



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

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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

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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 NL MIXED (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

core protein was re-suspended in 40 mM CAPS-NaOH, pH 10.5, 1 mM EDTA, 1 mM benzamidinium-HCl, and fractionated on a Q-XL anion exchange column to remove smaller peptide fragments. The core protein fractions were dialyzed against 50 mM carbonate/bicarbonate buffer, pH 10.5 and concentrated (~ 35 mg, $> 90\%$ purity, ~ 6 mg/ml) for crystallization. The resulting Cry1A.105 tryptic core protein was crystallized using the technique of vapor diffusion by hanging drops in a 24-well VDX plate (Hampton Research, Aliso Viejo, CA) at room temperature.

Numerous crystallization conditions were screened using the protein sample and a variety of commercially-available protein crystallization reagents. Rectangular, plate-like crystals were obtained using a precipitant/reservoir solution of 5% (w/v) PEG 4000, 0.1 M sodium citrate buffer, pH 6, and 10% isopropanol. Microseeding (Bergfors, 2003) was required to obtain crystals sizable enough for X-ray intensity data collection at the synchrotron. The best-quality crystals (McPherson and Cudney, 2014) were obtained from hanging drops that were composed of 2 μ l protein solution, 4 μ l reservoir solution and 0.5 μ l of a 1:100 dilution of the crystal microseed stock.

Crystals of Cry1A.105 tryptic core were prepared for low temperature (~ 100 K) X-ray intensity data collection by dipping them in cryo-solution of 7% PEG 4000, 0.1 M sodium citrate, pH 6, 25% glycerol and 0.15 mM sodium azide. Crystals were dipped in the cryo-solution for ~ 5 s prior to plunge-cooling in liquid nitrogen. X-ray diffraction data were collected in-person at the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source (APS), at Argonne National Laboratory (Argonne, IL). The X-ray data set used for structure determination was collected on a cryo-cooled crystal, at a wavelength of 1.0 Å, using a MAR300 CCD detector. One hundred and eighty degrees of data were collected using 2 s exposures, an oscillation angle of 1.0 degree, and a crystal-to-detector distance of 250 mm. These data were processed using the HKL2000 package (Otwinowski and Minor, 1997).

The Cry1A.105 tryptic core structure was solved by molecular replacement (MR) methods using the Phaser program (McCoy et al., 2007) as implemented in the CCP4 package (Winn et al., 2011), and using the Cry1Aa crystal structure (Grochulski et al., 1995) as a phasing template. Iterative map-fitting and refinement were performed to improve the structure. Crystallographic refinement was conducted initially using CNX2002 (Brunger et al., 1998), followed by refmac5 (Murshudov et al., 1997) and finally with refinement protocols in the Phenix package (Adams et al., 2010). Model-building and map-fitting were performed with the program O (Jones et al., 1991) at first, and later with the Coot program (Emsley and Cowtan, 2004).

2.4. Bioinformatic assessment of Cry1A.105 proteins

Bioinformatic assessments and thresholds used to determine structural similarity to known allergens and toxins were derived from those described previously by Wang et al. (2015) and as outlined by Codex Alimentarius (2009), the exceptions being that 2018 versions of databases were used and the full 35% identity over 80 amino acid threshold searches could be conducted because the query sequences were of sufficient length to apply this criterion. The database used to represent all known proteins was GenBank release 223. An updated toxin database (herein described as TOX_2018) was compiled in a similar manner to that described in Wang et al. (2015) whereby sequence descriptions were screened for the keywords “toxi” or “ricin”. Further post-processing was conducted on these sequences whereby differences between the 2017 iteration of toxin database and the pool for inclusion in the 2018 toxin database were manually inspected and sorted by keyword followed by the removal of duplicate or fully contained subsequences. Two final filters were applied to remove any sequences sourced from a group of known insecticidal proteins that might be erroneously included by keyword alone. The end result is the TOX_2018 toxin database which contains 28,344 sequences. The allergen database (herein

described as AD_2018) utilized was the “COMprehensive Protein Allergen Resource” (COMPARE) database as generated and maintained by the Health and Environmental Sciences Institute (HESI) (www.comparedatabase.org), and contains 2,038 sequences. Alignments were generated using FASTA v36.3.5d run with an E-score cutoff of 1, and a threshold of $\leq 1e-5$ (1×10^{-5}) was used as an initial threshold for alignment significance. This is the same threshold used previously (Wang et al., 2015; Wang et al., 2016) and is recognized as being a conservative threshold for the identification of homologous proteins (Pearson, 2003).

2.5. Assessment of Cry1A.105 protein susceptibility to pepsin and pancreatin

The susceptibility of Cry1A.105 protein to degradation by pepsin was assessed following a standardized protocol (Thomas et al., 2004). Briefly, the purified Cry1A.105 protein in an appropriate buffer was mixed with high purity pepsin to a final protein-to-pepsin ratio of 1 μ g total protein:10 U of pepsin. The reaction mixture tube was immediately placed in a 37 ± 2 °C water bath. Samples were removed at 0, 0.5, 2, 5, 10, 20, 30 and 60 min and were immediately quenched by the addition of 0.7 M sodium carbonate and 5X SDS-PAGE sample loading buffer. Protein only and pepsin only experimental controls were also prepared and incubated for 60 min in a 37 ± 2 °C water bath. All resulting samples were heated at 95–100 °C for 5–10 min, frozen on dry ice, and stored in a -80 °C freezer prior to SDS-PAGE analysis.

Cleavage by digestive enzymes was also assessed by pepsin treatment followed by pancreatin treatment. Pancreatin (Sigma) was dissolved in 50 mM potassium phosphate buffer (pH 7.5) to a concentration of 10 mg of pancreatin powder/ml as described in The United States Pharmacopoeia (Delaney et al., 2008; USP, 1995). The pancreatin solution was formulated so that 55.3 μ g of pancreatin powder would be present per μ g of Cry1A.105 protein in the reactions. The pepsin reaction was halted after 2 min of incubation by adding 0.7 M sodium carbonate buffer. The 2 min time point was selected to insure that the protein fragment of interest was readily detectable on SDS-PAGE. The quenched pepsin reaction mixture was added to a pre-heated (37 °C, 5 min) aliquot of pancreatin mixture. Samples were removed at 0, 0.5, 2, 5, 10, 30, 60 and 120 min and quenched with SDS-PAGE loading buffer. The resulting solutions were analyzed by SDS-PAGE.

