Food and Chemical Toxicology 166 (2022) 113187

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Safety assessment of the insecticidal protein IPD079Ea from the fern, *Ophioglossum pendulum*

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ARTICLE INFO

Handling Editor: Dr. Jose Luis Domingo

Keywords:
Biotechnology
Ophioglossum pendulum
IPD079Ea
PIP
Insecticide
Western corn rootworm

ABSTRACT

As agricultural biotechnology continues to develop solutions for addressing crop pests through newly expressed proteins from novel source organisms, with different modes or sites of action and/or different spectra of activity, the safety of these proteins will be assessed. The results of hazard-identification and characterization studies for the insecticidal protein IPD079Ea, which is derived from a fern (*Ophioglossum pendulum*) and active against the maize pest western corn rootworm (*Diabrotica virgifera virgifera*, Coleoptera: Chrysomelidae) are provided. Collectively these results indicate that IPD079Ea is unlikely to present a hazard to human or animal health and support the safety of genetically modified maize expressing IPD079Ea.

1. Introduction

Regulatory agencies require certain studies and data as a part of the weight-of-evidence approach to support the safety assessment of newly expressed proteins in genetically modified (GM) crops. The newly expressed protein weight-of-evidence studies use well-characterized protein lots to evaluate digestibility in simulated gastric and/or intestinal fluid assays, post-translational modifications such as glycosylation, the potential for acute toxicity in 14-day mouse studies, and depending on the nature of the protein, biological activity after heat treatment (Brune et al., 2021; Carlson et al., 2019; Delaney et al., 2008b; Mathesius et al., 2009). Corresponding protein sequences are used to bioinformatically evaluate their potential for allergenicity and toxicity based on similarity to known allergen and toxin sequences (Brune et al., 2021; Alimentarius Commission, 2009; Delaney et al., 2008a; Ladics et al., 2011; McClain et al., 2021; Roper et al., 2021). Although not all the information required by regulatory agencies is needed to assess safety, a subset of this information is still useful (Brune et al., 2021; McClain et al., 2021; Roper et al., 2021; Waters et al., 2021). While these studies may be routine in nature, it is important to continue to supplement the peer-reviewed literature with evidence that proteins introduced into GM crops are safe, especially as the agricultural biotechnology industry continues to identify active proteins from alternative sources, with different modes or sites of action, and/or different spectra of activity.

One such target for active proteins is novel control of the significant maize pest western corn rootworm (WCR; *Diabrotica virgifera virgifera*). The root damage caused by larvae of WCR can lead to significant yield losses in commercial maize, therefore different sites or modes of action other than current plant incorporated pesticide (PIP) actives such as those developed from *Bacillus thuringensis* (*Bt*) to which WCR has developed resistance will be needed to manage this pest (Barry et al., 2022; Carlson et al., 2019). IPD079Ea, a protein derived from a fern (*Ophioglossum pendulum* or *O. pendulum*), contains a Membrane Attack Complex/Perforin and Cholesterol-Dependent Cytolysin domain (MACPF/CDC). The MACPF/CDC proteins are widespread in nature and are found across multiple kingdoms including Eukaryotes and Bacteria (Anderluh et al., 2014; Crickmore et al., 2022). In plants, MACPF proteins are understood to play a role in development and response to environmental stresses (Yu et al., 2020). IPD079Ea has demonstrated WCR protection in GM maize including WCR strains resistant to insecticidal proteins derived from *Bt*. This activity is likely due to a different binding site in the insect midgut epithelium but occurs via a similar pore

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https://doi.org/10.1016/j.fct.2022.113187
Received 6 January 2022; Received in revised form 5 May 2022; Accepted 25 May 2022
Available online 7 June 2022

formation mode of action (Barry et al., 2022). From a functional perspective IPD079Ea has a narrow spectrum of activity with specificity to certain coleopteran pests and related insects, with the highest activity against WCR (Boeckman et al., 2022). The results of hazard-identification and characterization studies conducted for the new insecticidal protein IPD079Ea are described herein.

2. Materials and methods

2.1. Production and characterization of purified microbially derived IPD079Ea

IPD079Ea was expressed in an *Escherichia coli* system as a fusion protein with an N-terminal His tag and was purified using Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography. The fusion tag was cleaved with thrombin, which was then removed using heparin Sepharose column chromatography. The buffer was exchanged to 50 mM ammonium bicarbonate by tangential flow filtration and the protein was lyophilized. Characterization of the microbially derived IPD079Ea included amino acid composition analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, protein glycosylation analysis, peptide mapping and mass determination by mass spectrometry (MS), N-terminal amino acid sequencing, and a bioactivity assay.

2.1.1. Amino acid composition analysis and molecular mass determination

Lyophilized microbially derived IPD079Ea samples were first solubilized in 70% formic acid, then hydrolyzed with a final concentration of 6N hydrochloric acid under argon at approximately 110 °C for 24 h. Hydrolyzed samples were diluted and mixed with isotopically labeled amino acid internal standards at a final concentration of 0.5 μg/ml. Amino acid calibration solutions were prepared at concentration levels ranging from 25 ng/ml to 2.5 μg/ml of amino acids and contained 0.5 μg/ml each of isotopically labeled amino acid. Diluted hydrolysate samples and calibration solutions were separated by gradient elution on an ACQUITY UPLC (ultra-performance liquid chromatography) (Waters Corporation; Milford, MA) fitted with an Acquity UPLC HSS C18 T3 1.8 μm Column (Waters Corporation; Milford, MA). Eluent from the column was directed into an electrospray source on a Xevo TQ (Waters Corporation; Milford, MA) mass spectrometer operating in positive mode. Multiple Reaction Monitoring transitions were collected for each of the following non-labeled amino acids and the isotopically labeled internal standards. Quantitation was performed using QuanLynx (Version 4.1) software (Waters Corporation; Milford, MA). The ratio of each non-labeled amino acid peak area to the labeled internal standard area was calculated, and each amino acid concentration (ng/ml) was determined using linear regression. Individual amino acid molar amounts were calculated by multiplying the concentration by the dilution factor then dividing by the amino acid molecular weight. Based on the frequency (number of residues) of each amino acid in the protein sequence, the molar amount of each amino acid was used to calculate the protein concentration in each sample. For each sample, the two highest and lowest concentration values were discarded, and then any value generated by an amino acid that was not represented in each sample was discarded. The average of the remaining concentration values was calculated to determine the protein concentration (nmol) in each subsample. Results were converted to μg/μl by multiplying by the protein molecular weight and then dividing by the sample volume. Sample concentration values in μg/μl were converted to mg of protein per mg of lyophilized powder by multiplying by the sample volume and dividing by the sample weight. Sample values were averaged to calculate the IPD079Ea concentration (mg/mg) in the lyophilized test substance. For molecular mass determination, a lyophilized microbially derived IPD079Ea sample was solubilized in 10 ml of a 2% acetonitrile/0.1% formic acid buffer, further diluted, and analyzed with UPLC-MS. The diluted sample was separated by gradient elution on an ACQUITY UPLC fitted with an Acquity UPLC BEH C4, 300 Å, 1.7 μm Column (Waters Corporation; Milford, MA). Eluent from the column was directed into an electrospray source, on a TripleTOF 5600+ (AB Sciex, currently Sciex; Framingham, MA) MS operating in positive mode. Ions were collected from 500 to 4000 Da and the resulting data were processed using BioPharmaView (version 2.1) (Sciex; Farmingham, MA) to produce an intact protein molecular weight.

