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The mutation $R_{423}S$ in the *Bacillus thuringiensis* hybrid toxin CryAAC slightly increases toxicity for *Mamestra brassicae* L.

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Abstract

Bacillus thuringiensis Cry1Ac toxin is 100 times less toxic than Cry1C to *Mamestra brassicae*. An $R_{423}S$ mutation abolishes Cry1Ac toxin proteolysis in *M. brassicae* gut juice but does not increase its toxicity to this insect. The CryAAC hybrid toxin (1Ac/1Ac/1Ca) is toxic to *M. brassicae* but is susceptible to gut protease digestion at the R_{423} residue. Accordingly we have investigated the effect of the $R_{423}S$ mutation in CryAAC on its toxicity for *M. brassicae* and *Pieris brassicae*. Bioassays demonstrated that the $R_{423}S$ mutation slightly increased the toxicity of CryAAC for *M. brassicae* by having a significantly inhibitory effect on the growth of surviving larvae. The mutant hybrid was still highly toxic to *P. brassicae*. Features of CryAAC$R_{423}S$ such as, (1) stability in *M. brassicae* gut juice and (2) crystal solubility were investigated. Computer simulations suggest that a possible major increase in flexibility in the CryAAC loop β7/β8 (G_{391}–P_{397}) caused by the $R_{423}S$ substitution could be a reason for the increase in *M. brassicae* toxicity.

Keywords: *Bacillus thuringiensis*; *Mamestra brassicae* L.; Cry toxins; proteolysis; Bioassays; β7/β8 loop

1. Introduction

Bacillus thuringiensis is a spore-forming bacterium widely used in agriculture as a biological pesticide because it produces at least three groups of proteins (Cry, Cyt and VIP) that are toxic for insect pests and disease vectors (de Maagd et al., 2003; Crickmore et al., 2005). Cry and Cyt delta-endotoxins are produced during sporulation as cytoplasmic parasporal bodies and VIP toxins (Vegetative Insecticidal Proteins) are secreted from the cells during vegetative growth (Schnepf et al., 1998). Cry toxins have three structural domains of approximately 200 residues each. Domain I is formed by a bundle of seven anti-parallel α-helixes where a central amphipatic helix (α-5) is surrounded by the six others. The function of this domain has been associated with membrane lytic pore formation (Grochulski et al., 1995; Gazit et al., 1998). Domain II consists of three anti-parallel β-sheets folded in a "Greek key" topology adopting a so-called β-prism conformation. Domain III is formed by two β-sheets in a β-sandwich structure with a "jelly roll" topology. Both domain II and III have been associated with the recognition and binding of a receptor in midgut cells (Ge et al., 1991; de Maagd et al., 1996, 2000). Domain III has been also associated with regulation of the pore activity (Schnepf et al., 1998).

Among Cry toxins, the Cry1-class contains several that are highly specific for lepidopteran pests. The primary site of Cry1 toxin action is the brush border membrane of midgut epithelia. Once ingested, crystals are solubilized in the alkaline and reducing environment of the midgut lumen as 130–135 kDa protoxins that are rapidly proteolysed and activated by midgut proteases to remove approximately 600 residues from the C-terminus and the first 28 N-terminal residues to produce a 62 kDa protease resistant core (Lightwood et al., 2000). Activated toxins then cross the peritrophic membrane and

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bind specific receptor(s) on the epithelial cell membrane, where further proteolysis may take place before the toxin inserts into the membrane to form cytolytic pores (Bravo et al., 2002, 2004).

Since Cry toxins act in the digestive tract of insects, their toxicity and potency might be influenced by the identity and specificity of midgut proteases. It has been reported that variations in gut protease expression patterns have severe consequences on the appearance of insect resistance to Cry toxins either because of a lower protoxin activation or a complete toxin degradation (Oppert et al., 1994; Inagaki et al., 1992). Resistance to Cry1A toxins in *Plodia interpunctella* (Indian meal moth) has been found to be in part related to the alteration of gut protease expression (Oppert et al., 1996; Candas et al., 2003). In one report the proteolysis regime was found to determine toxin specificity. A *B. thuringiensis* var. *colmeri* insecticidal δ-endotoxin (Cry1Ab7) activated first with lepidopteran gut juice and shown toxic to lepidoptera was converted to a slighter smaller dipteran active toxin when subsequently activated a second time with dipteran gut juice (Haider et al., 1986).