The extent of test protein digestion in pepsin and in the pepsin/pancreatin sequential assay was assessed by Brilliant Blue G staining of SDS-PAGE gels. In each case, a standard curve was generated on second gels to determine the limit of detection (LOD) of the test proteins.

2.6. Heat lability of Cry1A.105 proteins

The heat stability of Cry1Ac, maize Cry1A.105 and SIP2 Cry1A.105 proteins was tested in separate experiments that each followed the same procedure. In each experiment, the test protein was exposed to five temperatures from 25 °C to 95 °C for 15 min, with a control sample incubated at 0 °C. Samples were placed on wet ice following incubation. A control sample aliquot of each protein was also maintained on wet ice throughout the course of the heat treatment incubation period. Each heat-treated sample was used to test for functional stability using a CEW assay, as described above (section 2.2). Each protein concentration contained one replicate with a target number of 16 individually housed insect larvae, and the buffer control contained three replicates each with a target number of 16 individually housed insect larvae.

2.7. Acute oral toxicity assessment

An acute oral toxicity study with SIP2 Cry1A.105 was conducted in CD-1 mice. The study was performed according to Good Laboratory Practices (GLPs) using a study design adapted from the EPA OPPTS

Guideline 870.1100. Oral exposure was chosen as it is the most relevant route of human exposure and this study was conducted as an acute high dose oral toxicity study based on the principle that a majority of toxic proteins elicit toxicity acutely, i.e. with a short duration to onset of effects (Hammond and Fuchs, 1998). The Cry1A.105 dose solution was formulated in a vehicle buffer (1 mM carbonate/bicarbonate buffer, pH 10.15–10.30) at dose concentration of ~30.0 mg SIP2 Cry1A.105 protein/ml to enable a targeted dose level of 2000 mg SIP2 Cry1A.105 protein/kg body weight. In addition to the test dosing solution, a protein control (bovine serum albumin, BSA) dosing solution in 1 mM carbonate/bicarbonate buffer, pH 10.15–10.30 was prepared to enable a targeted dose level comparable to that of the analytically confirmed dose level for SIP2 Cry1A.105 protein (36.8 mg/ml). Prior to the start of the study, the dosing solutions were analyzed to confirm pH, concentration, and homogeneity (data not shown). Stability was confirmed by analyzing protein concentration and integrity of pre- and post-dosing solutions on a SDS-PAGE gel (data not shown). Insecticidal efficacy was also confirmed for pre- and post-dosing solutions in a corn earworm insect bioassay as described above (data not shown).

Male and female CD-1 mice were purchased from Charles River Laboratories (Portage, MI). Following a 2–3 h fast, mice were given two oral gavage doses (about 4 h apart) of test or control dosing solution on study day 0 with a dose volume of 33.3 ml/kg body weight. Ten male and ten female mice, at approximately 8 weeks of age at dosing, were administered Cry1A.105 protein (males, Day 0 fasted body weight range 29–37 g; females, Day 0 fasted body weight range 21–27 g). A 14-day observation period was conducted following dosing. Endpoints evaluated during the dosing and observation periods included: clinical observations, survival, body weights/body weight changes, and food consumption. After the observation period, all surviving animals were euthanized by carbon dioxide inhalation and necropsied. The endpoints evaluated at necropsy included macroscopic examination of: the carcass and musculoskeletal system, all external orifices and surfaces, cranial cavity and external brain surfaces, and the abdominal, pelvic, and thoracic cavities and their associated organs and tissues. Necropsies and macroscopic examinations were conducted under the supervision of a board-certified veterinary pathologist. To ensure animal welfare, all work was conducted in an AAALAC accredited laboratory and the study protocol was reviewed and approved by the test facility IACUC committee prior to study initiation.

Statistical analysis was conducted on body weight, body weight gain, and food consumption data obtained in the acute toxicology study conducted with Cry1A.105. Data were subjected to a statistical decision tree built into the laboratory information system with data first analyzed for homogeneity of variance using Levene's test (Levene, 1960) followed by the Shapiro-Wilk test for normality (Shapiro and Wilk, 1965). If neither Levene's test nor the Shapiro-Wilk test were significant (at $p < 0.001$), a single-factor parametric analysis of variance (ANOVA) was applied with animal grouping as the factor, using a $p < 0.05$ level of significance. Dunnett's test was used ($p < 0.05$) to identify statistically-significant differences between the control and test substance-treated group (Dunnett, 1955, 1964; Snedecor and Cochran, 1998). A Kruskal-Wallis non-parametric ANOVA (Kruskal and Wallis, 1952) was applied if either Levene's test and/or the Shapiro-Wilk test were significant. Dunn's test (Dunn, 1964) was used ($p < 0.05$) if the non-parametric Kruskal-Wallis ANOVA was significant ($p < 0.05$) to identify statistically-significant differences between the control and test substance-treated group.

3. Results and discussion

3.1. Cry1A.105 shows high bioinformatics relationship to its donor proteins

Cry1 proteins consist of three core domains (I, II, and III) with a protease-cleaved protoxin C-terminal domain (or protoxin domain). The origin of each domain of Cry1A.105 and the identity of each

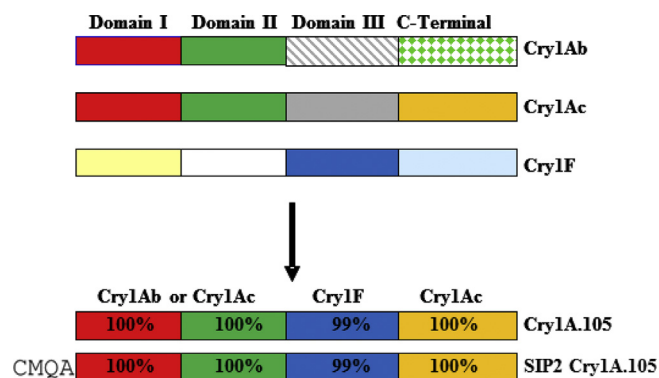


Fig. 1. Schematic representation of the origin of Cry1A.105 protein domains. Different colors and patterns illustrate the origin of each domain in Cry1A.105. For simplicity, the lengths of domains in this illustration are not in proportion to the lengths of amino acid sequence of the respective domains.

domain to the counterpart from other proteins is shown schematically in Fig. 1. To further illustrate the level of similarity [percent similarity of two sequences is the sum of both identical and similar amino acid (residues conserved with similar physicochemical properties, e.g. arginine and lysine) matches in the alignment] between Cry1A.105 and its source proteins, a FASTA36 search using the chimeric SIP2 Cry1A.105 sequence (1181 amino acids) as a query was conducted against each of the domain source proteins, and this analysis showed that each of the various proteins displayed high levels of sequence identity (the percentage of amino acids with an identical match in the alignment) ranging from 76.6% to 100% across their alignment lengths (Fig. 1, Supplemental Table 1).