2.1.2. SDS-PAGE, western blot, and glycosylation analysis

Microbially derived IPD079Ea sample preparation for SDS-PAGE (Laemmli, 1970) involved solubilization in 1X LDS sample buffer, heating, and then loading on 4–12% Bis-Tris gels, which were also loaded with pre-stained molecular weight markers (Precision Plus Protein Dual Xtra Standards). Upon completion of electrophoresis using a Mini-Cell Electrophoresis System, the gels were removed and used for Coomassie staining, western blot analysis, protein glycosylation analysis, or sample preparation for mass spectrometry of tryptic and chymotryptic peptides. For Coomassie staining, the gel was washed, stained with GelCode Blue Stain Reagent, and de-stained. The gel image was captured electronically, and imaging software was used for densitometry analysis to determine the microbially derived IPD079Ea purity. For western blot, following SDS-PAGE, an iBlot Gel Transfer Device was used to transfer the proteins to a nitrocellulose membrane. After transfer, the membrane was washed and blocked before a series of alternating washes and incubations: the first incubation used an IPD079Ea-specific monoclonal antibody; the second incubation used a secondary antibody conjugated with horseradish peroxidase; the final incubation used a chemiluminescent substrate. The chemiluminescent signal and the pre-stained markers were detected and captured using ChemiDoc MP Imaging System (Bio-Rad; Hercules, CA). The Pierce Glycoprotein Staining Kit was used to determine whether the microbially derived IPD079Ea protein was glycosylated. Following electrophoresis, the gel was washed with water, fixed with 50% methanol, and washed before and after incubation in an oxidizing solution with 3% acetic acid. The prepared gel was incubated with glycoprotein staining reagent followed by reducing reagent incubation, then washed with 3% acetic acid and rinsed in water. Glycoproteins, if present, were detected as magenta-colored bands on the gel. Following glycoprotein detection, the gel image was captured electronically. The same gel was then stained with GelCode Blue stain reagent, washed with water to reveal all protein bands and the resulting image captured electronically using ChemiDoc MP Imaging System (Bio-Rad; Hercules, CA).

2.1.3. MS analysis of tryptic and chymotryptic peptides

Following SDS-PAGE, Coomassie staining, and gel imaging, bands of IPD079Ea were excised from a gel, reduced with dithiothreitol, alkylated with iodoacetamide, and subsequently digested with trypsin or chymotrypsin. The digested samples were separated by gradient elution on an ACQUITY UPLC fitted with a Cortecs UPLC C18 1.6 μm Column (Waters Corporation; Milford, MA). Eluent from the column was directed into an electrospray source operating in positive mode on a TripleTOF 5600+ hybrid quadrupole-TOF mass spectrometer (MS) (AB Sciex; Farmingham, WA). The resulting MS data were processed using MS Data Converter (Beta 1.3) (AB Sciex; Farmingham, WA) to produce a peak list from which an MS/MS ion search (Mascot Software, version 2.6.1; Matrix Science; Boston, MA) was performed to match peptides from the expected IPD079Ea sequence (Perkins et al., 1999). The Mascot-generated peptide ion score threshold was >13 which indicated identity or extensive homology ($p < 0.05$). The combined sequence coverage was calculated with GPMAW software (version 12.1) (Lighthouse Data; Odsense, Denmark).

2.1.4. N-terminal sequencing

Following SDS-PAGE, the resulting gel was incubated in cathode buffer and a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell system was used to transfer the proteins from the gel to a prepared

Immobilon-P PVDF membrane. After protein transfer, the membrane was washed, stained with GelCode Blue stain reagent, and destained with water to reveal the protein bands. The bands of IPD079Ea were excised, and one band was analyzed using a Shimadzu PPSQ-51A (Shimadzu; Columbia, MD) sequencer. Ten cycles of Edman sequencing (Edman, 1950) were performed. During each cycle, the N-terminal amino acid was sequentially derivatized with phenylisothiocyanate, cleaved with trifluoracetic acid, and converted to phenylthiohydantoin amino acid which was identified through chromatography. LabSolutions Software (Shimadzu; Columbia, MD) was used to automatically identify the N-terminal sequence.

2.1.5. Biological activity assay

The biological activity of the microbially derived IPD079Ea was evaluated using a 7-day bioassay with WCR (*D. virgifera virgifera*) neonates (within 24 h of hatching). A generalized randomized block design containing three blocks, each consisting of a 24-well bioassay plate with 10 replicates of each treatment for a total of 30 replicates was used. Diets either containing 50 ng IPD079Ea/mg diet wet weight or the negative control diet containing ultrapure water, were dispensed into individual wells of 24-well bioassay plates and a single neonate was placed in each well containing diet. The plates were sealed with ventilated heat-sealing film and placed into an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. After 4 days, new bioassay plates were prepared with fresh diet and living WCR larvae were transferred to the new plates. Missing or dead larvae were recorded, and the freshly prepared plates were placed into the environmental chamber. After seven days, mortality was assessed, and surviving larvae were individually weighed.

2.2. In silico similarity assessment of IPD079Ea sequences to known or putative allergens and to a proprietary toxin database

2.2.1. Allergen similarity

Bioinformatics assessment of the IPD079Ea protein sequence for potential cross-reactivity with known or putative allergens was performed according to relevant guidelines (Alimentarius Commission, 2009). The two separate searches for the IPD079Ea sequence used the peer-reviewed Comprehensive Protein Allergen Resource (COMPARE) database which was updated in January 2020 and is maintained by the Protein Allergens, Toxins and Bioinformatics (PATB) Committee of the Health and Environmental Sciences Institute (HESI) (van Ree et al., 2021). A FASTA (v35.4.4; Pearson and Lipman, 1988) search queried the IPD079Ea sequence against the COMPARE database sequences using the default parameters except the *E*-score threshold was set to 10^{-4} which has been shown to minimize false positives without increasing false negatives (Mirsky et al., 2013; Silvanovich et al., 2009). This first search produced alignments which were then assessed to identify those $\geq$80 in length and with a sequence identity >35%. The second search used the FUZZPRO program (Emboss Package v6.4.0) to identify any contiguous 8-residue identical matches between the IPD079Ea sequence and the allergen sequences.

2.2.2. Toxin similarity

Bioinformatics assessment of the potential toxicity of IPD079Ea was performed by comparing its sequence with the sequences in the 2020 version of the Corteva Agriscience toxin database which is produced by filtering the proteins in UniProtKB/Swiss-Prot (https://www.uniprot.org) database for molecular function by using keywords that may indicate possible toxic or adverse health effects (e.g., toxin, vasoactive, hemagglutinin). A BLASTP 2.9.0+ search involved comparing the IPD079Ea sequence with the sequences in the database using default parameters except the *E*-value threshold was set to 10^{-4}, low-complexity filtering was turned off, and the number of returned alignments was set to "unlimited." Setting the *E*-value threshold to 10^{-4}, rather than a higher value, yields results more likely to indicate true homology (Pearson, 2000). The output was then evaluated to determine if any resulting alignments were suggestive of putative toxicity of IPD079Ea.

