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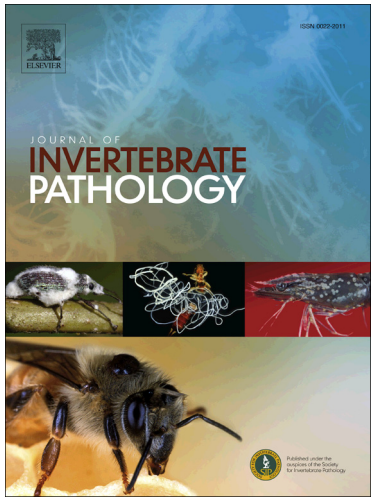

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Specificity determinants for Cry insecticidal proteins: insights from their mode of action

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Abstract

Insecticidal proteins from the bacterium *Bacillus thuringiensis* (Bt) are used as active components of biopesticides and as plant incorporated protectants in transgenic crops. One of the most relevant attributes of these Bt protein-based insecticidal technologies is their high specificity, which assures lack of detrimental effects on non-target insects, vertebrates and the environment. The identification of specificity determinants in Bt insecticidal proteins could guide risk assessment for novel insecticidal proteins currently considered for commercialization. In this work we review the available data on specificity determinants of crystal (Cry) insecticidal proteins as the Bt toxins most well characterized and used in transgenic crops. The multi-step mode of action of the Cry insecticidal proteins allows various factors to potentially affect specificity determination and here we define seven levels that could influence specificity. The relative relevance of each of these determinants on efficacy of transgenic crops producing Cry insecticidal proteins is also discussed.

Introduction

Among the insecticidal proteins produced by the bacterium *Bacillus thuringiensis* (Bt), the crystal (or Cry) proteins are the most well studied and produced by currently commercialized transgenic crops (Bt crops). Apart from their efficacy in controlling targeted pest species, Bt crops are also recognized for their environmental safety as a result of their high specificity (Koch et al., 2015). As with other proteins, specificity of Cry toxins is determined by the different steps involved in their mode of action, which are in part reflected in the three dimensional (3D) structure of the protein. Among the currently >350 holotype Cry toxins, the most common 3D structure in the active toxin form involves three domains (reviewed in Xu et al., 2014). Domain I is composed of seven amphipathic alpha helices organized in a bundle with helix alpha-5 located centrally. Its structural similarity with pore-forming domains of alternative bacterial toxins and currently available experimental evidence supports a role for domain I in insertion in cell membranes. Domain II presents the highest diversity (suggestive of a role in specificity), and is composed of three antiparallel beta sheets arranged in a beta prism, displaying structural similarities with lectins in the jacalin family (Burton et al., 1999; Xu et al., 2014). In these lectins, three loops protruding from the beta prism structure determine specificity for carbohydrate binding (Meagher et al., 2005). Similar protruding loops in domain II have been shown to be involved in determination of binding specificity to host midgut proteins (Dean et al., 1996; Pigott et al., 2008), although their potential role in recognizing glycan moieties has not been experimentally tested. The three dimensional structure of domain III, also composed of beta sheets but arranged in a jelly roll topology, displays morphological similarities with cellulose binding domains of cellulolytic enzymes (Xu et al., 2014), supporting a role in recognizing specific carbohydrate moieties on proteins. In some cases, specific carbohydrate-

binding regions in domain III have been detected and shown to be critical in determining specificity, as in the case of the N-acetylgalactosamine (GalNAc) binding pocket in Cry1Ac (Burton et al., 1999; Jurat-Fuentes and Adang, 2004).

As noted above, three dimensional protein structures give clues to specificity determinants. In the case of three domain Cry toxins, their structural features suggest a mode of action (reviewed in Adang et al., 2014) that includes interactions with midgut proteins (domains II and III) and insertion in cell membranes (domain I). However, when examined, there is a lack of direct correlation between structure and activity against specific targets, i.e. the same Cry toxin can be active against taxonomically diverse insects and Cry toxins with diverse binding determinants (domains II and/or III) may be active against the same insect (Palma et al., 2014). Consequently, Cry protein structure is generally not predictive of specificity and additional determinants, probably provided by the host, need to be considered.