3.2. The X-ray crystal structure of the tryptic core of Cry1A.105 is highly similar to tryptic core structures of the donor proteins

Proteins that share similar secondary and tertiary structures tend to possess the same overall mode of action and similar biological function (Hammond et al., 2013). Therefore, determination of the crystal structure of an introduced, exogenous protein can be a valuable tool for understanding its mode of action, which is an essential component of a weight-of-evidence based safety assessment. Producing sizable, single crystals of the Cry1A.105 tryptic core domain for crystallography studies proved to be challenging. Most of the crystallization attempts resulted in twinned or multi-crystal aggregates that were intergrown in the drop and could not be easily separated. Additionally, most cryo-cooled crystals tested at the synchrotron were not of sufficient quality to permit collection of data that were usable for three-dimensional structure analysis.

Despite the technical challenges associated with producing suitable Cry1A.105 crystals, Phaser-based molecular replacement using the 3.0 Å data sets and a phasing template based on the protein-atom only Cry1Aa protein database (PDB) structure (entry 1CIY.pdb) yielded strong rotation function ($Z = 25.0$) and translation function ($Z = 26.6$) peaks, indicative of an unambiguous solution. The Cry1A.105 and Cry1Aa tryptic cores share approximately 75% sequence identity, and, as presented in the Materials and Methods section, map-fitting and refinement using the programs noted were used to deliver a final structure. The final Cry1A.105 tryptic core (often referred to as the three-domain structure) is displayed in Fig. 2A, and its refinement statistics are presented in supplemental Table 2.

The Cry1A.105 tryptic core structure, shown as a ribbons rendition in Fig. 2A, is colored based on its characteristic domain structure: domain I, a seven α -helical bundle domain, extends from Tyr33 to Thr264, and is colored red; domain II, a β -prism domain, extends from Arg265 to Asn463, and is colored green; domain III, a lectin-like β -sandwich fold, extends from Asn464 to Ala609, and is colored cyan (Morse et al.,

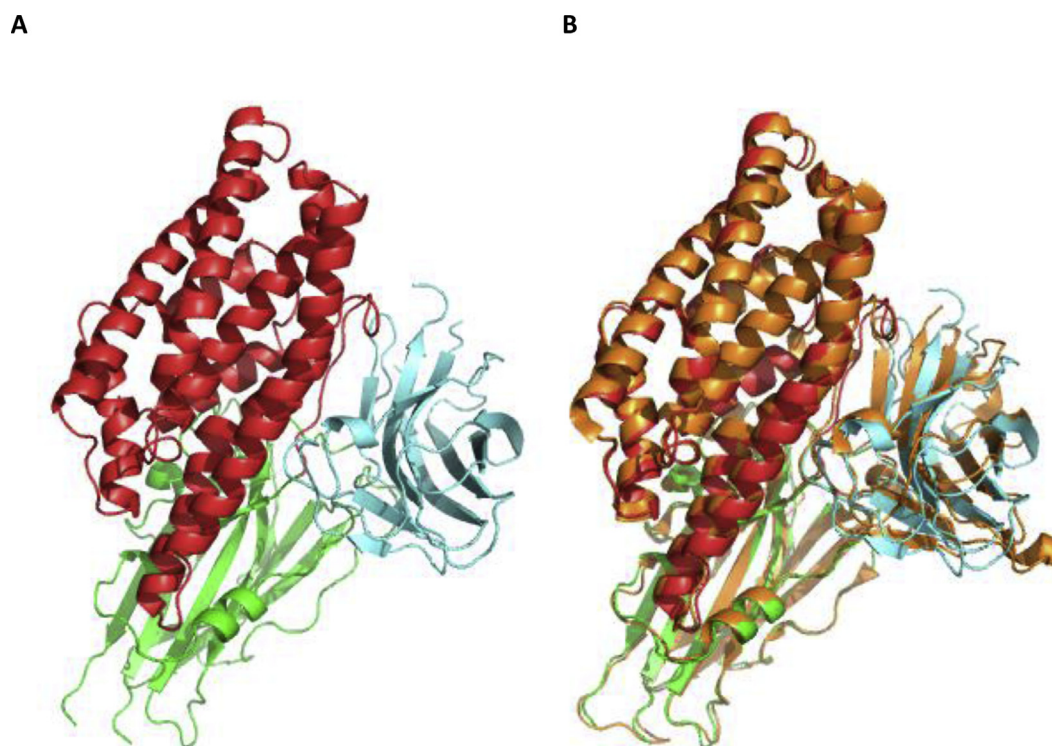


Fig. 2. The Cry1A.105 tryptic core crystal structure alone, and with the Cry1Ac structure aligned to it. **A:** Ribbons figure of the Cry1A.105 crystal structure color-coded by domain: I (red), II (green), III (cyan); **B:** Cry1A.105 structure aligned to ribbons figure of Cry1Ac tryptic core structure, in orange (PDB entry 4ARX). Figures were rendered using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

Table 1

Characterization of Cry1A.105 and Cry1Ac proteins.