2.3. Characterization of microbially derived IPD079Ea digestibility through an in vitro pepsin resistance assay

In vitro digestion of microbially derived IPD079Ea in simulated gastric fluid (SGF) containing pepsin was conducted over a 60-min time course, and the conditions and digestion reactions were similar to those described previously (Thomas et al., 2004; Carlson et al., 2019). Briefly, the protein digestion reaction mixture for the microbially derived IPD079Ea was prepared by mixing 1895 μl SGF solution with 105 μl IPD079Ea stock solution.

2.4. Assessment of microbially derived IPD079Ea acute oral toxicity in mice

The potential toxicity of microbially derived IPD079Ea was evaluated by acute exposure of six male and six female Crl:CD1(ICR) mice (Charles River Laboratories International, Inc., Raleigh, NC) to 5000 mg/kg body weight IPD079Ea via oral gavage. The study was conducted in general accordance with the prescriptive Standard of the People's Republic of China (MOA, 2016), which requires large group sizes and inclusion of concurrent control groups, and exceeds the requirements of the Organisation for Economic Cooperation and Development for a limit test (OECD, 2001). The study was conducted in accordance with appropriate animal welfare guidance (NRC, 2011) and the study design and protocol were reviewed and approved by the Institutional Animal Care and Use Committee at the Test Facility (Haskell R&D Center an AAALAC-accredited test facility, Newark, DE). In consideration of dose volume limitations for mice, the IPD079Ea target dose (adjusted for the concentration of IPD079Ea in the lyophilized material) was achieved by administering the specified dose volume two times with 4 h between each dose. Concurrent control groups comprising six male and six female mice administered deionized water (vehicle control), and six male and six female mice administered 5000 mg/kg body weight of bovine serum albumin (BSA control) were dosed at an equivalent dose volume on a comparable schedule. All mice were fasted approximately 4 h prior to the first dose administration, and food was returned approximately 2 h after second dose administration. Individual body weights were recorded at the start of fasting, after approximately 4 h of fasting prior to administration of the first dose (test day 1), and on test days 2, 3, 5, 8, and 15 and day of sacrifice. All animals were observed for survival and moribundity twice daily, and clinical observations were recorded prior to fasting, prior to the administration of each dose, approximately 30 min and 2 h after each dose, and daily throughout the in-life phase. After a 2-week non-dosing observation period, all surviving animals were humanely euthanized and examined grossly.

2.5. Assessment of biological activity after heat treatment using microbially derived IPD079Ea

WCR neonates (within 24 h of hatch) were used in a seven-day bioassay conducted in an environmental chamber set at 21 °C, 65% relative humidity, and continuous dark. The larvae were fed a negative control diet containing ultrapure water, a positive control diet containing unheated 50 ng IPD079Ea per mg diet, or test diets containing 50 ng IPD079Ea per mg diet. Test dosing solutions were incubated at 25, 50, 75, or 95 °C for 30–35 min prior to use in the bioassay. Each test group contained a target of 30 larva. WCR larvae were refed on Day 4. After seven days mortality was assessed, and surviving organisms were individually weighed.

3. Results

3.1. Microbially derived IPD079Ea characterization

3.1.1. Amino acid composition analysis and molecular mass determination

Amino acid composition analysis determined that the protein concentration of the test substance was 0.77 mg of microbially derived IPD079Ea per mg of lyophilized powder (Table 1).

3.1.2. SDS-PAGE, western blot, and glycosylation analysis

SDS-PAGE analysis demonstrated that the microbially derived IPD079Ea migrated as a band consistent with the expected molecular weight of approximately 52 kDa (Fig. 1). Densitometry analysis indicated that the purity of microbially derived IPD079Ea was a single band and is therefore estimated as >95% on a total protein basis. The mass determined by LC-MS analysis of the protein was 51,896.42 Da, consistent with the expected mass of 51,896.50 Da (calculated using BioPharmaView, version 2.1; Sciex, Farmingham, VA) for microbially derived IPD079Ea.

Western blot analysis demonstrated that microbially derived IPD079Ea was immunoreactive to an IPD079Ea monoclonal antibody and visible as a single band consistent with the expected molecular weight of approximately 52 kDa (Fig. 2).

Protein glycosylation was not detected for microbially derived IPD079Ea in a glycoprotein staining assay (Fig. 3). The horseradish peroxidase positive control was stained as a magenta-colored band and the soybean trypsin inhibitor negative control was not stained by the glycoprotein stain.

3.1.3. MS analysis of tryptic and chymotryptic peptides and N-terminal sequencing

The matched peptides identified with the LC-MS analysis of the trypsin and chymotrypsin-digested microbially derived IPD079Ea account for 96% (463/481) of the expected IPD079Ea amino acid sequence (Tables 2 and 3). N-terminal amino acid sequence analysis identified a sequence (GSMAEPNKGG) matching amino acid residues 1–10 of the expected protein sequence.

3.1.4. Biological activity

Bioactivity analysis demonstrated that microbially derived IPD079Ea had insecticidal activity toward the target insect, western corn rootworm, with 100% mortality at 50 ng IPD079Ea per mg diet.

3.2. IPD079Ea sequence in silico similarity with allergens and toxins

Results of the IPD079Ea sequence searches against the COMPARE database of known and putative allergen sequences (van Ree et al., 2021) found no alignments that were a length of 80 or greater with a sequence identity of ≥35%, and the second search found no contiguous 8-residue matches between the IPD079Ea sequence and the allergen sequences. No alignments with an *E*-value $\leq 10^{-4}$ were returned between the IPD079Ea sequence and the protein sequences in the internal toxin database.

Table 1
Amino acid analysis results of the microbially derived IPD079Ea by LC-MS.

Amino Acids	Sample 1 (nmol)	Sample 2 (nmol)	Sample 3 (nmol)
Glutamine/Glutamic Acid	3.65	3.50	3.53
Isoleucine	3.81	3.58	3.65
Leucine	3.94	3.80	3.96
Lysine	3.82	3.60	3.90
Phenylalanine	3.66	3.52	3.71
Proline	3.81	3.61	3.62
Valine	3.85	3.65	3.82
Average (nmol)	3.79	3.61	3.74
Protein Concentration of Analytical Sample (μg/μl)	0.787	0.749	0.777
Protein Concentration of Lyophilized Powder (mg/mg)	0.787	0.749	0.777
Overall Average (mg/mg)	0.771		

Note: Mass spectrometer readings were converted to protein concentration values.

