Regulatory Toxicology and Pharmacology 99 (2018) 50–60

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Safety of the *Bacillus thuringiensis*-derived Cry1A.105 protein: Evidence that domain exchange preserves mode of action and safety

Cunxi Wang*, Wenze Li, Colton R. Kessenich, Jay S. Petrick, Timothy J. Rydel, Eric J. Sturman, Thomas C. Lee, Kevin C. Glenn, Thomas C. Edrington**

Bayer U.S., Crop Science Division, 700 Chesterfield Pkwy West, Chesterfield, MO 63017, USA

ARTICLE INFO

Keywords:
Bacillus thuringiensis
Chimeric protein
Cry1A.105
Domain exchange
History of safe use
Safety assessment
Soybean insect protection

ABSTRACT

The lepidopteran-active Cry1A.105 protein is a chimeric three-domain insecticidal toxin with distinct structural domains derived from the naturally occurring Cry1Ab, Cry1Ac and Cry1F proteins from the soil bacterium *Bacillus thuringiensis* (*Bt*). The X-ray crystal structure of the Cry1A.105 tryptic core at 3.0 Å resolution demonstrates its high structural similarity to the tryptic core of Cry1Ac. Bioinformatics analyses demonstrate that Cry1A.105 has no significant amino acid sequence similarity to known allergens or mammalian toxins, which is the same conclusion reached for its component domains. Like its intact donor proteins, Cry1A.105 was heat labile at temperatures ≥75 °C and degraded upon exposure to gastrointestinal proteases. No adverse effects were observed in mice when Cry1A.105 was dosed orally at 2451 mg/kg body weight. Therefore, the weight of evidence supports that Cry1A.105 is safe for human and animal consumption. These results support the conclusion that the safety of a chimeric protein for human or animal consumption can be evaluated in the context of the safety of its donor proteins.

1. Introduction

Both insect-protected GM (genetically modified) crops and those commercial biopesticides from *Bacillus thuringiensis* (*Bt*) typically contain active insecticidal crystal (Cry) proteins. Although other classes of *Bt* insecticidal proteins have been discovered (e. g., Cyt and Vip), Cry proteins are by far the most well characterized (Koch et al., 2015). More than 700 *cry* gene sequences that code for Cry proteins have been identified and many Cry proteins can be utilized for the control of insect pests in agriculture (Koch et al., 2015; Palma et al., 2014).

The most common agriculturally employed Cry proteins have a characteristic three-domain structure and exert their insecticidal activity through a shared mode of action: Following ingestion of the protein by the target insect pest, exposure to the alkaline conditions of the insect midgut and proteolytic cleavage (i.e. cleavage by trypsin-like insect midgut proteases) of the protoxin yields a bioactive, protease-resistant core protein (or tryptic core) comprised of three-domains. The core protein interacts with specific receptors located on the target insect pest cell surface to form an oligomeric structure, leading to insertion into the plasma membrane and pore formation (Pigott and Ellar, 2007). Each of the three domains in Cry proteins can be ascribed a role in facilitating insecticidal activity: domain I is primarily responsible for membrane-insertion and pore formation; domain II facilitates receptor interactions, and domain III aids in oligomeric structural integrity and receptor specificity/binding (Deist et al., 2014). Importantly, for most Cry proteins, proteolytic cleavage of the protoxin includes removal of a fourth, large C-terminal domain which results in the solubilization of the protein that is implicated in crystal formation (de Maagd et al., 2001) and is required for insecticidal efficacy (Deist et al., 2014). Target insect specificity of insecticidal Cry proteins is mediated in part by the specific insect midgut proteases that activate the cry protein upon exposure to the alkaline conditions of the insect midgut. However, more importantly, target insect specificity is determined by specific toxin-receptor interactions that occur along the insect midgut brush border membrane (Bravo et al., 2007). Their insecticidal activity can be described by a shared insecticidal activation process through interaction between the Cry protein and susceptible pests.