Characteristic	Method	MON 87701 Cry1Ac	Maize Cry1A.105	SIP2 Cry1A.105	
		<i>E. coli</i> -produced	<i>E. coli</i> -produced	Soybean-produced	<i>E. coli</i> -produced
Percent Purity	SDS-PAGE/Densitometry	80%	92%	60%	93%
Activity (EC ₅₀ , ng/ml diet)	Insect bioassay	2.2	5.8	3.5	3.2
Immunoreaction	Immunoblot	Confirmed	Confirmed	Confirmed	Confirmed
Glycosylation	GE Glycoprotein Detection Module	none	none	none	none
Apparent molecular weight (kDa)	SDS-PAGE/Densitometry	131.7	131.5	132.9	130.8
Protein identity	N-terminal sequence	CMQAMDNN	MDNNPNINECIPYN	CMQAMDNNP	CMQAMDNNPNIN
	Tryptic Mass Fingerprint	43%	43%	54%	57%

2001). Moreover, in Fig. 2B, the Cry1Ac tryptic core structure (PDB entry 4ARX), colored orange, is aligned onto the Cry1A.105 tryptic core structure, showing that the two share highly similar structural shapes. The alignment was done using with the PyMOL program align utility. A pair-wise root-mean-square difference (r.m.s.d.) in α -carbon positions between the two molecules is 0.62 Å using 447 α -carbons, indicating high-structural similarity overall between the two structures. The structures are nearly identical in domains I and II; the main region of structural difference is in domain III, which is the domain responsible for receptor-specificity and contains the most amino acid sequence divergence. However, the domain III still shares ~44% sequence identity and the overall structural similarity. A sequence identity of 44% over a region involving two proteins which are essentially identical over the rest of the proteins is significant. The overall protein folding is maintained in both proteins with the β -strand positions being slightly off-set from one another. The high structural similarity between Cry1A.105 and Cry1Ac supports the conclusion that the domain exchange strategy utilized to engineer the Cry1A.105 protein preserves the Cry1 mode of action, which was functionally confirmed by insect bioassays (Table 1). Thus, the safety of the Cry1A.105 protein is supported by its structural homology to one of its source proteins, Cry1Ac.

3.3. Plant and *E. coli*-produced Cry1A.105 have the identical amino acid sequence, similar immunoreactivity and functional activity, and absence of glycosylation

The Cry1A.105 expression level in dry soybean grain was estimated by enzyme-linked immunosorbent assay (ELISA) to be 2.4 ppm. To obtain quantities of SIP2 Cry1A.105 protein sufficient for a globally relevant regulatory safety assessment (e.g., ~5 g), it was necessary to produce the protein in a high-expressing recombinant host system such as *E. coli*. To this end, the SIP2 Cry1A.105 protein was expressed and purified from *E. coli* to a purity of > 90% (Table 1, supplemental Figure 1). To demonstrate that the heterologously expressed Cry1A.105 is equivalent to the Cry1A.105 protein present in soybean, the SIP2 Cry1A.105 protein was also purified directly from SIP2 seed (Table 1, supplemental Figure 1).

The identities of both soybean- and *E. coli*-produced Cry1A.105 were confirmed by Edman sequencing and peptide mass fingerprinting analyses. Edman sequencing confirmed that soybean produced a Cry1A.105 protein that contains an additional four amino acids derived from the C terminal end of CTP preceding the N-terminus of the maize Cry1A.105 protein (Table 1). Alternative cleavage of CTP from a

Table 2

Summary of alignments for the FASTA searches of the AD_2018 database using SIP2 Cry1A.105, Cry1Ab, Cry1Ac and Cry1F proteins.

Sequence Name	Sequence Accession	Search of the AD_2018 Sequence Database							
		FASTA search							
		Sliding Window	35% ID 80 aa	# of Returned Alignments	Aligned Sequence Accession	Description	Identity	aa Overlap	E-score
SIP2 Cry1A.105	XXXXXX*	No	No	2	AAK67492.1	Allergen cytochrome c [Curvularia lunata] (108 aa)	24.4%	86	0.34
Cry1Ab	AAA22330.1	No	No	8	ABZ81040.1	Allergen pollen allergen Car b 1 isoform [(160 aa)	27.6%	98	0.3
Cry1Ac	AAA22331.1	No	No	1	CAA50328.1	Allergen major allergen [Corylus avellana] (160 aa)	27.6%	98	0.87
Cry1F	AAA22348.1	No	No	0	–	–	–	–	–

*The sequence used for searching the database was the [CAS91272.1](#) sequence plus four amino acids (CMQA) on its N-terminal (see [Fig. 1](#)).

transgenic protein in *planta* by a general stromal processing peptidase is a common process ([Richter and Lamppa, 1998](#)). The N-terminal sequence of *E. coli*-produced SIP2 Cry1A.105 protein was consistent with the N-terminal sequence of the soybean-produced SIP2 Cry1A.105 protein, which was engineered to contain a cysteine as the first amino acid ([Table 1](#)). The N-terminal methionine of the *E. coli*-produced SIP2 Cry1A.105 was removed during expression in *E. coli*. Removal of the N-terminal methionine by methionine aminopeptidase is a common modification that occurs co-translationally in *E. coli* before completion of the nascent protein chain ([Arfin and Bradshaw, 1988](#); [Bradshaw et al., 1998](#); [Polevoda and Sherman, 2000](#)). Mass fingerprint analysis provided 54% and 57% coverage of the amino acid sequences for the soybean-produced and *E. coli*-produced Cry1A.105 proteins, respectively. These sequence coverages are sufficient to confirm their identity as protein identification is considered reliable if the coverage is at least 15% of the sequence ([Jensen et al., 1997](#); [Raybould et al., 2013](#)).

Both soybean - and *E. coli*-produced SIP2 Cry1A.105 proteins had comparable immunoreactivity to Cry1A.105-specific antibody ([supplemental Figure 2](#)). No glycosylation was detected for Cry1A.105 purified from either soybean or *E. coli* ([supplemental Figure 3](#)). The soybean-produced Cry1A.105 displayed equivalent functional activity with the *E. coli*-produced Cry1A.105, showing comparable EC₅₀ values in a corn earworm bioassay of 3.5 and 3.2 ng Cry1A.105 protein/ml diet, respectively ([Table 1](#)).

Cry1A.105 expressed in maize and Cry1Ac expressed in soybean were also produced from *E. coli* and characterized ([Table 1](#)). It is noteworthy that Cry1A.105 isolated from different sources possesses similar functional activity to Cry1Ac when the functional activity is tested against corn earworm. However, when functional activity is tested using a different insect species, these proteins display different degrees of insecticidal activity although Cry1A.105 and its donor proteins compete with high affinity for the same binding sites in both insect species ([Hernandez-Rodriguez et al., 2013](#)).