It is well established that Cry toxins target host midgut cells and that they need to be ingested to reach the midgut epithelium. If the toxin is ingested as a parasporal crystalline body it must undergo solubilization to liberate a protoxin form. This protoxin form has been recently suggested to display toxicity through an alternative pathway (Tabashnik et al., 2015), but given the lack of direct experimental evidence for this process we focus our analysis of specificity determinants on the activated toxin, which is generated after sequential proteolysis of the protoxin form. The resulting activated toxin core must then traverse the peritrophic matrix and bind to receptors on the surface of midgut cells. Interaction between Cry toxin and midgut receptors is considered the main step dictating specificity of the toxin, although there are cases of high affinity binding not being associated with toxicity (Wolfersberger, 1990). While the specific mechanism responsible for enterocyte death by Cry toxins is still a matter of debate

(Vachon et al., 2012), it is generally accepted that the toxin forms a pore that kills the cell by osmotic shock. Massive enterocyte death disrupts integrity of the midgut epithelial layer, allowing Bt and potentially other resident gut bacteria to invade the nutrient-rich hemocoel where they proliferate leading to septicemia and death of the insect (Raymond et al., 2010).

The goal of this manuscript is to review available information on the mode of action of Cry toxins that identifies potential specificity determinants of these proteins as relevant models of highly specific insecticidal proteins. For the purpose of this work, we define specificity as the condition of Cry proteins being toxic to a particular insect. We predict that since most Cry proteins produced by transgenic *Bt* crops are soluble, their specificity is not affected by the crystal solubilization step described below. However, all the specificity levels described below and in Figure 1 would have a significant effect on specificity of *Bt* pesticides.

Specificity level I: Exposure to the insecticidal protein

An obvious first step determining specificity is the probability of the particular insecticidal protein encountering a host. The presentation of most of the Cry toxins as insoluble crystals limits their availability to certain hosts, for example sap feeding hemipterans. The poor ability of Bt to colonize various habitats including plant surfaces (Maduell et al., 2008), would also seem to limit the extent to which insects in those environments are exposed to Bt, unless transmission is primarily through insect-to-insect interactions (Milutinovic et al., 2015). Various interactions between Bt and nematodes have also been proposed as a mechanism by which the bacterium and its Cry toxins can be delivered to a susceptible host (Ruan et al., 2015). The specificity of certain Cry toxins (parasporins) towards human cancer cells (Mizuki et al., 2000) is particularly difficult to explain in evolutionary terms. In this case, it is possible that specificity

determinants for interaction between the Cry toxins and these tumor cells are actually shared with as yet unidentified targeted gut insect cells.

Another interesting ecological observation is that some Cry toxins present inter-order activity, which has been documented for 6 of the 68 Cry families. Maybe an extreme example is Cry2Aa, which has been described as active against species of Lepidoptera, Diptera, and Hemiptera (van Frankenhuyzen, 2009). In this case, given the distinct ecological niches of each host, one would expect that the toxin contains specificity determinants for each of the orders, as most Cry toxins display activity against species within a single taxonomic order.

Specificity level II: Crystal solubilization

Packaging of proteins in a parasporal crystal is expected to increase stability of these proteins in the environment. However, this crystal must undergo solubilization so the Cry proteins are liberated into solution to initiate the intoxication process. Early work established that parasporal crystal solubilization was favored by the physicochemical conditions in the host digestive fluids, most notably the pH (Angus, 1954). Consequently, it was reported that only insects producing digestive fluids capable of crystal solubilization were susceptible to the particular *Bt* strain producing the crystal (Du et al., 1994). The crystals from the non-insecticidal *Bt* strains were active when pre-solubilized, supporting a critical role for crystal solubilization in determining specificity. Disparity in *Bt* crystal solubility has been attributed to differences in the disulphide bridges (Du et al., 1994) and blocks of amino acids highly conserved among similar Cry toxins (Wang et al., 2012) that contribute to the stability of the *Bt* crystal structure. In another example of the importance of crystal solubilization, activity of Cry1B toxin crystals against coleopteran larvae was only detected after *in vitro* solubilization (Bradley et al., 1995).