In previous work, we investigated the role of proteolysis in determining the potency of Cry1Ac towards *Pieris brassicae* L. (large white butterfly) and *Mamestra brassicae* L. (cabbage moth), two of the most important cabbage pests in Europe (Lightwood et al., 2000). *M. brassicae* is less sensitive to Cry1Ac than *P. brassicae*, and gut juice from *Mamestra* cleaves Cry1Ac *in vitro* and *in vivo* into two insoluble and non toxic polypeptides at arginine 423 (R_{423}). When this arginine in Cry1Ac was replaced by serine, the cleavage was abolished but the toxicity was not significantly affected. By contrast, Cry1Ca is highly toxic to *M. brassicae* but its potency toward *P. brassicae* is not comparable with Cry1Ac. A recombinant hybrid CryAAC combining the Cry1Ac first and second domain with the Cry1Ca third domain showed some of the toxicity to *M. brassicae* of Cry1Ca and was as efficient as Cry1Ac against *P. brassicae* (Lightwood, 1999). However, the hybrid was still susceptible to proteolysis at the R_{423} residue.

Rational protocols for improvement in Cry toxin potency may require not only major mutagenesis such as hybrid construction, but also the additive effect of relatively minor incremental modifications at widely separated positions in the structure. The aim of this study was to determine whether substituting $R_{423}S$ in the dual acting CryAAC hybrid could enhance its potency to *M. brassicae* without affecting its toxicity to *P. brassicae*. We also sought to predict the structural consequences of the $R_{423}S$ mutation through computer simulations.

2. Materials and methods

2.1. Strains, plasmids and site-specific mutagenesis

Escherichia coli XL-1 blue strain (Bullock et al., 1987) was used for all standard molecular biology techniques. All toxin genes used in this study were cloned into the shuttle vector pHY300PLK (Takara Bio Inc., Japan). The *cry1Ca* gene was sub cloned from pMSV1Ca (Gilliland et al., 2002) into pHY300PLK using a 5.8-kb *Eco*RI fragment encoding the full-length gene to produce the plasmid pHY-1Ca. The source for *cry1Ac* gene was the plasmid pSVC10, which is a derivative of pHY300PLK and was kindly provided by S. Vilchez, (Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom). The construction of the pMSVAAC plasmid encoding the *cryAAC* hybrid gene was previously reported (Lightwood, 1999). For *cryAAC* gene subcloning, a unique *Kpn*I restriction site was previously introduced into the pHY300PLK just before the *Pst*I and after the *Bgl*II sites of the multicloning region to produce the pHY300PLK1 plasmid. In a triple-ligation strategy a 1.6-kb *Kpn*I–*Sac*I fragment and a 3.5-kb *Sac*I–*Eco*RI fragment both from pMSVAAC and encoding the full-length gene were sub cloned into the corresponding sites of pHY300PLK1.

To make the $R_{423}S$ mutation in the *cryAAC* gene, a site-specific mutagenesis reaction was performed on plasmid pHY-AAC using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Mutagenic oligonucleotides (5′–CGTGCCA CCTTCCCAAGGATTTAGTC–3′; 5′–TTGTTATTCTG TGGCGGTATTTCATCC–3′) were synthesized by The Protein and Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom. The introduced mutation was checked by DNA sequencing. This was done by the DNA Sequencing Facility, Department of Biochemistry, University of Cambridge, Cambridge, United Kingdom.

Plasmids pSVC-10, pHY-1Ca, pHY-AAC, and pHY-AAC$R_{423}S$ were introduced by electroporation into the acrystalliferous strain *B. thuringiensis* var. *israelensis* IPS-78/11 (Ward and Ellar, 1983), and crystals containing toxin were recovered using sucrose density gradients by the method of Thomas and Ellar (Thomas and Ellar, 1983). Toxin yield was quantified by the method of Lowry (Lowry et al., 1951) using bovine serum albumin (BSA) as the standard. Crystals were solubilized in 50 mM Na_2CO_3–10 mM dithiothreitol (DTT) (pH 10.5) at 37 °C for 60 min and the concentration subsequently adjusted to 1 mg/ml using a Bio-Rad protein assay (Bio-Rad).

2.2. Insects

Pieris brassicae and *M. brassicae* were reared from eggs kindly supplied by Warwick Horticulture Research International, Wellesbourne, Warwickshire, United Kingdom and the Centre for Ecology and Hydrology, N.E.R.C., Oxford, United Kingdom, respectively. *M. brassicae* (David and Gardiner, 1965) and *P. brassicae* larvae were reared on artificial diets at 25 °C using cycles consisting of 16 h of light and 8 h of darkness (16 L:8D). *P. brassicae* was raised on the *M. brassicae* diet supplemented with 16.2 g/l of dried organic cabbage powder.