Protein glycosylation has been evaluated in the context of potential for allergenicity, but recent reports have drawn the relevance of protein glycosylation in allergenicity into question ([Goodman et al., 2008](#); [van Ree, 2002](#)). This characteristic has a more significant impact on the physicochemical properties of a protein (e.g., function, half-life). Additionally, comparison of these results to characterization data for the Cry1A.105 expressed in maize indicates that the minor difference in the N-terminal amino acid sequence has negligible impact on the efficacy of the protein in insect diet bioassays ([Table 1](#)). Thus, from a holistic perspective, the three proteins (*E. coli*-produced SIP2 Cry1A.105, soybean-produced SIP2 Cry1A.105 and *E. coli*-produced maize Cry1A.105) can be considered indistinguishable in a weight-of-evidence based protein safety assessment. These analyses support the conclusion that the *E. coli*-produced soybean Cry1A.105 was a suitable surrogate for assessing safety of the soybean-produced Cry1A.105 protein ([Raybould et al., 2013](#); [Wang et al., 2015](#)). Therefore, all safety assessments herein

were conducted using the equivalent *E. coli*-produced protein.

3.4. Neither Cry1A.105, nor its donor proteins display homology to known allergens and toxins

A key element of the safety assessment of proteins introduced into GM crops is a bioinformatic analysis to determine whether the introduced protein shows similarity to known toxins or allergens ([Hammond et al., 2013](#); [Kier and Petrick, 2008](#)). The degree of sequence homology (i.e. those derived from a common ancestor having similar functions) between a protein of interest and sequences of protein allergens and toxins in databases can be assessed using the FASTA sequence alignment software which returns both identity and similarity values and calculates a corresponding expect value (*E*-score). While the FASTA program compares amino acid sequences (i.e., primary protein structure) and provides optimum local sequence alignments, the results can be used to infer as to any conservation of higher order structure, and then of functional properties ([Randhawa et al., 2011](#)). The bioinformatics analyses performed on SIP2 Cry1A.105 and each of the donor Cry proteins demonstrated highly congruent results. Using an *E*-score significance threshold of $\leq 1e-5$ (1×10^{-5}), which has traditionally been used as a conservative threshold ([Pearson, 2003](#); [Wang et al. 2015, 2016](#)), there were no significant alignments between the Cry1A.105 protein or its donor proteins with those contained within the COMPARE allergen database (AD_2018) or toxin database (TOX_2018). Alignments were observed to the COMPARE allergen database for each of the proteins except Cry1F, however none of these alignments displayed levels of identity indicative of homology. The best alignment to each of the aligning sequences was to regions of less than 100 amino acids in length (despite the query proteins being over 1000 amino acids in length), each of which displayed relatively low levels of identity ($< 30\%$) and had observed *E*-scores well below the significance threshold of $\leq 1e-5$ ([Table 2](#)). These results are not indicative of homology and are in line with expectations for random, non-homologous matches ([Pearson, 2000](#)) ([Table 2](#)). Likewise, searches against a toxin database (TOX_2018) resulted in the same observation for each of the query sequences and did not display levels of identity indicative of functional homology ([Table 3](#)). The only alignment to each of the variants was a heavily gapped length of low-level identity to a sequence described as 81-kDa leukemia toxin from *B. thuringiensis*. Further investigation into this protein reveals that it is, in fact, classified as a non-insecticidal Cry protein, which accounts for the low levels of identity observed across the protein length, but is in no way indicative of a potential for toxicity ([Crickmore et al., 2017](#); [Mizuki et al., 2000](#)). These data demonstrate the lack of sequence similarities between Cry1A.105 and any of its donor proteins to any known allergenic or toxic proteins that would be harmful to human or animal health. This is not surprising given that the previous bioinformatics analysis demonstrated no potential risk of allergenic cross-reactivity for the Cry1A.105

Table 3

Summary of alignments for the FASTA searches of the TOX_2018 database using SIP2 Cry1A.105, Cry1Ab, Cry1Ac and Cry1F proteins.

Sequence Name	Sequence Accession	Search of the TOX_2018 Sequence Database					
		# of Returned Alignments	Aligned Sequence Accession	Description	Identity	aa Overlap	E-score
SIP2 Cry1A.105	XXXXXX*	1	BAB11757.1	81-kDa leukemia toxin** [Bacillus thuringiensis (723 aa)]	22.1%	633	0.00016
Cry1Ab	AAA22330.1	1	BAB11757.1	81-kDa leukemia toxin [Bacillus thuringiensis (723 aa)]	23.1%	632	6.3e-05
Cry1Ac	AAA22331.1	1	BAB11757.1	81-kDa leukemia toxin [Bacillus thuringiensis (723 aa)]	22.0%	633	0.00055
Cry1F	AAA22348.1	1	BAB11757.1	81-kDa leukemia toxin [Bacillus thuringiensis (723 aa)]	21.3%	621	8.5e-05

*The sequence used for searching the database was the [CAS91272.1](#) sequence plus four amino acids (CMQA) on its N-terminal (see [Fig. 1](#)). **Due to the lack of standardized nomenclature, this was named as leukemia toxin in the database but it is a Cry protein (see [3.4](#) section).

donor proteins, Cry1Ab, Cry1Ac and Cry1F ([Randhawa et al., 2011](#)), from which the Cry1A.105 protein was derived. These protein sequences are sourced from the naturally occurring, soil-borne *Bt* microorganism, which has been safely used commercially for over 50 years in microbial pesticides and the Cry1Ab, Cry1Ac and Cry1F proteins had been previously deemed safe for consumption ([EPA, 2007](#); [FDA, 2007](#); [Gao et al., 2006](#); [James, 2006](#); [Koch et al., 2015](#); [USDA, 2007](#)). Thus, bioinformatic analyses indicate a consistent safety profile for the chimeric protein with that of its parental proteins. Additionally, these findings further indicate that small differences in SIP2 Cry1A.105 sequence ([Fig. 1](#)) have little to no impact on the conclusions from this type of bioinformatic assessment that these proteins lack any similarity to known allergens or toxins.

