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Safety assessment of coleopteran active IPD072Aa protein from *Psuedomonas chlororaphis*

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ABSTRACT

The *ipd072Aa* gene from *Pseudomonas chlororaphis* encodes the IPD072Aa protein which confers protection against certain coleopteran pests when expressed in genetically modified (GM) plants. A weight of evidence approach was used to assess the safety of the IPD072Aa protein. This approach considered the history of safe use of the source organism and bioinformatic comparison of the protein sequence with known allergenic and toxic proteins. The IPD072Aa protein was assessed for resistance to degradation in the presence of simulated gastric fluid containing pepsin as well as heat stability. There was no hazard identified with the IPD072Aa protein. Furthermore, an acute oral toxicity study found no evidence of adverse effects. Collectively, these studies support the human health safety assessment of the IPD072Aa protein.

1. Introduction

Western corn rootworms (WCR) (*Diabrotica virgifera virgifera* LeConte) are a particularly destructive insect pest of maize, and it has been estimated that WCR are responsible for greater than a billion dollars of yield loss and control costs annually (Andow et al., 2016). Historically, chemical pesticides including pyrethroids, organophosphates, and neonicotinoids were used to control WCR and other coleopteran insects (Ritter and Richter, 2013). In 2003, corn that was genetically modified to express the coleopteran-specific pesticidal crystalline (Cry) protein Cry3Bb1 from *Bacillus thuringiensis* (*Bt*) was commercialized offering an alternative approach for controlling insect damage (Vaughn et al., 2005). Since that time, additional genetically modified (GM) maize crops expressing different coleopteran specific proteins including mCry3A, eCry3.1Ab, and Cry34Ab1/Cry35Ab1 have also been commercialized (Frank et al., 2013; Narva et al., 2013). While these GM traits have protected yield in maize fields under WCR pressure (Fernandez-Cornejo et al., 2014), populations of *Bt*-resistant WCR populations have recently been identified in several Midwestern states (Gassmann et al., 2014; Zukoff et al., 2016). Further, WCR have a well-established history of developing resistance to multiple pest management strategies including crop rotations (Gray et al., 2009) and other pesticides (Meinke et al., 1998). These observations highlight the need to identify proteins with new modes of action against WCR. One such newly identified insecticidal protein is IPD072Aa, which was isolated from *Pseudomonas chlororaphis*, demonstrates selective insecticidal activity toward WCR when expressed in corn (Schellenberger et al., 2016). The approximately 10 kDa protein also demonstrated insecticidal activity toward WCR populations that are resistant to the *Bt* proteins mCry3A and/or Cry34Ab1/Cry35Ab1 (Schellenberger et al., 2016).

Multi-tiered weight-of-evidence testing paradigms are used to assess the safety of newly expressed proteins in GM crops (Delaney et al., 2008a; EFSA, 2006; FAO/WHO, 2001; Hammond et al., 2013). Among other considerations in the first tier (i.e., hazard identification framework) are the history of safe use (HOSU) of the source organism, mode of action and/or selectivity of the protein, sequence similarity to known toxic and allergenic proteins, and *in vitro* studies to determine if the protein is sensitive to degradation in the presence of digestive enzymes. If no evidence of hazard was identified from Tier I testing, then toxicology studies with laboratory animals for hazard characterization (i.e., Tier II) may not be justified (Delaney et al., 2008a). Nevertheless, acute toxicology studies with purified proteins often have been conducted because they are required by some global regulatory agencies (Mendelsohn et al., 2003). Previous studies have reported the application of the testing methods to establish the safety of numerous

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proteins expressed in GM crops (Cao et al., 2010, 2012; Delaney et al., 2008b; Herouet-Guicheney et al., 2009; Hérouet et al., 2005; Juberg et al., 2009; Mathesius et al., 2009; Stagg et al., 2012; Xu et al., 2009). The comprehensive results of safety testing that was conducted with the IPD072Aa protein are reported in this paper.

2. Materials and methods

2.1. Microbially derived IPD072Aa protein: production and purification

The IPD072Aa protein was expressed in an *Escherichia coli* protein expression system as a fusion protein with an N-terminal His tag. The fusion tagged protein was purified using Ni-NTA affinity chromatography. The His tag was cleaved with immobilized trypsin and then removed using Ni-NTA affinity chromatography. The IPD072Aa protein was further purified by anion exchange chromatography followed by buffer exchange to 50 mM ammonium bicarbonate and lyophilization.