The modular architecture of three-domain Cry proteins has led to a

Abbreviations: bw, body weight; BSA, Bovine Serum Albumin; *Bt*, *Bacillus thuringiensis*; Cry, crystal; *E. coli*, *Escherichia coli*; GM, genetically modified; MALDI-TOF MS, Matrix assisted laser desorption ionization - time of flight mass spectrometry; SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SIP2, soybean insect-protected product

* Corresponding author.
** Corresponding author.
E-mail addresses: Cunxi.wang@bayer.com (C. Wang), thomas.edrington@bayer.com (T.C. Edrington).

https://doi.org/10.1016/j.yrtph.2018.09.003
Received 31 July 2018; Received in revised form 1 September 2018; Accepted 4 September 2018
Available online 06 September 2018

systematic nomenclature that provides a standard framework for understanding the level of amino acid similarity between the Cry protein family members (Crickmore et al., 1998). Additionally, the modular architecture provides a repository of linkable structures for creating chimeric proteins composed of structural and functional domains sourced from different Cry proteins. The combination of domains from different Cry proteins, along with the ability to optimize key amino acids of the Cry protein that are involved in receptor interactions, can yield chimeric Cry proteins that possess greater insecticidal efficacy and/or altered insecticidal specificity while maintaining the common mode of action (Deist et al., 2014; Gatehouse, 2008; Sanahuja et al., 2011). For example, the coleopteran-active eCry3.1Ab protein was generated by combining specific regions of the lepidopteran-active proteins Cry1Ab and Cry3A to achieve activity against western corn rootworm larvae (Walters et al., 2010). While modern biotechnology has been successfully used to alter the insecticidal properties of Cry proteins as exemplified above, evidence from phylogenetic assessments suggest that domain swapping among Cry proteins is also an active, naturally occurring and biological process (Bravo, 1997; de Maagd et al., 2001).

The Cry1A.105 protein evaluated herein is an engineered three-domain toxin that is active against major lepidopteran pests. It is comprised of domains I and II from Cry1Ab or Cry1Ac (*Bt ssp. kurstaki*), domain III from Cry1F (*Bt. ssp. aizawai*) and the C-terminal domain from Cry1Ac. The Cry1A.105 protein is expressed in the current commercialized GM maize products YieldGard VT PRO® and Genuity® SmartStax® (Head et al., 2014). The next generation insect protected soybean product (SIP2), Intacta 2 Xtend™, expresses the Cry1A.105 protein from the MON 87751 soybean (SIP2 Cry1A.105). However, the soybean version of the protein differs from the version used in maize by four N-terminal amino acids due to incomplete processing of the C-terminal chloroplast transit peptide (CTP) in soybean, which facilitates Cry1A.105 targeting to the host chloroplast to minimize the evolution of target insect resistance (Muzaffar et al., 2015). The minor N-terminal amino acid sequence difference between the maize and SIP2 Cry1A.105 proteins has no observable impact on the functional activity (as shown herein).

In this study, the structure of the tryptic core domain (the activated toxin) (Grochulski et al., 1995) of the chimeric Cry1A.105 protein and the safety of the Cry1A.105 protein for human and animal consumption were evaluated to determine if these results parallel those observed for Cry1Ab, Cry1Ac and Cry1F proteins, from which structural and functional domains were derived to engineer the chimeric Cry1A.105 protein or if a comprehensive weight-of-evidence tiered approach (Delaney et al., 2008) was needed for safety assessment of a homologous protein with a history of safe use (HOSU). These results support the conclusion that HOSU of donor proteins or domains can be considered as part of the weight-of-evidence for the safe consumption of food or feed products derived from GM crops expressing a chimeric protein of interest. Since HOSU of proteins in food and feeds is a central facet of the harmonized paradigm for assessing the safety of proteins introduced into GM crops (Delaney et al., 2008; Hammond et al., 2013; OECD, 2002), including the HOSU of donor protein domains in newly engineered chimeric proteins is relevant.