3.5. Cry1A.105 and its donor proteins show similar levels of denaturation by temperatures common to food processing and cooking conditions

The stability of a protein after being subjected to temperatures encountered during processing or preparative cooking is a key endpoint in determining the potential for human or animal exposure to a natively folded and active protein ([Hammond et al., 2013](#)). The potential for a protein to be denatured by heat treatment can be assessed *in vitro* by heating it in aqueous solution at temperatures ranging from 25 to 95 °C for 15–30 min, conditions commonly found in the kitchen and in the processing of maize and soybean into human food products ([Hammond and Jez, 2011](#)). For example, a protocol for measuring the heat stability of proteins has been proposed by the Indian Department of Biotechnology ([Hammond et al., 2013](#)). Following internationally accepted GM safety guidance ([Codex Alimentarius, 2009](#); [Wang et al., 2016](#)), the SIP2 Cry1A.105, maize Cry1A.105 and Cry1Ac proteins were incubated at temperatures ranging from 25 to 95 °C and the activity of the proteins relative to a control sample incubated at 0 °C was assessed by insect diet bioassay ([Table 4](#)). The results indicate that SIP2 Cry1A.105, maize Cry1A.105 and Cry1Ac retained functional activity after incubation at temperatures up to 55 °C. When incubated at temperatures of 75 and

Table 4

Heat stability of Cry1A.105 and Cry1Ac proteins.

Temperature (°C)	Relative activity (%) ^a		
	SIP2 Cry1A.105	Maize Cry1A.105	Cry1Ac
0 (control)	100	100	100
25	130	169	100
37	108	147	108
55	105	92	82
75	< 3	< 19	< 2
95	< 3	< 19	< 2

^a Relative activity = Treatment EC₅₀ value/control EC₅₀ value x 100. EC₅₀ value of Cry1A.105 activity following heat treatment for 15 min.

Table 5

Summary of Cry1A.105 donor protein heat stability.

Test Protein	Temperature to denature protein functional activity	Reference
Cry1Ab	80 °C, 10 min	(de Luis et al., 2009)
Cry1F	75 °C, 30 min	(Baktavachalam et al., 2015)

95 °C for 15 min, the relative EC₅₀ values of SIP2 Cry1A.105, maize Cry1A.105 and Cry1Ac were reduced to < 3%, < 19% and < 2% of initial activity, respectively.

These results are consistent with those obtained from previous studies on Cry1Ab and Cry1F ([Table 5](#)) showing heat lability at temperatures ≥ 80 °C. Thus, the chimeric Cry1A.105 protein and its donor proteins, like the majority of dietary proteins, are denatured by temperatures commonly encountered during food processing and cooking.

3.6. Both Cry1A.105 and its donor proteins are readily degraded by gastrointestinal proteases

Most dietary proteins are rapidly degraded by digestive proteases to aid in the absorption of nutritional amino acids, which consequently minimizes exposure to intact proteins following consumption ([Hammond et al., 2013](#); [Kier and Petrick, 2008](#)). Assessing susceptibility of a protein in a GM crop to proteolytic degradation, therefore, is a key study towards establishing the weight of evidence for safety of a protein ([Codex Alimentarius, 2009](#)). To this end, the intact SIP2 Cry1A.105 protein was incubated with pepsin at 37 ± 2 °C and assessed for intactness by SDS-PAGE at specific defined time intervals ([Fig. 3](#)). The results indicate that the intact, full-length SIP2 Cry1A.105 protein (~130 kDa) was degraded to below the LOD of the Coomassie-stained SDS-PAGE gel after 0.5 min. This represented > 98.7% of the initial protein sample. A band of ~60 kDa was visible at 0.5 min and disappeared at 2 min, and a band of ~5 kDa presented at 20 min but disappeared by 30 min ([Fig. 3A](#)). There was no change in the protein banding pattern for the protein when incubated at 37 °C in the absence of pepsin, indicating that the observed protein degradation was a direct result of the proteolytic activity of pepsin and not due to instability of the protein when incubated at 37 °C ([Fig. 3A](#), Lanes 3 and 12). Additionally, there was no change in the protein band corresponding to pepsin (~38 kDa) when incubated at 37 °C in the absence of SIP2 Cry1A.105, indicating that pepsin was stable at 37 °C over the course of the experiment ([Fig. 3A](#), Lanes 2 and 13).

To better understand the fate of the transiently stable fragment, an assessment of the susceptibility of this fragment to digestion by pancreatin was conducted ([Fig. 3B](#)). After SIP2 Cry1A.105 treatment with pepsin for 2 min, the reaction was quenched and the transiently stable ~5 kDa fragment was exposed to pancreatin. In the pepsin–pancreatin

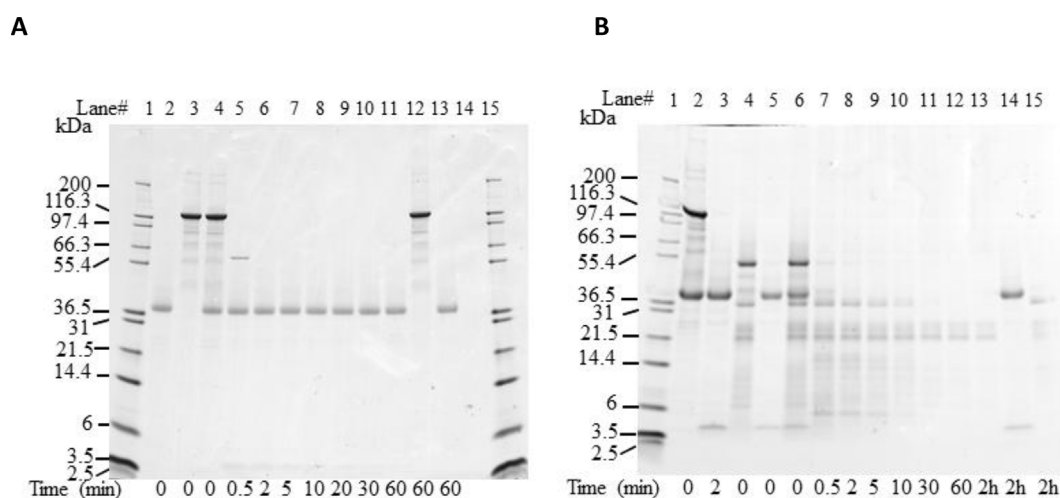


Fig. 3. SDS-PAGE analysis of SIP2 Cry1A.105 digestion.