2.2. Purified microbially derived IPD072Aa protein: characterization

Characterization of the purified IPD072Aa protein included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis, amino acid composition analysis, mass determination and peptide mapping by mass spectrometry, N-terminal sequencing, glycoprotein staining, bioactivity analysis, and endotoxin determination. For SDS-PAGE, aliquots of the lyophilized IPD072Aa protein were solubilized in 1X LDS sample buffer, heated at 90–100 °C for 5 min prior to loading into 4–12% Bis-Tris gels. Each gel was also loaded with pre-stained molecular weight markers (Precision Plus Protein Dual Xtra Standards). Electrophoreses were performed using the Mini-Cell Electrophoresis System. Gels intended for Coomassie staining were washed, stained with Gelcode Blue Stain Reagent and then destained. The resulting gel-images were captured electronically and the purity of IPD072Aa protein was determined by densitometry. For Western blot analyses, protein transfer from a SDS-PAGE gel to a nitrocellulose membrane was performed using the iBlot Gel Transfer device. After transfer, wash, and blocking the membrane was sequentially incubated with an IPD072Aa-specific polyclonal antibody, a secondary antibody conjugated with horseradish peroxidase and a chemiluminescent substrate. The image of the chemiluminescent signal was captured electronically along with the pre-stained markers. For protein glycosylation analysis, a gel from SDS-PAGE was washed with ultrapure water (hereafter referred to as water), fixed with 50% methanol, and washed with 3% acetic acid. The gel was incubated with oxidizing solution, washed with 3% acetic acid, and incubated with the glycoprotein staining reagent. After staining, the gel was incubated in a reducing agent, washed with 3% acetic acid, and rinsed with water. To visualize all protein bands, the same gel was then stained with GelCode Blue and washed with water. Gel images were captured electronically both immediately before and after the GelCode Blue staining step.

For amino acid analysis, aliquots of the lyophilized IPD072Aa protein samples were solubilized in water and subjected to acid hydrolysis under reduced pressure in an argon atmosphere. Individual amino acid analysis was then conducted using ion exchange chromatography, post-column derivatization with ninhydrin, and quantification with a BioChrom 30 amino acid analyzer. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) was used for intact protein molecular weight determination. The IPD072Aa protein was solubilized in 0.5 mM ammonium acetate and 3% methanol. The sample was purified using Millipore Ziptips, mixed with matrix, and analyzed with Bruker Autoflex Speed MALDI TOF/TOF MS in linear mode using Compass 1.4 control and processing software. For peptide mapping, an IPD072Aa protein band was excised from a Coomassie stained SDS-PAGE gel and carbamidomethylated followed by digestion with trypsin and chymotrypsin. Analyses of peptides were performed on a Bruker Autoflex Speed MALDI TOF/TOF mass spectrometer. Mascot software was used to search for the matching peptides with the protein sequence.

The IPD072Aa protein biological activity was evaluated by conducting a 7-day WCR bioassay. The bioassay used a randomized complete block design containing four blocks each of which consisted of a 24-well tissue culture plate containing eight wells of each artificial insect diet. WCR neonates were exposed to three artificial insect diets: control diet containing a dosing solution of water, test diet containing 100 ng IPD072Aa protein/mg diet wet weight, and positive control diet containing 4285 ng boric acid/mg diet wet weight. WCR eggs were incubated in an environmental chamber and neonate larvae were infested within 24 h of hatch. On bioassay day 0, test, control, and positive control dosing solutions were prepared and combined with a dry carrier. Approximately 300 µl of freshly prepared diet was dispensed into individual wells and infested with a neonate WCR larva. Each plate was sealed with a ventilated lid. The infested plates were then placed in a dark environmental chamber at 21 °C and 65% relative humidity. On bioassay day 3, new tissue culture plates were prepared using the day 0 procedures, living WCR larvae were transferred to the new plates, and the plates were returned to the dark environmental chamber. After 7 days, mortality was assessed and surviving organisms were individually weighed.