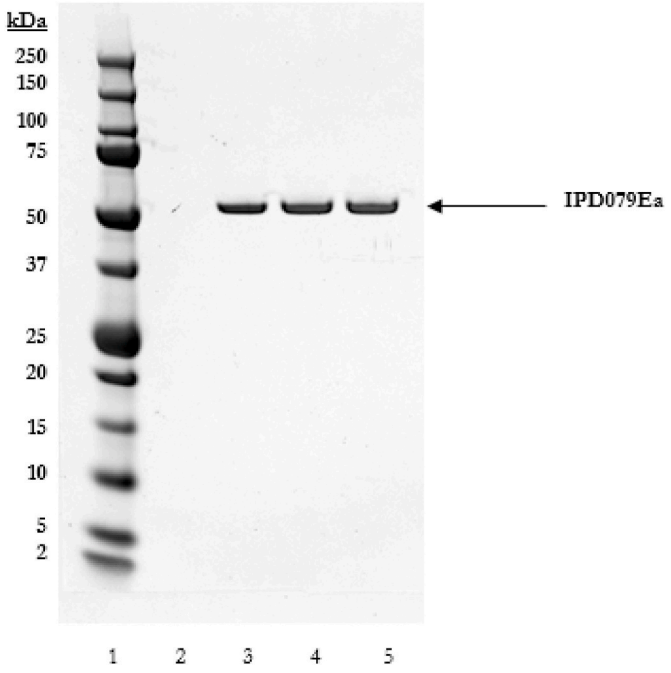

Fig. 1. Characterization of microbially derived IPD079Ea by SDS-PAGE. Lane 1 contained pre-stained molecular weight markers; lane 2 contained buffer only; lanes 3–5 contained microbially derived IPD079Ea samples.

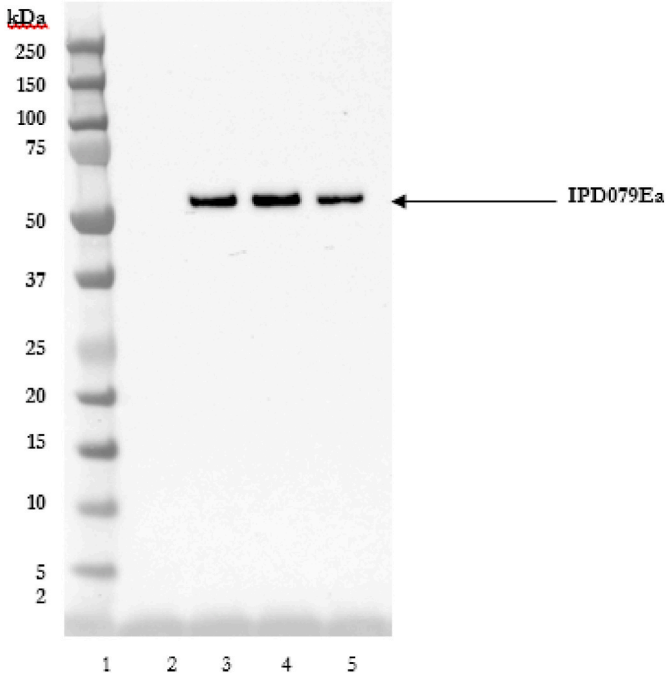

Fig. 2. Characterization of microbially derived IPD079Ea by western blot. Lane 1 contained pre-stained molecular weight markers; lane 2 contained buffer only; lanes 3–5 contained microbially derived IPD079Ea samples.

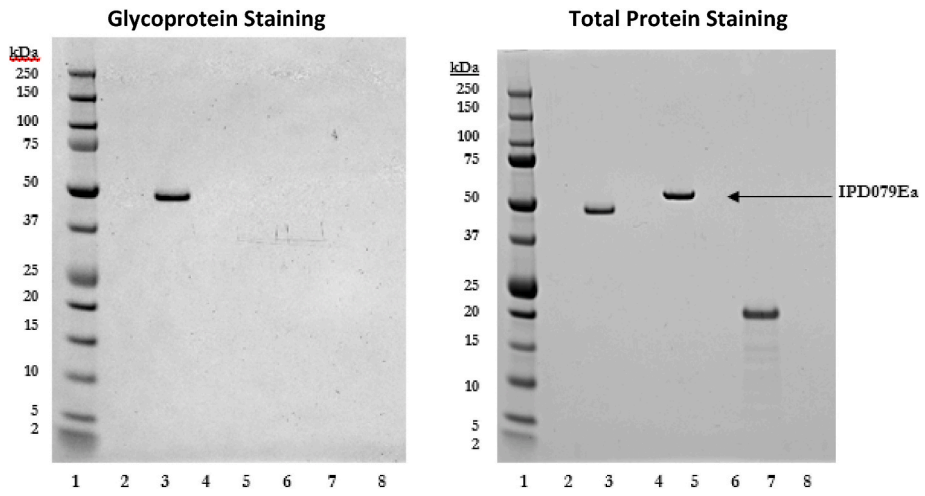

Fig. 3. Characterization of microbially derived IPD079Ea by glycosylation staining. Lane 1 contained pre-stained molecular weight markers; Lanes 2, 4, 6, and 8 contained buffer only; Lane 3 contained the positive control horseradish peroxidase (1 μg); Lane 5 contained microbially derived IPD079Ea (1 μg); Lane 7 contained the negative control soybean trypsin inhibitor (1 μg).

Table 2
Identified tryptic peptides of microbially derived IPD079Ea by LC-MS.

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
1–8	832.37	832.37	GSMAEPNK
1–15	1444.68	1444.68	GSMAEPNKGGAPAMK
9–15	630.31	630.32	GGAPAMK
16–23	843.48	843.48	NVAKPSTK
24–65	4324.12	4324.14	RLIPSSIAASSQTSANALTEPLPGSDAIGQSYDAFGFFANPR
25–65	4168.02	4168.04	LIPSSIAASSQTSANALTEPLPGSDAIGQSYDAFGFFANPR
100–108	994.45	994.46	DTETSTVSR
110–116	855.39	855.40	TKDDYSK
112–121	1166.58	1166.58	DDYSKELAVK
124–155	3427.50	3427.52	LSGSYGYFSASVESDFSQSISDATDTTYTSVR
163–173	1185.66	1185.67	LSLKDDVGALR
167–173	744.37	744.38	DDVGALR
174–181	840.54	840.54	SKLLPGVK
182–209	3042.46	3042.48	QALATMDATQLFDTFGTHYVSEVLVGGR[c]
210–217	867.43	867.43	ADYVATTK
218–248	3018.41	3018.43	TSAFSSSTSISVAAEASFQSIAGGEVSPESK
249–255	830.47	830.47	VLAEMLR
302–312	1175.57	1175.58	SISELADSAQR
302–313	1331.68	1331.68	SISELADSAQRR
319–354	3901.91	3901.94	ASQSYIPSYVTRPAVVGLEVIISDSNSESPPYGYTR
355–361	907.44	907.44	IDYDLNR
367–373	991.48	991.48	YVFLCYK
376–397	2262.10	2262.11	NISVGGDADAITDVLVVYGNDR
376–407	3292.60	3292.62	NISVGGDADAITDVLVVYGNDRNPSVPSGYTK
398–407	1048.51	1048.52	NPSVPSGYTK
408–419	1173.60	1173.60	IDKDLNSGAGGK
411–419	817.39	817.39	DLNSGAGGK
420–427	1142.51	1142.51	YIYFCYSK
431–439	1068.59	1068.59	KQEEGLPIR
432–439	940.49	940.50	QEEGLPIR
443–458	1641.84	1641.85	VVGPHPTSVAPYGFSK
459–476	2013.94	2013.95	IDIDLNMGAGGDFIYLCK

[a] The experimental mass is the uncharged mass calculated from the mass to charge ratio of the observed ion.
[b] The theoretical mass is the *in silico* generated mass that matches closest to the experimental mass.
[c] This peptide was modified by methionine oxidation (Oxidation-M).