The observation that the same Cry1B crystals were highly active when applied directly to lepidopteran larvae further supports a critical role for the physicochemical conditions in the host digestive system in determining Cry specificity.

Specificity level III: Toxin processing and stability

Once the Cry protoxin form is liberated from the crystal it becomes susceptible to proteolytic cleavage by digestive enzymes present in the host digestive fluids. Depending on the Cry toxin family, this proteolytic processing may be more or less extensive. For instance, most Cry1 protoxins are ~120kDa in size, while Cry3 or Cry2 protoxins do not contain an extensive C terminus protoxin domain and are much smaller (~70-73kDa). Processing of the protoxin domain yields an active toxin core of ~55-60kDa in size. Production of a stable toxin core has been shown to determine or significantly contribute to specificity in multiple cases. For instance, comparison of processing in Cry1A susceptible (*Pieris brassicae*, *Bombyx mori*) and tolerant (*Mamestra brassicae*, *Spdoptera litura*) hosts identified reduced production of the toxin form associated with susceptibility in the tolerant larvae (Inagaki et al., 1992; Lightwood et al., 2000). Degradation of Cry toxins by midgut proteases has been associated to reduced susceptibility in mosquito larvae (Elleuch et al., 2015). In Coleoptera, the Cry3Aa protein was found to be very slowly processed to an active 55-kDa toxin core in the midgut of corn rootworm (*Diabrotica virgifera virgifera*) larvae, suggesting an involvement of slow processing in the low activity of Cry3Aa against rootworms. Introduction of a chymotrypsin/cathepsin G site to create a modified Cry3Aa protein (mCry3Aa) resulted in faster processing to the 55-kDa form and a consequent increase in toxicity against rootworms (Walters et al., 2008). Similarly, altered and/or slower protoxin processing has been associated with reduced susceptibility and resistance in

Lepidoptera (Zalunin et al., 2015), supporting that both appropriate processing and kinetics of toxin core production may have an effect on specificity. Although the smaller toxins such as Cry2 and Cry3 do not undergo extensive processing at the C-terminus, they are cleaved at the N-terminus and there is evidence that this cleavage is required for toxicity and could potentially determine specificity (Bravo et al., 2002; Morse et al., 2001). An additional N-terminal cleavage is proposed to occur as part of the mechanism of action of some toxins, and mutants where toxins have been pre-cleaved at this site show an altered specificity in that they can partially overcome a resistant phenotype (Soberón et al., 2007). It is noteworthy that although the larger toxins expressed in transgenic crops are usually truncated at the C-terminus they are often not truncated at the N-terminus and so cannot be considered as the fully activated form.

Specificity level IV: Toxin sequestration

Once processed, the resulting toxin form must remain stable in the gut fluids to be active. For instance, gut fluids from *Choristoneura fumiferana* larvae contain an elastase capable of specifically binding and precipitating Cry1A protoxin and toxin forms, contributing to reduced activity against this insect (Milne et al., 1998). Similarly, toxin sequestration by esterases or hexamerins in the host gut fluids that prevented advancement of the intoxication process have been associated to Cry1Ac-tolerance in *Helicoverpa armigera* (Gunning et al., 2005; Ma et al., 2005). The existence of similar sequestering mechanisms in digestive fluids of other insects needs to be explored.