Panel A corresponds to the SIP2 Cry1A.105 protein digestion by pepsin. Based on pre-digestion protein concentrations, 1 μ g SIP2 Cry1A.105 protein was loaded in lanes containing SIP2 Cry1A.105 protein. The incubation times are indicated. Lanes 1 & 15: molecular weight markers (values shown at left of image indicate molecular weight), lanes 2 & 13: pepsin only, lanes 3 & 12: SIP2 Cry1A.105 only, lanes 4 to 11: a time course of pepsin digestion. **Panel B:** After digestion of the SIP2 Cry1A.105 protein by pepsin for 2 min, the reaction was quenched and the transiently stable SIP2 Cry1A.105 protein fragment was exposed to further digestion by pancreatin. Based on protein concentrations before digestion by pepsin, 1 μ g of total protein was loaded per lane containing SIP2 Cry1A.105 protein. Lanes 1: molecular weight markers (values shown at left of image indicate molecular weight), lane 2: pepsin reaction mixture, lane 3: 2 min after digestion of SIP2 Cry1A.105 by pepsin, lanes 4 & 15: pancreatin mixture only, lanes 5 & 14: no pancreatin added to reaction, lanes 6 to 13: a time course of pancreatin digestion. 2h: 2 h.

sequential digestion, the band corresponding to full-length protein was no longer visible after 2 min digestion in pepsin, as expected from the pepsin-only assay (Fig. 3A). Furthermore, the ~5 kDa band present transiently on the SDS-PAGE gel in samples digested with pepsin alone was no longer visible after 0.5 min digestion in pancreatin (Fig. 3B).

The Cry1Ac protein displayed a similar digestive fate as Cry1A.105, that is the intact, full-length Cry1Ac protein was rapidly digested by pepsin and a transient stable fragment (~5 kDa) from pepsin digestion no longer existed after 0.5 min sequential digestion in pancreatin. The other Cry1A.105 donor proteins, Cry1Ab and Cry1F, have also been shown to be susceptible to pepsin degradation (Table 6) (Baktavachalam et al., 2015; Guimaraes et al., 2010; Hammond, 2016). Thus, the chimeric Cry1A.105 protein and its donor proteins possess similar susceptibility to gastrointestinal proteases.

3.7. Cry1A.105 and its donor proteins show no observable acute toxicity in mice, even at limit dosage levels

An acute toxicity study was conducted with the Cry1A.105 protein to provide further assurance that the protein does not present a hazard when consumed. However, this higher tier hazard assessment was not scientifically warranted because the previously described structural, bioinformatic and stability assessments (tier 1 weight of the evidence as defined by (Delaney et al., 2008)) indicated a lack of apparent hazards. The test group of mice were dosed at 2,451 mg SIP2 Cry1A.105/kg body weight, a physiologically unrealistic dose, given that similar exposure to humans would require a 70 kg person to consume approximately 70,000 kg of soybean grain (2.4 ppm expression) in a single day.

Table 6
Summary of Cry1A.105 donor protein susceptibility to pepsin.

Test Protein	Time (min) to complete the digestion of a full-length protein	Reference
Cry1Ab	0.5	(Guimaraes et al., 2010)
Cry1Ac	0.5	(Betz et al., 2000)
Cry1F	< 1	(Baktavachalam et al., 2015)
Maize Cry1A.105	0.5	(U.S. EPA, 2010a)

Dietary consumption of the grain containing Cry1A.105 is irrelevant from a food consumption context due to the low level of expression in grain. Therefore, the acute exposure in mice represents an extreme exposure condition relative to food and feed safety. BSA was administered in the vehicle at a dose level comparable to the Cry1A.105 dose level (2,584 mg/kg) to serve as a control group. This BSA control group accounts for any potential impacts of the high dose bolus administration of protein in this study. All animals were healthy, and no mortality or Cry1A.105-related clinical signs were observed. Cry1A.105 did not affect weekly body weights in males or females (Table 7), however, weekly body weight gains were lower in test males in the intervals from study days 0–7 and 0 to 14 but not days 7–14 (Table 8). These differences were not considered treatment related as: 1) body weight gain in the control males was at the high end of the normal range for this endpoint and 2) body weight gain of the test substance-treated males during the Day 0–7 and Day 7–14 intervals was within the normal range, as defined by historical control data at the Testing Facility (data not shown). Body weight gain differences were therefore attributed to biological variation. There was no impact of the test substance on food consumption (Table 9) and there were no abnormal gross necropsy findings present at study completion. Therefore, the SIP2 Cry1A.105 did not exhibit toxicity at 2451 mg/kg body weight, the highest dose level tested.

An acute toxicity assessment, like the assessment reported herein, can be used to assess protein toxicity because most known protein toxins exert toxicity through acute mechanisms (Pariza and Johnson, 2001; Sjoblad et al., 1992). There was no hypothesis of toxicity based on the prior safety testing of Cry1A.105 component domains, known safety of Cry proteins in general and the lack of similarity in sequence with known toxins and allergens. The study resulted in no adverse impacts on the treatment animals. These results are consistent with those obtained from previous studies on Cry1Ab, Cry1Ac, Cry1Ab/Ac fusion protein, Cry1F, and maize Cry1A.105 conducted at dose levels of 4,000, 5,000, > 5,000, 576, and 2,072 mg of protein/kg body weight, respectively as described in Table 10. These results support the conclusion that Cry1A.105, like its donor proteins, presents no hazard to humans or animals when consumed in food or feed.

Table 7
Summary of acute toxicity study body weights for SIP2 Cry1A.105.

Sex	Mean BW (g) $\pm$ SD Day 0		Mean BW (g) $\pm$ SD Day 7		Mean BW (g) $\pm$ SD Day 14	
	Cry1A.105	BSA	Cry1A.105	BSA	Cry1A.105	BSA
Males	32.2 $\pm$ 2.4	31.9 $\pm$ 1.8	33.9 $\pm$ 2.7	35.1 $\pm$ 2.6	35.4 $\pm$ 2.8	36.3 $\pm$ 2.7
Females	24.0 $\pm$ 1.7	23.9 $\pm$ 1.6	25.7 $\pm$ 1.6	26.3 $\pm$ 1.8	27.2 $\pm$ 2.0	27.5 $\pm$ 1.6

No statistically significant differences by ANOVA at $p < 0.05$. $N = 10$.