3.3. Digestibility of microbially derived IPD079Ea

Microbially derived IPD079Ea migrated at ~52 kDa and was digested below the level of detection within 30 s in SGF (Fig. 4). Observed faint lower molecular bands were rapidly digested by sequential exposure to simulated intestinal fluid (USP, 2006; unpublished). As expected, the BSA positive control was digested below the level of detection in 1 min and the β-lactoglobulin negative control was detectable after 60 min (Fig. 5).

3.4. Microbially derived IPD079Ea acute oral toxicity

All animals administered 5000 mg/kg body weight microbially derived IPD079Ea and BSA control survived to the scheduled sacrifice. One female in the vehicle-control group was sacrificed on test day three due to clinical signs related to injury consistent with intubation error.

Table 3
Identified chymotryptic peptides of microbially derived IPD079Ea by LC-MS.

Matched Residue Position	Experimental Mass[a]	Theoretical Mass[b]	Identified Peptide Sequence
42–55	1433.66	1433.67	TEPLPGSDAIGQSY
62–72	1304.68	1304.69	ANPRSIMKELF
73–87	1762.79	1762.81	EFSPQEEIVVEGNTW
132–151	2109.87	2109.89	SASVESDFSQSISDATDTTY
140–151	1287.54	1287.55	SQSISDATDTTY
152–161	1226.65	1226.65	TSVRTHVNKW
162–172	1185.66	1185.67	RLSLKDDVGAL
164–172	916.48	916.49	SLKDDVGAL
173–184	1308.82	1308.82	RSKLLPGVKQAL
177–184	824.51	824.51	LPGVKQAL
185–193	996.45	996.46	ATMDATQLF
185–196	1359.59	1359.60	ATMDATQLFDTF
194–200	839.34	839.34	DTFGTHY
194–205	1366.63	1366.64	DTFGTHYVSEVL
197–205	1003.49	1003.50	GTHYVSEVL
197–212	1721.83	1721.84	GTHYVSEVLVGGRADY
201–212	1263.64	1263.65	VSEVLVGGRADY
206–212	736.35	736.35	VGGRADY
206–221	1642.82	1642.83	VGGRADYVATTKTSAF
213–221	924.49	924.49	VATTKTSAF
222–235	1342.62	1342.63	SSSTSISVAAEASF
222–250	2824.38	2824.40	SSSTSISVAAEASFQSIAGGEVSPESKVL
236–250	1499.77	1499.78	QSIAGGEVSPESKVL
236–254	1943.97	1943.99	QSIAGGEVSPESKVLAEML
251–262	1405.69	1405.70	AEMLRENSSTRL
251–263	1568.75	1568.76	AEMLRENSSTRLY
255–263	1124.55	1124.56	RENSSTRLY
263–279	1722.84	1722.85	YALGGSALPNITDPATY
264–279	1559.77	1559.78	ALGGSALPNITDPATY
266–279	1375.65	1375.66	GGSALPNITDPATY
283–292	1132.60	1132.60	LESIDTIPVF
283–295	1496.71	1496.72	LESIDTIPVFCGF
293–300	925.39	925.40	CGFTQNSL
296–306	1218.64	1218.65	TQNSLKSISEL
296–316	2302.18	2302.19	TQNSLKSISELADSAQRRDAL
301–306	675.38	675.38	KSISEL
301–316	1758.91	1758.92	KSISELADSAQRRDAL
301–323	2494.26	2494.28	KSISELADSAQRRDALAKASQSY
307–323	1836.90	1836.91	ADSAQRRDALAKASQSY
317–323	753.36	753.37	AKASQSY
324–336	1370.78	1370.79	IPSYVTRPAVVGL
351–357	886.42	886.42	GYTRIDY
353–367	1754.86	1754.87	TRIDYDLNRNAGGKY
358–367	1106.55	1106.55	DLNRNAGGKY
358–369	1352.68	1352.68	DLNRNAGGKYVF
360–367	878.43	878.44	NRNAGGKY
371–393	2527.25	2527.26	CYKQKNISVGGDADAITDVLVVY
373–390	1842.96	1842.97	KQKNISVGGDADAITDVL
373–393	2204.15	2204.17	KQKNISVGGDADAITDVLVVY
394–405	1261.56	1261.57	GNDRNPSVPSGY
406–420	1565.80	1565.80	TKIDKDLNSGAGGKY
406–422	1841.94	1841.95	TKIDKDLNSGAGGKYIY
426–441	1853.04	1853.05	SKDKRKQEEGLPIRGL
442–454	1378.73	1378.74	RVVGPHPTSVAPY
475–481	928.45	928.45	CKSRHLE

[a] The experimental mass is the uncharged mass calculated from the mass to charge ratio of the observed ion.
[b] The theoretical mass is the *in silico* generated mass that matches closest to the experimental mass.

There were no differences in body weights (Table 4) or clinical observations considered to be related to administration of IPD079Ea. One male in the BSA group had a swollen abdomen on test day 15 and was observed grossly to have large preputial glands filled with viscous green fluid. Under conditions of this study, no test-substance related mortality or other evidence of acute oral toxicity was observed, based on evaluation of body weight, clinical signs and gross pathology. Therefore, the oral LD_{50} of microbially derived IPD079Ea in male and female mice was greater than 5000 mg/kg.

3.5. Assessment of biological activity after heat treatment using microbially derived IPD079Ea

Mortality data indicated that heating IPD079Ea for approximately 30 min at 50 °C–95 °C effectively inactivated the protein against WCR. Statistically significant differences were observed in mortality for WCR provided diets containing IPD079Ea incubated at 50 °C, 75 °C, and 95 °C (3.57%, 3.45%, and 0%, respectively; P-values < 0.0001) when compared with those provided the unheated IPD079Ea control diet (95.2% mortality). Mortality was not significantly different for WCR provided the test diet containing IPD079Ea incubated at 25 °C (96.4% mortality; P-value $= 0.8214$) in comparison to those provided the unheated IPD079Ea positive control diet. Mortality was 3.45% in the

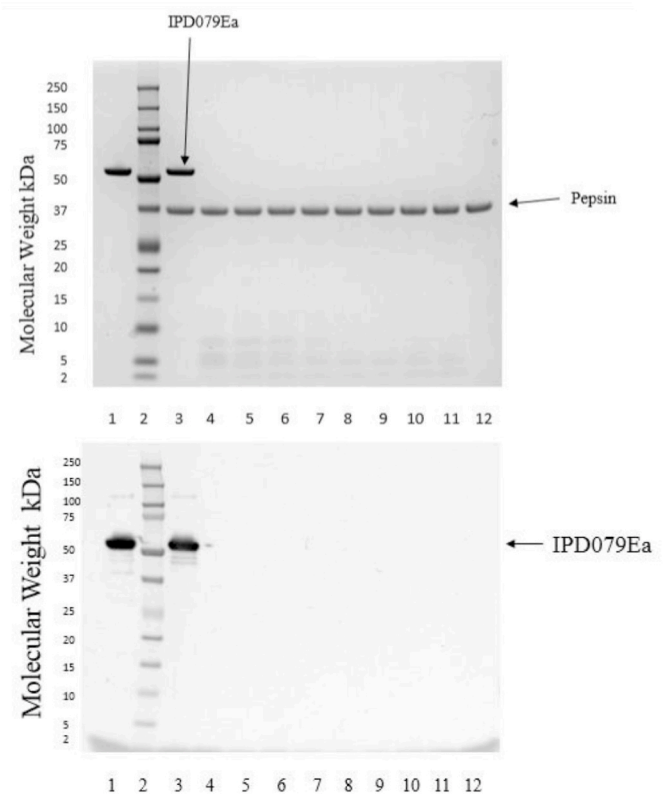

Fig. 4. SDS-PAGE analysis (top) and western blot analysis (bottom) of microbially derived IPD079Ea in SGF digestion time-course. Lane 1: IPD079Ea in water, time 0; Lane 2: Pre-stained molecular weight marker; Lanes 3 to 11: IPD079Ea in SGF for 0, 0.5, 1, 2, 5, 10, 20, 30 and 60 min; Lane 12: SGF control for 60 min.