Endotoxin content of the lyophilized IPD072Aa protein test was determined using a gel clot assay (Limulus Amebocyte Lysate Endosafe diagnostic kit, Charles River Laboratories, Charleston, South Carolina).

2.3. Bioinformatics: in silico comparison of IPD072Aa protein to known and putative allergen sequences in the COMPARE database and to an internal toxin database

An *in silico* assessment of potential cross-reactivity with known or putative allergens and IPD072Aa protein was performed according to well-established guidelines (Codex Alimentarius Commission, 2003; FAO/WHO, 2001). Two individual searches were conducted using the peer reviewed Comprehensive Protein Allergen Resource (COMPARE) 2017 database (updated February 2017), which was created through a collaborative effort of the Health and Environmental Sciences Institute (HESI) Protein Allergens, Toxins and Bioinformatics Committee (PATB) and is available at http://comparedatabase.org. The first search, which used the IPD072Aa protein sequence as a query in a FASTA v35.4.4 (Pearson and Lipman, 1988) search against the COMPARE database allergen sequences, used default parameters except the *E*-score threshold was set to 10^{-4}. An *E*-score threshold value set to 10^{-4} minimizes the number of false positive results without any increase in false negatives and is considered an appropriate setting for these types of searches (Mirsky et al., 2013; Silvanovich et al., 2009). Any resulting alignment was assessed for ≥35% sequence identity with 80 residues or greater (Codex Alimentarius Commission, 2009). The second search used a Corteva Agriscience™ developed Perl script to find contiguous 8-residue matches between the IPD072Aa protein sequence and the COMPARE database allergen sequences.

The potential toxicity of IPD072Aa protein was assessed *in silico* by comparing its sequence with those in the Corteva Agriscience™ toxin database that is comprised of a subset of sequences found in the UniProtKB/Swiss-Prot (http://www.uniprot.org) database. To produce this database, the manually annotated proteins from UniProtKB/Swiss-Prot database were evaluated for function by searching for keywords that could imply toxic or adverse health effects (e.g. toxin, hemagglutinin, vasoactive, etc.). The IPD072Aa protein sequence comparison was performed with BLASTP 2.2.26 using default parameters with the exception of the *E*-value (expectation value) set to 10^{-4}, turning off the low complexity filter, and returning unlimited alignments. Any alignments produced from this search were then reviewed to determine its toxic effect potential.

2.4. Digestibility assay: characterization of in vitro pepsin resistance of IPD072Aa protein

The *in vitro* digestion of the IPD072Aa protein in simulated gastric fluid (SGF) containing pepsin was conducted with a similar time course and formulations described in the multi-laboratory evaluation (Thomas et al., 2004). Pepsin digestion solution was prepared on the day of use by solubilizing pepsin (Sigma Aldrich, St. Louis, Missouri) in the gastric control solution (G-Con, Ricca Chemical Company, Arlington, Texas) for a final concentration of 2500 pepsin activity units per microgram test or control protein in the final digestion mixture. The lyophilized IPD072Aa protein powder and control proteins (BSA, β-lactoglobulin) were solubilized in water to a target concentration of 5 mg/ml. The IPD072Aa protein digestion reaction mixture was prepared by mixing 1900 µl SGF solution with 100 µl IPD072Aa protein stock solution. The IPD072Aa protein digestion reaction mixture was mixed continuously throughout the sampling period and a sub-sample was taken from the vial at several time points (± 10 s): 0.5, 1, 2, 5, 10, 20, 30, and 60 min. Sub-samples were neutralized with a pre-mixed neutralizing solution and heated for 5 min at 90–100 °C. Several control digestion reactions were run: SGF alone (no IPD072Aa protein for 60 min), BSA in SGF (1 min and 60 min), β-lactoglobulin in SGF (1 min and 60 min), IPD072Aa in water (no SGF for 60 min), and IPD072Aa in gastric control solution (no pepsin for 60 min). The IPD072Aa SGF digestion time-course samples were analyzed by SDS-PAGE/Coomassie staining and Western blot. The control digestion samples were analyzed by SDS-PAGE and Coomassie staining.