Specificity level V: Crossing the peritrophic matrix

The insect gut epithelium is protected from abrasion and some pathogens by a mucus-like layer called the peritrophic matrix, although its pore size does not preclude passage of Cry proteins (Brandt et al., 1978). This layer is mostly composed of chitin but also includes heavily glycosylated proteins (Moskalyk et al., 1996). Lectin-like folds in two of the three domains of most Cry toxins can recognize these glycosylic residues on the peritrophic matrix proteins, thereby sequestering the toxin to the matrix and stalling the intoxication process. For instance, in the Douglas fir tussock moth (*Orgyia pseudotsugata*), Cry1A-binding proteins in the peritrophic matrix were identified as highly glycosylated peritrophins containing GalNAc (Valaitis and Podgwaite, 2013), which is known to interfere with binding of Cry1Ac toxin to midgut receptors (Burton et al., 1999). In *Agrotis ipsilon* and *Mamestra brassicae* larvae, the Cry1Ac toxin is retained in the peritrophic matrix and then eliminated in the frass, which prevents interactions with midgut cells and explains tolerance to the toxin (Rees et al., 2009). Similarly, pretreatment with GalNAc was shown to reduce Cry1Ac sequestration to the peritrophic matrix and increase susceptibility in *Bombyx mori* larvae (Hayakawa et al., 2004). Reduced activity of *Bt* strains in the presence of chitinase inhibitors (Ding et al., 2008; Lertcanawanichakul et al., 2004; Regev et al., 1996; Sampson and Gooday, 1998), further supports that the ability to evade the peritrophic matrix may greatly contribute to determine specificity. The specific composition of the peritrophic matrix in distinct insects, especially in terms of protein glycosylation, would be expected to have a relevant impact on its effect on *Bt* toxin specificity.

Specificity level VI: Binding to receptors

Since the highest changes in Cry toxin activity have been obtained through alterations in binding to midgut receptors, this step is considered critical, although not sufficient

(Wolfersberger, 1990), to determine activity. A number of functional Cry toxin receptors have been identified, including proteins and glycolipids (Pigott and Ellar, 2007), and it has been proposed that they may interact sequentially with the toxin (Pacheco et al., 2009). Protein-protein and protein-carbohydrate interactions have been proposed between Cry toxin domains and receptors, and recognition of unique carbohydrate structures present in invertebrates has been proposed to explain specificity of Cry toxins to insects and nematodes but not to vertebrates (Griffitts et al., 2003). In this regard, it is plausible that specificity of Cry toxins (parasporins) against mammalian tumor cells may be dictated by carbohydrate structures shared with targeted invertebrate cells but not present in healthy mammalian cells.

A number of diverse studies identify domains II and III as the main determinants of Cry toxin binding specificity (reviewed in Dean et al., 1996; Xu et al., 2014). Cross-resistance between Cry toxins is often associated with sequence similarities in these domains (Carriere et al., 2015). Within domain II, three protruding loops heavily influence binding specificity. Amino acid substitutions in these loops allowed introduction of mosquitocidal activity in Cry4Ba (Abdullah et al., 2003) and Cry19A (Abdullah and Dean, 2004) toxins. Loop 2 seems critical for recognition of Cry receptors (Arenas et al., 2010; Jiménez et al., 2012; Pigott et al., 2008), through interactions proposed to involve hydropathic complementarity (Gómez et al., 2002).

There is also clear evidence supporting a role for domain III in determining Cry binding specificity. A clear example is the introduction of *Spodoptera exigua* toxicity in a Cry1Ab hybrid toxin by substitution of domain III for that of Cry1Ca (de Maagd et al., 1996). The sequence determinants for *S. exigua* specificity in domain III of Cry1Ca have been identified (de Maagd et al., 1999). In the case of Cry1Ac, binding specificity governed by domain III is dependent on recognition of GalNAc residues in receptor proteins (Burton et al., 1999; Jurat-

Fuentes and Adang, 2004). A clear example of domain III determining specificity comes from introduction of rootworm specificity in Cry3Aa by replacing domain III with the same domain from Cry1Ab (Walters et al., 2010). This observation would be unexpected based on the specificity of Cry1Ab, which is inactive against coleopteran larvae, and suggests that significant similarity in domains II and/or III may not be an appropriate predictor of specificity for engineered toxins.

There has been much interest recently in the interaction of Cry toxins with members of the ATP binding cassette (ABC) protein family, particularly subfamilies C2 and A. Alterations in these ABC proteins are linked with resistance against Cry1 toxins (Heckel, 2012; Park et al., 2014; Xiao et al., 2014) and Cry2Ab (Tay et al., 2015). Although the exact role of these proteins remains unclear it has been proposed that they may act as receptors but only when in a particular conformational state (Heckel, 2012). This possibility opens up a potentially complex sub-level of receptor-binding based specificity where binding is dependent on the particular state of the receptor. This state could be an open/closed configuration as proposed for the ABC proteins or could represent a particular post-translational modification or even association with a ligand or some other binding partner.