bioassay negative control group.

4. Discussion

4.1. History of safe use

The IPD079Ea source organism is a fern, *O. pendulum*, from the Ophioglossaceae (adder's-tongue) family which is native to India, Australia, parts of Africa and southeast Asia and introduced in the United States (Kew Science, 2020; USDA, 2022). While specific food use of *O. pendulum* has not been identified, consumption of several other Ophioglossum fern species have been documented including fronds for small ruminant animal fodder (*O.* grande L.), leaves as vegetable or salad (*O. lusoafricanum* Prantl, *O. ovatum* Bory, *O. polyphyllum* A. Braun, *O. reticulatum* L., *O. vulgatum* L.) in Sub-Saharan Africa (Maroyi, 2014), fronds as vegetable or salad in Malaysia, India, Nepal and Indonesia (*O. reticulatum* L., *O. nudicaule* L.) (Mannan et al., 2008) or fronds used in soups in Tibet (*O. polyphyllum* A. Braun in Seub.) (Lognay et al., 2008).

Table 4
Acute oral toxicity study with microbially derived IPD07Ea: Mean mouse body weights (g ± SD).

Treatment	n	Day 1[a,b]	Day 2	Day 3	Day 5	Day 8	Day 15
Males							
Vehicle	6	32.1 ± 2.8	33.3 ± 3.2	33.8 ± 3.1	34.3 ± 3.5	34.7 ± 3.2	35.6 ± 3.3
BSA[c]	6	32.3 ± 2.2	33.0 ± 2.4	33.4 ± 2.4	34.2 ± 2.3	34.5 ± 2.4	35.7 ± 2.0
IPD079Ea[c]	6	32.0 ± 2.1	33.3 ± 2.4	33.5 ± 2.4	34.1 ± 2.2	34.1 ± 2.2	35.5 ± 2.4
Females							
Vehicle	6	24.2 ± 1.0	24.5 ± 1.7	24.5 ± 2.3	26.3 ± 0.7 [d]	26.4 ± 1.0 [d]	28.1 ± 1.4 [d]
BSA[c]	6	23.9 ± 1.6	24.3 ± 1.3	25.2 ± 0.9	25.3 ± 1.1	26.1 ± 0.7	27.0 ± 1.0
IPD079Ea[c]	6	23.8 ± 1.8	24.5 ± 2.1	25.1 ± 1.7	25.8 ± 1.8	25.8 ± 2.0	27.4 ± 2.4

[a] Days Relative to Start Date.
[b] Fasted weight.
[c] Dose: 5000 mg/kg.
[d] n = 5.

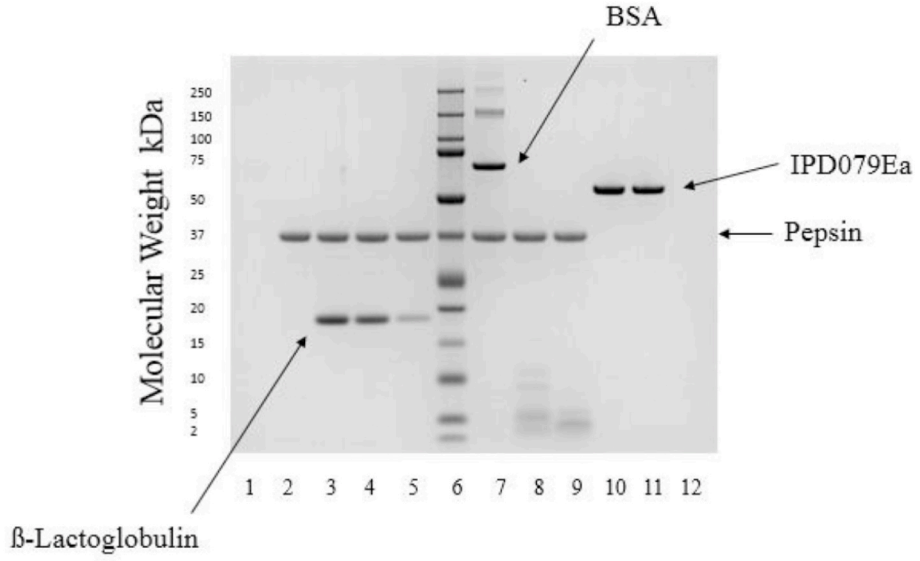

Fig. 5. SDS-PAGE analysis of control digestion samples. Lane 1: 1X LDS sample buffer blank; Lane 2: SGF control; Lanes 3–5: β-lactoglobulin in SGF for 0, 1, and 60 min; Lane 6: pre-stained protein molecular weight marker; Lanes 7–9: BSA in SGF for 0, 1, and 60 min; Lane 10: microbially derived IPD079Ea in water (no SGF) for 60 min; microbially derived IPD079Ea in gastric control solution (no pepsin) for 60 min; Lane 12: 1X LDS sample buffer blank.

4.2. Identity, concentration, and activity of the microbially derived IPD079Ea

Amino acid composition analysis indicated that the microbially derived lot of IPD079Ea contained 0.77 mg of IPD079Ea per mg of lyophilized powder, and SDS-PAGE analysis demonstrated the microbially derived IPD079Ea migrated as a band consistent with the expected molecular weight of approximately 52 kDa. Western blot analysis confirmed that the microbially derived IPD079Ea was immunoreactive to an IPD079Ea monoclonal antibody and visible as a single band consistent with the expected molecular weight of approximately 52 kDa. Glycoprotein staining did not detect glycosylation for IPD079Ea. LC-MS analysis and Edman sequencing confirmed the expected amino acid sequence for IPD079Ea, and bioassay results with western corn rootworm confirmed the insecticidal activity of the protein lot. Together these results confirmed the identity and activity of IPD079Ea and determined its concentration in the protein lot.

4.3. Toxicity and allergenicity potential of IPD079Ea

Bioinformatic analysis indicated no meaningful similarity between the IPD079Ea amino acid sequence and that of known toxins and allergens. IPD079Ea was rapidly degraded in simulated gastric fluid and inactivated with heat treatment. As expected based on a lack of identified hazard, acute oral exposure of male and female mice to IPD079Ea at a dose of 5000 mg/kg did not result in mortality or any evidence of toxicity.