2.5. Acute oral toxicity: IPD072Aa protein in mice

A 14-day acute oral toxicity study with the microbially derived IPD072Aa protein was conducted. Study design was based on OECD, Section 4 (Part 423): Acute Oral Toxicity – Acute Toxic Class Method, *Guideline for the Testing of Chemicals* (2001) with exception of the number of animals, the addition of the vehicle control and BSA control groups, and a male test system group (OECD, 2001). A single 2000 mg/kg, purity adjusted dose of IPD0720Aa protein was administered to six male and six female Crl:CD1(ICR) mice (Charles River Laboratories International, Inc., Raleigh, North Carolina) via oral gavage. Two additional groups of six male and six female mice were dosed via oral gavage with either the vehicle control (deionized water) or 2000 mg/kg BSA control. The mice were fasted 4 h prior to and throughout the dosing procedure. Body weights were collected on test days 1 (both prefast and shortly prior to dosing), 2, 3, 5, 8, and 15. Clinical observations were made prior to fasting on test day 1, prior to dosing, approximately 30 min after dosing, approximately 2 h after dosing, and once daily thereafter, along with twice daily mortality/moribundity checks. All animals were subjected to a gross pathological examination at the end of the in-life phase.

2.6. Bioassay: biological activity of heat-treated IPD072Aa protein

WCR larvae (< 24-hr old) were exposed to diet containing an unheated IPD072Aa protein dosing solution, diet containing IPD072Aa protein dosing solutions that had been incubated for 30–35 min at 25, 50, 60, or 95 °C, and a test diet containing an IPD072Aa protein dosing solution autoclaved at 121 °C. WCR bioassay methodology followed that described above except WCR were moved to plates containing freshly prepared diet on bioassay day 4.

3. Results

3.1. Characterization of the microbially derived IPD072Aa protein

SDS-PAGE analysis confirmed that the IPD072Aa protein migrated as a predominant band consistent with the expected molecular weight of approximately 10 kDa (Fig. 1). The purity of the microbially derived IPD072a was determined to be > 95% on a total protein basis.

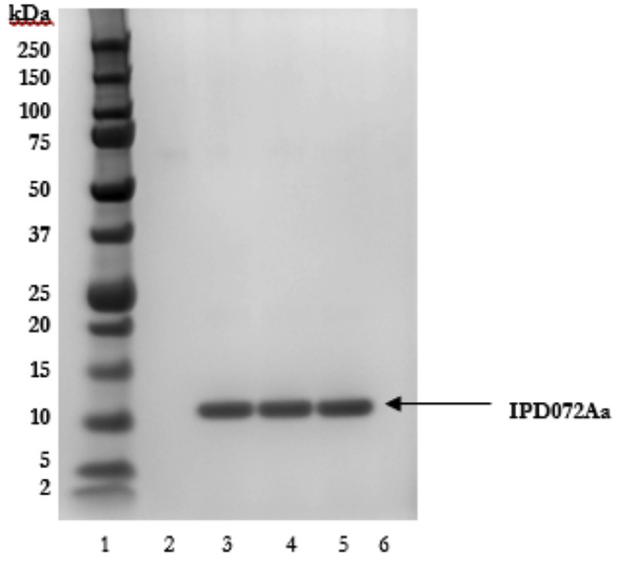

Fig. 1. Characterization of the IPD072Aa protein by SDS-PAGE analysis. Lane 1 is pre-stained molecular weight markers; lanes 2 and 6 are loaded with buffer only; lanes 3–5 are samples of the IPD072Aa protein.

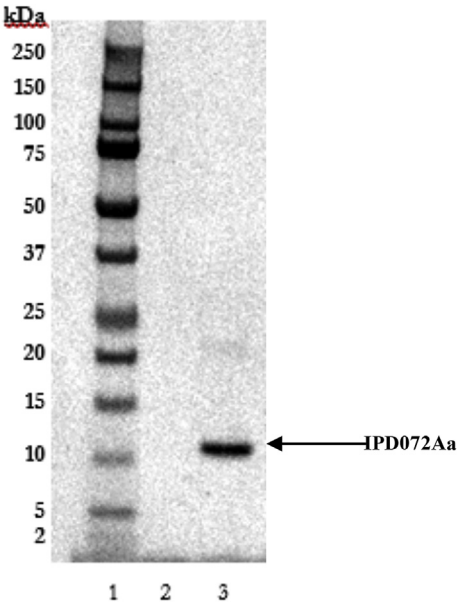

Fig. 2. Characterization of the IPD072Aa protein by Western blot analysis. Lane 1 is pre-stained molecular weight markers; lane 2 is buffer only; lane 3 is IPD072Aa protein.