Specificity level VII: Post-binding events

Lack of direct correlation between Cry toxin binding affinity and toxicity in some cases (Garczynski et al., 1991; Wolfersberger, 1990) suggests the involvement of post-binding events in determining specificity. For instance, Cry toxins do bind to mammalian proteins *in vitro* (Shimada et al., 2006b; Vazquez-Padron et al., 2000), but this interaction is mostly non-specific (Hofmann et al., 1988) and thus is not conducive to formation of toxin pores (Shimada et al.,

2006a). More difficult to explain are reports of high affinity toxin binding, which suggests toxin insertion in the membrane (Liang et al., 1995), associated with low susceptibility, as in the case of Cry1Ac and *Spodoptera frugiperda* (Garczynski et al., 1991) or *Lymantria dispar* (Wolfersberger, 1990). While these observations may be explained by pre-binding specificity determinants, it is also plausible that high affinity binding (i.e. toxin insertion in the membrane) may not be sufficient for toxicity. Evidence for post-binding specificity determinants is provided by the effect of toxin oligomerization on specificity. Mutant Cry1Ab toxins incapable of oligomerization lost specificity to *Manduca sexta* (Jiménez-Juárez et al., 2007). Moreover, modified Cry1A toxins that form toxin oligomers in solution overcome dependency of interactions with cadherin for specificity (Porta et al., 2011) and are active against Cry1A-resistant insects (Soberón et al., 2007). However, the binding specificity determinants affecting these modified toxins are not known.

Other potential post-binding specificity determinants may include differences in activation of intracellular cell death pathways or differential gut defensive responses to intoxication. Intracellular oncotic cell death pathways were proposed to be responsible for enterocyte killing by Cry proteins (Zhang et al., 2006). Considering this possibility, it is plausible that differences in the control and activation of these cell death pathways between hosts may affect Cry toxin specificity. Similarly, differences in the gut defensive response to Cry intoxication among hosts may contribute to determine Cry specificity. For instance, enhanced repair of damaged gut epithelium during Cry1Ac intoxication has been proposed to be responsible for resistance to this toxin in strains of *Heliothis virescens* (Forcada et al., 1999; Martinez-Ramirez et al., 1999). Consequently, differences in this gut defensive response during intoxication may contribute to specificity of Cry toxins.

Conclusions and future prospects

Based on the multi-step mode of action of Cry insecticidal proteins we have described seven different levels that can influence specificity. This list could be expanded as molecular details of toxin interaction with host proteins are further identified. Some of these specificity levels may be less relevant for Cry proteins produced by transgenic crops, as they usually produce a soluble, partially-truncated form of the protein. Binding to midgut receptors has been shown to be the specificity level with highest potential to engineer susceptibility in Cry proteins. Recognition of selected carbohydrates on receptor proteins and glycolipids appears critical to binding reactions conducive to toxicity, although mutations in regions relevant to toxin stability are also relevant. Cry proteins have evolved to target selected organisms, yet Cry protein similarity does not always predict specificity. Considering the drastic effect of toxin-receptor interactions on specificity, refined Cry toxin-receptor models should facilitate specificity prediction based on relevant regions of the protein. However, available data supports the premise that steps other than binding specificity may impact susceptibility to Cry proteins and that host-dependent factors are greatly relevant to specificity.

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Figure Legends

Figure 1.- Dichotomous flow chart detailing seven steps in the mode of action of Cry insecticidal proteins that determine toxin specificity. Each specificity determining step is shown as a dichotomous key in roman numeral. Cry proteins produced by transgenic Bt crops are not subjected to the two first specificity determinants.

Figure

Bt crystal

Insect not exposed

I

Insect exposed

Crystal solubilized

II

Crystal insoluble

Transgenic Bt plant

Protoxin processed to activated toxin

III

Protoxin/toxin precipitation or degradation

Toxin in circulation

IV

Toxin sequestration

Toxin crosses peritrophic matrix

V

Toxin retained by peritrophic matrix

Toxin binding to receptors

VI

No receptors for toxin present
Toxin receptors altered (no binding)

Toxin pore formation
Gut epithelium collapse

VII

No functional toxin pores formed
Healing of gut epithelium

Graphical abstract