While no hazard was detected for microbially derived IPD079Ea, lability to heat involved with processing and susceptibility to digestion will lower exposure in the mammalian gut further reducing the risk of toxicity. While these studies are often used to inform the allergenicity assessment of newly expressed proteins in GM crops, neither heat lability nor digestion correlate with the allergenic status of proteins (GMO Panel, 2021; Privalle et al., 2011).

Not all the studies required by regulatory agencies are necessary to demonstrate safety for every protein, and protein safety assessments should follow a tiered approach (Brune et al., 2021). When sufficient information from lower-tier studies such as: history of safe use information (HOSU), bioinformatics assessments for similarity to known allergens and toxins, information about mechanism of action and functional specificity, stability when exposed to conditions of simulated digestion, and stability to heat and or processing do not elucidate hypotheses suggestive of a hazard potential, then there is limited value and necessity for conducting higher-tiered studies such as *in vivo* acute and repeated-dose toxicity tests (Delaney et al., 2008a; Roper et al., 2021). Although there was no identified direct consumption of the source organism or IPD079Ea by humans or animals, there was absence of similarity to known toxins and allergens, rapid degradation under simulated conditions of digestion, loss of functional activity against the target species (WCR) following heat exposure, and a demonstrated functional specificity restricted primarily to certain coleopteran pests and related species (Boeckman et al., 2022). As such, it could reasonably be considered that higher tiered *in vivo* toxicity testing was unnecessary for the overall safety assessment.

However, based on a fundamental understanding of global regulatory requirements, an *in vivo* acute oral toxicity study was conducted as confirmation of IPD079Ea safety. Additionally, because the overall weight-of-evidence did not demonstrate a hazard potential for IPD079Ea, a human dietary exposure assessment was not needed to assess risk, which is a function of both hazard and exposure. This work presents some of the studies required by regulatory agencies to demonstrate the safety of a newly expressed protein in a GM crop and further supports the established weight-of-evidence protein safety testing strategies (Brune et al., 2021; Alimentarius Commission, 2009; Delaney et al., 2008a).

5. Conclusions

The results of these hazard-identification and characterization studies with the fully characterized microbially derived IPD079Ea inform the food and feed safety assessment for the insecticidal protein IPD079Ea from the fern *O. pendulum*. Collectively these studies indicate through a weight-of-evidence that IPD079Ea is unlikely to present a hazard and therefore is of negligible risk to human or animal health. This protein, sourced from a novel donor organism (*O. pendulum*) and with a different site of action from that of *Bt*-derived proteins will provide another agricultural biotechnology tool for growers to address WCR damage and to extend the efficacy of PIP products currently in use for control of this pest. As regulatory requirements continue to evolve around GM crops, adding safety data on newly expressed proteins to the public literature can help to gain acceptance of the much-needed solutions offered by agricultural biotechnology.

CRediT authorship contribution statement

Anne B. Carlson: Investigation, Visualization, Writing – original draft, Writing – review & editing. **Carey A. Mathesius:** Supervision, Writing – original draft, Writing – review & editing, Project administration. **Stephen Ballou:** Investigation. **Melissa N. Fallers:** Investigation. **Tim A. Gunderson:** Investigation. **Aideen Hession:** Investigation. **Henry Mirsky:** Investigation. **Brian Stolte:** Investigation. **John Zhang:** Supervision, Writing – original draft, Writing – review & editing, Project administration. **Rachel M. Woods:** Investigation. **Rod A. Herman:** Writing – original draft, Writing – review & editing. **Jason M. Roper:** Supervision, Writing – original draft, Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anne B. Carlson, Carey A. Mathesius, Stephen Ballou, Melissa N. Fallers, Tim A. Gunderson, Aideen Hession, Henry Mirsky, Brian Stolte, John Zhang, Rachel M. Woods, Rod A. Herman and Jason M. Roper report a relationship with Corteva Agriscience that includes: employment and equity or stocks for Anne B. Carlson and Jason M. Roper.

Acknowledgements

The authors thank all reviewers for their insightful input as well as Nancy Wilmeth and Brenda Smith for formatting assistance. Corteva Agriscience™ funded this research.

References

Alimentarius Commission, Codex, 2009. Foods Derived from Modern Biotechnology, 2. Food and Agriculture Organization of the United Nations, World Health Organization, Rome.

Anderluh, G., Kisovec, M., Kraševec, N., Gilbert, R.J.C., 2014. Distribution of MACPF/CDC proteins. In: Anderluh, G., Gilbert, R. (Eds.), MACPF/CDC Proteins - Agents of Defence, Attack and Invasion. Springer Netherlands, Dordrecht, pp. 7–30.

Barry, J., Schepers, E., English, J., Udranszky, I., Fengler, K., Kassa, A., Sethi, A., Zhao, J.-Z., Diehn, S., Crane, V., Sandahl, G., Mabry, T., Perez Ortega, C., Nelson, M.E., Liu, L., Lu, A.L., 2022. A Novel Family of Insecticidal Proteins from Vascular Plants with Potent Activity against Western Corn Rootworm (*Diabrotica Spp.*). Scientific Reports Submitted.

Boeckman, C., Ballou, S., Gunderson, T., Huang, E., Linderblood, C., Olson, T., Stolte, B., LeRoy, K., Walker, C., Wang, Y., Woods, R., Zhang, J., 2022. Characterization of the spectrum of activity of IPD079Ea: a protein derived from Ophioglossum pendulum (Ophioglossales: Ophioglossaceae) with activity against Western Corn Rootworm [Diabrotica virgifera virgifera (Coleoptera: Chrysomelidae)]. J. Econ. Entomol. In press https://academic.oup.com/jee/advance-article/doi/10.1093/jee/toac079/6594673.

Brune, P., Chakravarthy, S., Graser, G., Mathesius, C., McClain, S., Petrick, J.S., Sauve-Ciencewicki, A., Schafer, B., Silvanovich, A., Brink, K., Burgin, K., Bushey, D., Cheever, M.L., Edrington, T., Fu, H., Habex, V., Herman, R., Islamovic, E., Lipscomb, E.A., Motyka, S., Privalle, L., Ranjan, R., Roper, J., Song, P., Tilton, G.,