Western blot analysis demonstrated the expected immunoreactivity for the IPD072Aa protein (Fig. 2). A predominant band was recognized by a specific polyclonal antibody with a size consistent with the expected molecular weight of approximately 10 kDa.

Protein glycosylation staining analysis indicated no glycosylation for the IPD072Aa protein derived from a genetically modified maize line (data not shown). Similarly, no glycosylation was detected for the microbially derived IPD072Aa protein (Fig. 3).

Amino acid composition analysis determined that the microbially derived IPD072Aa protein has a concentration of 0.82 mg of protein per mg of lyophilized powder. Molecular mass determination by MALDI-MS obtained a mass of 9549.8 Da which is consistent with the expected mass of 9548.9 Da based upon the protein sequence. The chymotrypsin-

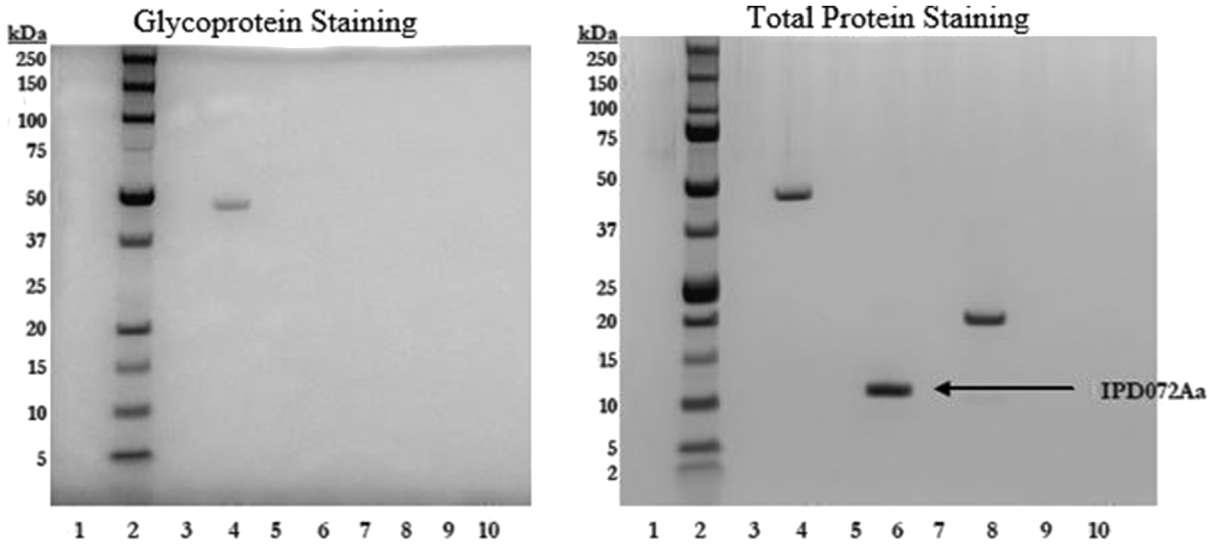

Fig. 3. Characterization of IPD072Aa protein by glycosylation staining. Lanes 1, 3, 5, 7, 9, and 10 are buffer only. Lane 2 is pre-stained molecular weight markers; Lane 4 is the positive control horseradish peroxidase (1 μg); Lane 6 is the microbially derived IPD072Aa protein (1 μg); Lane 8 is the negative glycosylation control soybean trypsin inhibitor (1 μg).

Table 1
Chymotryptic peptides of the IPD072Aa protein identified using MALDI-MS analysis.