2. Materials and methods

2.1. Expression and purification of Cry1A.105 proteins

The coding sequence corresponding to the maize or MON 87751 soybean-produced Cry1A.105 protein (hereafter referred as SIP2 Cry1A.105) was ligated into pET24b (Novagen, Madison, WI) and expressed in BL21 (*DE3*) *E. coli* (Invitrogen, Carlsbad, CA). Fermentation of the transfected cells was performed in the presence of kanamycin. The resulting fermentation product(s) were collected and resuspended in neutral buffer containing protease inhibitors and benzonase nuclease. The fermentation slurries were then lysed using a cell disrupter and the insoluble fractions (e.g., the inclusion bodies) were harvested. Following extensive washing in neutral buffer, inclusion body pellets were solubilized in alkaline (pH 10.8) sample buffer (at a mass:volume ratio of about 1:100) and incubated in a cold room (~4 °C) overnight with slow stirring. The SIP2 Cry1A.105 protein was purified from the solubilized inclusion bodies using a multi-step process including anion exchange chromatography, ceramic hydroxyapatite chromatography and diafiltration. Proteins (the maize Cry1A.105 and Cry1Ac) evaluated herein were generated following a similar procedure to what is outlined above.

To demonstrate physicochemical and functional equivalence between the *E. coli*-produced and plant-produced proteins, isolation of the SIP2 Cry1A.105 was conducted utilizing defatted seed powder as the starting material. Briefly, the plant-produced SIP2 Cry1A.105 protein was purified using a combination of ammonium sulfate fractionation, anion exchange chromatography, and immunoaffinity chromatography.

2.2. Characterization of Cry1A.105 proteins

Protein characterization is part of the weight of evidence for protein safety assessment. All methods used to characterize proteins that are introduced into GM crops have previously been reported (Wang et al., 2015) and were similarly used to characterize Cry1A.105 and Cry1Ac proteins in this study. The concentration of total protein was determined using quantitative amino acid compositional analysis or Bio-Rad protein assay. Purity and apparent molecular weight of proteins were determined using densitometric analysis of stained SDS–PAGE gels. For immunoblot analysis, each protein was subjected to SDS–PAGE and transferred to a nitrocellulose membrane. The blot was probed with a goat anti-Cry1A.105 specific polyclonal antibody. Glycosylation analysis was conducted following ECL Glycoprotein Detection method (GE Healthcare, Piscataway, NJ) and transferrin was used as a positive control. N-terminal sequence analysis was performed for 15 cycles using an Applied Biosystems 494 Procise- Sequencing System (Hunkapiller and Hood, 1983; Wang et al., 2015). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to confirm the identity of each protein by tryptic digest mapping (Wang et al., 2015). A total of 60 fragments identified by MALDI TOF from the soybean-produced Cry1A.105 matched the expected masses of the trypsin-digested peptides, providing coverage for 632 of the 1181 amino acids. The equivalence between soybean- and *E. coli*-produced proteins was evaluated using approaches described previously (Wang et al., 2015).

Each Cry1A.105 protein as well as Cry1Ac was tested for activity against corn earworm (CEW; *Helicoverpa zea*). CEW eggs were obtained from Benzon Research, Inc. (Carlisle, PA). CEW larvae (≤30 h old) were used to measure biological activity. Each protein was incorporated into artificial diet at six concentrations using 16 larvae for each concentration. Larvae were allowed to feed for ~7 days in an environmental chamber at 27 °C before the weight of the survivors was assessed. The bioassay was replicated three times on separate days, each with a separate batch of insects. SAS procedure PROC NLMIXED (SAS institute Inc., Cary, NC, USA) was used to fit the data separately for each bioassay using a 3-parameter logistic model to estimate the EC50 value, which is the estimated protein concentration that results in 50% growth inhibition relative to the control.

2.3. X-ray crystal structure determination of Cry1A.105 tryptic core

The Cry1A.105 tryptic core protein used for crystallization studies was generated by incubation of the full-length *E. coli*-produced maize Cry1A.105 protein with bovine pancreatic trypsin (Cry1A.105:trypsin ratio of about 10:1). Digestion to the trypsin-resistant core occurred in a dialysis bag placed in 4 L of a buffer solution containing 50 mM Bis-Tris-Propane, pH 9.25, and 1 mM CaCl2. After centrifugation, the pelleted