Zhang, J., Waters, S., Ramos, A., Culler, A.H., Hunst, P., Gast, R., Mahadeo, D., Goodwin, L., 2021. Core and supplementary studies to assess the safety of genetically modified (GM) plants used for food and feed. J. Regulat. Sci. 9, 45–60.
Carlson, A.B., Mathesius, C.A., Ballou, S., Boeckman, C.J., Gunderson, T.A., Mirsky, H.P., Mukerji, P., Roe, J.C., Schmidt, J.M., Zhang, J., Delaney, B., 2019. Safety assessment of coleopteran active IPD072Aa protein from *Psuedomonas chlororaphis*. Food Chem. Toxicol. 129, 376–381.
Crickmore, N., Berry, C., Panneerselvan, S., Mishra, R., Connor, T.R., Bonning, B.C., 2022. Bacterial Pesticidal Protein Resource Center.
Delaney, B., Astwood, J.D., Cunny, H., Conn, R.E., Herouet-Guicheney, C., MacIntosh, S., Meyer, L.S., Privalle, L., Gao, Y., Mattsson, J., Levine, M., 2008a. Evaluation of protein safety in the context of agricultural biotechnology. Food Chem. Toxicol. 46, S71–S97.
Delaney, B., Zhang, J., Carlson, G., Schmidt, J., Stagg, B., Comstock, B., Babb, A., Finlay, C., Cressman, R.F., Ladics, G., Cogburn, A., Siehl, D., Bardina, L., Sampson, H., Han, Y., 2008b. A gene-shuffled glyphosate acetyltransferase protein from *Bacillus licheniformis* (GAT4601) shows No evidence of allergenicity or toxicity. Toxicol. Sci. 102, 425–432.
Edman, P., 1950. Method for determination of the amino acid sequence in peptides. Acta Chem. Scand. 4, 283–293.
GMO Panel, E.F.S.A., Naegeli, H., Bresson, J.-L., Dalmay, T., Dewhurst, I.C., Epstein, M. M., Firbank, L.G., Guerche, P., Hejatko, J., Moreno, F.J., Mullins, E., Nogué, F., Rostoks, N., Sánchez Serrano, J.J., Savoini, G., Veromann, E., Veronesi, F., Dumont, A.F., 2021. Statement on *in vitro* protein digestibility tests in allergenicity and protein safety assessment of genetically modified plants. EFSA J. 19, e06350.
Kew Science, 2020. *Ophioglossum Pendulum* L. Plants of the World *Online*.
Ladics, G.S., Cressman, R.F., Herouet-Guicheney, C., Herman, R.A., Privalle, L., Song, P., Ward, J.M., McClain, S., 2011. Bioinformatics and the allergy assessment of agricultural biotechnology products: industry practices and recommendations. Regul. Toxicol. Pharmacol. 60, 46–53.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Lognay, G., Haubruge, E., Delcarte, E., Wathelet, B., Mathieu, F., Marlier, M., Malaisse, F., 2008. *Ophioglossum polyphyllum* A. Braun in Seub. (Ophioglossaceae, Pteridophyta) a rare potherb in south central Tibet (T.A.R., P.R.China). Geo-Eco-Trop 32, 9–16.
Mannan, M.M., Maridass, M., Victor, B., 2008. A review on the potential uses of ferns, 2008 Ethnobotanical Leaflets 33.
Maroyi, A., 2014. Not just minor wild edible forest products: consumption of pteridophytes in sub-Saharan Africa. J. Ethnobiol. Ethnomed. 10, 78.
Mathesius, C.A., Barnett Jr., J.F., Cressman, R.F., Ding, J., Carpenter, C., Ladics, G.S., Schmidt, J., Layton, R.J., Zhang, J.X.Q., Appenzeller, L.M., Carlson, G., Ballou, S., Delaney, B., 2009. Safety assessment of a modified acetolactate synthase protein (GM-HRA) used as a selectable marker in genetically modified soybeans. Regul. Toxicol. Pharmacol. 55, 309–320.
McClain, S., Herman, R.A., Islamovic, E., Ranjan, R., Silvanovich, A., Song, P., Goodwin, L., 2021. Allergy risk assessment for newly expressed proteins (NEPs) in genetically modified (GM) plants. J. Regulat. Sci. 9, 67–75.
Mirsky, H.P., Cressman Jr., R.F., Ladics, G.S., 2013. Comparative assessment of multiple criteria for the in silico prediction of cross-reactivity of proteins to known allergens. Regul. Toxicol. Pharmacol. 67, 232–239.
MOA, 2016. Standard of the People's Republic of China, Circular of the Ministry of Agriculture. Public Notice 2406-10-2016, Food Safety Detection of Genetically Modified Organisms and Derived Products – Oral Acute Toxicity Test of Protein.
NRC, 2011. Guide for the Care and Use of Laboratory Animals, 8. The National Academies Press, Washington, D.C.
OECD, 2001. OECD Guideline for Testing of Chemicals 423: Acute Oral Toxicity-Acute Toxic Class Method. Organisation for Economic Cooperation and Development.
Pearson, W.R., 2000. Flexible sequence similarity searching with the FASTA3 program package. Methods Mol. Biol. 132, 185–219.
Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. Unit. States Am. 85, 2444–2448.
Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567.
Privalle, L., Bannon, G., Herman, R., Ladics, G., McClain, S., Stagg, N., Ward, J., Herouet-Guicheney, C., 2011. Heat stability, its measurement, and its lack of utility in the assessment of the potential allergenicity of novel proteins. Regul. Toxicol. Pharmacol. 61, 292–295.
Roper, J., Lipscomb, E.A., Petrick, J.S., Ranjan, R., Sauve-Ciencewicki, A., Goodwin, L., 2021. Toxicological assessment of newly expressed proteins (NEPs) in genetically modified (GM) plants. J. Regulat. Sci. 9, 61-55.
Silvanovich, A., Bannon, G., McClain, S., 2009. The use of *E*-scores to determine the quality of protein alignments. Regul. Toxicol. Pharmacol. 54, S26–S31.
Thomas, K., Aalbers, M., Bannon, G.A., Bartels, M., Dearman, R.J., Esdaile, D.J., Fu, T.J., Glatt, C.M., Hadfield, N., Hatzos, C., Hefle, S.L., Heylings, J.R., Goodman, R.E., Henry, B., Herouet, C., Holsapple, M., Ladics, G.S., Landry, T.D., MacIntosh, S.C., Rice, E.A., Privalle, L.S., Steiner, H.Y., Teshima, R., van Ree, R., Woolhiser, M., Zawodny, J., 2004. A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. Regul. Toxicol. Pharmacol. 39, 87–98.
USDA, 2022. Ophioglossum Pendulum. United States Department of Agriculture.
USP, 2006. USP 23, Simulated Gastric Fluid and Simulated Intestinal Fluid, vol. 18. National Formulary, Rockville, MD. US Pharmacopeia.
van Ree, R., Sapiter Ballerda, D., Berin, M.C., Beuf, L., Chang, A., Gadermaier, G., Guevera, P.A., Hoffmann-Sommergruber, K., Islamovic, E., Koski, L., Kough, J., Ladics, G.S., McClain, S., McKillop, K.A., Mitchell-Ryan, S., Narrod, C.A., Pereira Mouriès, L., Pettit, S., Poulsen, L.K., Silvanovich, A., Song, P., Teuber, S.S., Bowman, C., 2021. The COMPARE database: a public Resource for allergen identification, adapted for continuous improvement. Front. Allergy 2, 700533.
Waters, S., Ramos, A., Hendrickson Culler, A., Hunst, P., Zeph, L., Gast, R., Mahadeo, D., Sottosanto, J., Huber, S., Shan, G., Chakravarthy, S., Goodwin, L., 2021. Recommendations for science-based safety assessment of genetically modified (GM) plants for food and feed uses. J. Regulat. Sci. 9, 16–21.
Yu, L., Liu, D., Chen, S., Dai, Y., Guo, W., Zhang, X., Wang, L., Ma, S., Xiao, M., Qi, H., Xiao, S., Chen, Q., 2020. Evolution and expression of the membrane Attack Complex and perforin gene family in the poaceae. Int. J. Mol. Sci. 21, 5736.