IPD072Aa Amino Acid Residue Position	IPD072Aa Theoretical Peptide Mass [M + H]	IPD072Aa Observed Peptide Mass [M + H] (input)	Identified Peptide Sequence
1–30	3247.47	3247.33	HMGITVTNNSSNPIEVAINHWGSDGDTSFF
31–40	1105.52	1105.51	SVGNGKQETW
31–48	2025.93	2025.91	SVGNGKQETWDRSDSRGF
31–50	2238.08	2238.05	SVGNGKQETWDRSDSRGFVL
41–48	939.42	939.42	DRSDSRGF
41–50	1151.57	1151.56	DRSDSRGFVL
49–61	1454.80	1454.79	VLSLKKNGAQHPY
51–61	1242.65	1242.64	SLKKNGAQHPY
62–87	2873.49	2873.46	YVQASSKIEVDNNAVKDQGRLIEPLS

Note: alanine (A), arginine (R), asparagine (N), aspartic acid (D), glutamic acid (E), glutamine (Q), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V).

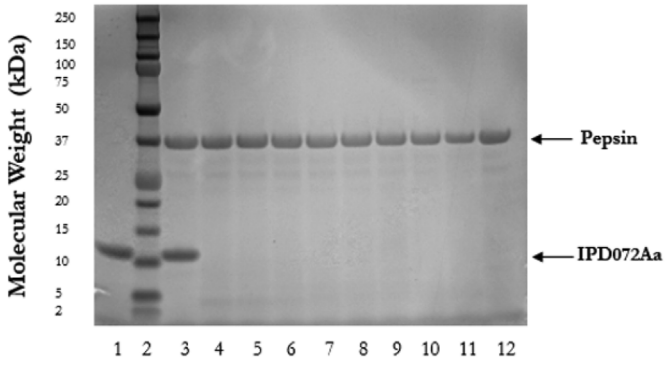

Fig. 4. Characterization of microbially derived IPD072Aa protein by digestion in SGF containing pepsin. Lane 1 is IPD072Aa in water, time 0; Lane 2 is pre-stained molecular weight markers; Lanes 3–11 are IPD072Aa in SGF for 0, 0.5, 1, 2, 5, 10, 20, 30, and 60 min, respectively; Lane 12 is SGF control (no IPD072Aa), 60 min.

digested IPD072Aa was analyzed with MALDI-MS and the identified matching peptides accounted for 100% of the expected IPD072Aa amino acid sequence (Table 1). N-terminal amino acid sequence analysis showed that the primary sequence was consistent with amino acid residues 1–10 of the expected IPD072Aa protein sequence.

The biological activity of the IPD072Aa protein was verified using a 7-day WCR bioassay. Mortality for the group fed a diet containing the microbially derived IPD072Aa protein was 90.3% compared with 18.8% for the group fed the bioassay control diet indicating activity on WCR.

3.2. Bioinformatics allergen and toxin comparisons

Results of the IPD072Aa protein sequence evaluation against the COMPARE database of known and putative allergens found no alignments that were 80 residues or longer with a sequence identity of ≥35% nor were there any contiguous 8-residue matches between the IPD072Aa protein sequence and the allergen sequences in the COMPARE database. No biologically relevant alignments were returned from the IPD072Aa protein sequence query against the Corteva Agriscience™ toxin database.

3.3. Assay: characterization of in vitro pepsin resistance of the IPD072Aa protein

The microbially derived IPD072Aa protein was rapidly digested in simulated gastric fluid (SGF). Both the Coomassie stained SDS-PAGE gel (Fig. 4.) and the Western blot analysis indicated that the IPD072Aa protein was not detectable within 0.5 min (Fig. 4). The BSA positive control was rapidly digested within 1 min and the β-lactoglobulin negative control persisted after 60 min in SGF as expected (data not

Table 2
Acute oral toxicity study with IPD072Aa protein: Mean mouse body weights (g + SD).

Days[a]	1	2	3	5	8	15
Males (n = 6)						
Vehicle	27.8 ± 2.2	28.7 ± 2.2	28.7 ± 2.1	29.6 ± 2.2	30.3 ± 2.6	32.4 ± 3.0
BSA[b]	28.0 ± 1.0	29.1 ± 1.0	29.2 ± 1.0	29.9 ± 0.9	30.2 ± 0.8	31.6 ± 1.1
IPD072Aa[b]	29.1 ± 2.3	30.3 ± 2.5	30.5 ± 2.5	30.2 ± 0.8	31.6 ± 1.1	33.9 ± 2.9
Females (n = 6)						
Vehicle	24.0 ± 1.1	24.8 ± 1.6	24.7 ± 1.5	25.2 ± 1.3	25.5 ± 1.3	27.2 ± 2.0
BSA[b]	24.1 ± 0.7	25.2 ± 1.0	25.4 ± 0.8	25.8 ± 0.9	25.6 ± 0.5	26.5 ± 0.8
IPD072Aa[b]	23.9 ± 1.0	25.4 ± 1.1	25.7 ± 1.0	25.3 ± 1.3	26.3 ± 1.1	26.5 ± 1.5

