase N (APN) (Burton et al., 1999; de Maagd et al., 1999b), to some, but not all APNs of H. virescens (Banks et al., 2001) as well as to a membrane-bound alkaline phosphatase of H. virescens (Jurat-Fuentes and Adang, 2004). Mutations affecting the GalNAc-binding pocket of domain III had no effect on toxicity for M. sexta (Burton et al., 1999), and a small (4-fold) but significant negative effect on toxicity for H. virescens (Lee et al., 1999). In the latter case, substantial (20-fold) decreases in affinity of binding to H. virescens gut membranes coincided with relatively little decrease in toxicity. We tested the triple Cry1Ac domain III mutant Tmut [N506D, Q509E, Y513A; Plasmid pMSV1AcTmut, kindly provided by Prof. D. Ellar; (Burton et al., 1999)] for toxicity against H. virescens and found it to be approximately 7-fold less active then the wild type Cry1Ac (Table 1). We transferred the same mutations to the Cry1Ca-Cry1Ac hybrid RK15 by replacing a PstI-fragment corresponding to bases 1428–1788 of Cry1Ac by the corresponding fragment from pMSV1AcTmut, encompassing the mutations, resulting in RK15mut. Although toxicity was still detectable at high concentrations, this mutation reduced toxicity more then 50-fold compared to pRK15 (Table 1).
The role of domain III of Cry1Ac in toxicity of different hybrids for *H. virescens* as described in this study shows interesting parallels with the role of Cry1Ca domain III in toxicity for *S. exigua*. We have shown previously that domain III of Cry1Ca is an important, but not universal determinant of *S. exigua* activity in different combinations with domains I and II of other toxins (de Maagd et al., 2000). Domain III mutations in a region similar to the GalNAc-binding pocket of Cry1Ac reduced toxicity of Cry1Ca for *S. exigua* approximately 30-fold and binding affinity 17-fold (Herrero et al., 2004). In the Cry1Ea-Cry1Ca hybrid G27 the same mutation completely destroyed activity (>200-fold reduction) (de Maagd et al., 1999a). Thus, in both that case as well as in the comparison of Cry1Ac and RK15 toxicity for *H. virescens* in this study, the effect of the domain III mutation is dependent on the origin of domains I and II as well.

The hypothesis that domains II and III can each function separately in a two-step mechanism of binding of Cry1Ac to APN (Jenkins et al., 2000) and that Cry1Ab binds to a cadherin-like protein as well as to APN (Bravo et al., 2004) could help to explain why novel combinations of domains II and III can have an altered toxicity. We have recently demonstrated that domain II function of Cry1Ca is required in an insect-specific manner for the oligomer formation that probably precedes or coincides with membrane insertion of the toxin (Herrero et al., 2004). Domain III substitution may increase toxicity if domain III binding is rate limiting. In the case of Cry1Ac and *H. virescens*, domain III mutations only affected toxicity if they strongly affected its contribution to binding (Lee et al., 1999). If the domain II-mediated binding or oligomerization step was rate limiting or absent, little or no increase in toxicity will occur even with domain III of Cry1Ac. This may explain the lack of activity of our Cry1Da-Cry1Ac and Cry1Ea-Cry1Ac hybrids. In this respect, it is interesting to note that Cry1Ea was shown not to bind to *H. virescens* midgut brush border membrane vesicles, whereas Cry1Ca did bind (Van Rie et al., 1990).

Acknowledgments

Part of this work was supported by EU-INCO project Contract No. ERB IC18-CT98-0303. Rummya Karlova was supported by an EU-TEMPUS fellowship. We thank Salvador Herrero for critically reading the manuscript.

References


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