BW = body weight; BSA = Bovine Serum Albumin protein control; SD = Standard Deviation.

Table 8
Summary of acute toxicity study body weight changes for SIP2 Cry1A.105.

Sex	Mean BW Change (g) $\pm$ SD Days 0–7		Mean BW Change (g) $\pm$ SD Days 7–14		Mean BW Change (g) $\pm$ SD Days 0–14	
	Cry1A.105	BSA	Cry1A.105	BSA	Cry1A.105	BSA
Males	1.6 $\pm$ 0.6 ^a	3.2 $\pm$ 1.4	1.5 $\pm$ 0.6	1.2 $\pm$ 0.9	3.1 $\pm$ 0.6 ^a	4.4 $\pm$ 1.6
Females	1.7 $\pm$ 1.0	2.5 $\pm$ 0.9	1.5 $\pm$ 0.8	1.2 $\pm$ 0.9	3.2 $\pm$ 0.9	3.7 $\pm$ 1.3

BW = body weight; BSA = Bovine Serum Albumin protein control. SD = Standard Deviation.

^a Statistically significant difference by ANOVA with Dunnett's Test at $p < 0.05$. $N = 10$.

Table 9
Summary of food consumption data for SIP2 Cry1A.105 toxicology study.

Group	Mean Food Consumption $\pm$ SD Days 0–7 (grams/day)		Mean Food Consumption $\pm$ SD Days 7–14 (grams/day)	
	Cry1A.105	BSA	Cry1A.105	BSA
Males				
Mean $\pm$ SD	8.9 $\pm$ 2.0	10.6 $\pm$ 4.9	9.0 $\pm$ 2.1	9.2 $\pm$ 2.6
N	10	10	10	9
Females				
Mean $\pm$ SD	6.6 $\pm$ 1.0	7.1 $\pm$ 1.5	6.7 $\pm$ 1.2	6.9 $\pm$ 0.9
N	10	10	10	9

No statistically significant differences by ANOVA at $p < 0.05$. $N = 10$.

$N = 10$, some cage data not collected due to excessive food spillage. SD = Standard Deviation.

Table 10
Summary of Cry1A.105 donor protein toxicity data.

Test Protein	Acute Non-Toxic Dose Level in Mice	Reference
Cry1Ab	4000 mg/kg	(Betz et al., 2000)
Cry1Ac	5000 mg/kg	(Betz et al., 2000)
Cry1Ac/Cry1Ab Fusion Protein	5000 mg/kg	(Xu et al., 2009)
Cry1F	576 mg/kg	(U.S. EPA, 2010b)
Maize Cry1A.105	2072 mg/kg	(U.S. EPA, 2010a)
SIP2 Cry1A.105	2451 mg/kg	Present Study

4. Conclusions

The safety of the Cry1A.105 protein itself has already been demonstrated and established in the currently approved and widely cultivated commercial GM maize products YieldGard VT PRO[®] and Genuity[®] SmartStax[®] (Head et al., 2014). These products have been safely cultivated on millions of acres across nine countries since their commercial introduction in 2009. Homologous proteins share related amino acid sequences and three-dimensional structures, as well as common function (Hammond et al., 2013). A transgenic protein that is homologous to proteins with a HOSU could be considered to be “as-safe-as” those that have a HOSU (Hammond et al., 2013). Because the Cry1A.105 has an established HOSU, a comprehensive safety assessment should not be needed to address the safety of SIP2 Cry1A.105,

which only differs by four amino acids from the safely consumed maize Cry1A.105. This facet is further supported by evidence that this minor amino acid difference does not interfere with the protein's functional activity. However, to meet the global requirements from regulatory agencies, the SIP2 Cry1A.105 protein was characterized and assessed using a weight of evidence approach (Codex Alimentarius, 2009; Delaney et al., 2008; Hammond et al., 2013; Raybould et al., 2013) as presented in this report.

Evidence for the safety of SIP2 Cry1A.105 was generated. As expected, those comprehensive safety assessments did not provide any more scientifically meaningful data than what we already knew about the safety of Cry1A.105. These results were also compared side by side to that of its donor proteins on multiple levels including, 1) bioinformatics analyses comparing the similarity of Cry1A.105 and its donor proteins to known allergens and toxins, 2) structural, functional and physiochemical characterization of Cry1A.105 and its donor proteins, 3) an assessment of the stability of Cry1A.105 and its donor proteins upon exposure to digestive enzymes and elevated temperatures encountered during processing and cooking, and 4) acute toxicity testing of the Cry1A.105 protein comparing the data to that of its donor proteins. The results from these analyses echo the observed structural homology between the tryptic core domains of Cry1A.105 and Cry1Ac. These results also are comparable to similar studies conducted with each of the donor proteins that contributed domains to the chimeric Cry1A.105 protein. Thus, the domain exchange strategy employed to generate the chimeric Cry1A.105 protein did not make non-toxic protein domains into a toxic protein from a mammalian safety perspective,

nor did these exchanges alter safety properties of this chimeric protein unexpectedly beyond that of the respective donor proteins. This comparison supports a central facet in evaluating the safety of a chimeric protein, which is that the HOSU and safety evaluation of a chimeric protein's donor protein(s) should be considered as an integral part of a weight of evidence based protein safety assessment. Bioinformatics analysis helps to identify homology to other proteins or functional domains, as well as whether the protein itself has characteristics indicative of potential toxicity or allergenicity. In case where a protein has a strong HOSU and the safety of its functional domains has been rigorously evaluated, a limited safety assessment that focuses on bioinformatics and structural/functional analyses would be sufficient for safety assessment of the homologous protein. Further characterization or toxicity testing of the protein beyond this preliminary assessment would only be confirmatory. Finally, this study provides an example validating the safety of a domain exchange strategy to develop new proteins for improvement of agricultural productivity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.yrtph.2018.09.003>.

Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.yrtph.2018.09.003>.

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