[a] Days Relative to Start Date.
[b] Dose: 2000 mg/kg.

shown).

3.4. Acute oral toxicity study with IPD072Aa protein

All animals survived to the scheduled sacrifice. There were no instances of mortality or gross lesions identified in any of the animals and no clinical abnormalities or overall body weight losses (days 1–15) observed in any of the animals during the study (Table 2). Under the conditions of this study, the oral LD50 of the microbially derived IPD072Aa protein in male and female mice was greater than 2000 mg/kg.

3.5. Biological activity of heat-treated IPD072Aa protein

Exposure of IPD072Aa protein to temperatures between 25 and 95 °C for 30–35 min had no significant effect on the bioactivity of the protein with WCR showing 83–90% mortality after 7 days in the heat-treated protein experiment. Autoclaving IPD072Aa protein at 121 °C, however, effectively eliminated biological activity to WCR (13.8% mortality) when compared with control diet (27.6% mortality).

4. Discussion

IPD072Aa protein from the source organism *Psuedomonas chlororaphis* has been identified as a new pesticidal tool to protect GM maize against WCR. Several *Pseudomonas* species, including *P. chlororaphis*, are ubiquitous in the environment and have a HOSU in agricultural food and feed crops as a biopesticide or as a gene source for certain GM crops (Anderson et al., 2018). Laboratory studies indicate that IPD072Aa protein disrupts the midgut epithelial cells of certain coleopteran insects and there is no cross-resistance with mCry3A and/or Cry34Ab1/Cry35Ab1 proteins (Boeckman et al., 2019; Schellenberger et al., 2016). Collectively, these results provide evidence that the source of the IPD072Aa protein, *P. chlororaphis,* has a HOSU, and that IPD072Aa protein displays a specific range of biological activity within the order Coleoptera. Following a weight of evidence approach to assess safety, bioinformatics analyses did not reveal any similarity between the IPD072Aa protein and any known allergenic protein or toxic proteins. Laboratory studies demonstrated that the IPD072Aa protein was rapidly degraded in SGF containing pepsin and was biologically active up to 95 °C but inactivated at the autoclave temperature of 121 °C. However, retention of biological activity after heat treatment is not necessarily predictive of potential allergenicity of a protein (Privalle et al., 2011). In addition, processing of maize into foodstuffs often involves extreme or harsh physical and environmental conditions that lead to loss of protein function or activity (Hammond and Jez, 2011). Finally, IPD072Aa protein produced no evidence of adverse effects following administration of 2000 mg/kg body weight/day to mice in an acute oral toxicity study. This hazard characterization (Tier II) study finding was expected since data from the hazard identification (Tier I) studies did not identify any hazard to investigate. The two-tiered approach was developed as a strategy to consider multiple factors for individual proteins rather than to subject them to guideline toxicology studies designed for chemical safety assessment (Delaney et al., 2008a). Support for this recommendation has been repeatedly confirmed for many proteins expressed in GM crops with no hazard identified and no evidence of adverse effects in acute oral toxicology studies (Cao et al., 2010, 2012; Delaney et al., 2008b; Juberg et al., 2009; Mathesius et al., 2009; Stagg et al., 2012; Xu et al., 2009). Collectively, these results suggest that default requirements from global regulatory agencies for acute oral toxicity studies may need to be updated.

5. Conclusions

This paper presents the results of a weight of evidence approach for a human health safety assessment for the coleopteran active IPD072Aa protein from *P. chlororaphis*. The source of this protein is not pathogenic and the IPD072Aa protein itself is unlikely to produce allergenic or toxic effects. Collectively these studies indicate that the IPD072Aa protein is unlikely to present a hazard to human health.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.04.055.

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