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Food safety assessment of Cry8Ka5 mutant protein using Cry1Ac as a control *Bt* protein

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ABSTRACT

Cry8Ka5 is a mutant protein from *Bacillus thuringiensis* (*Bt*) that has been proposed for developing transgenic plants due to promising activity against coleopterans, like *Anthonomus grandis* (the major pest of Brazilian cotton culture). Thus, an early food safety assessment of Cry8Ka5 protein could provide valuable information to support its use as a harmless biotechnological tool. This study aimed to evaluate the food safety of Cry8Ka5 protein following the two-tiered approach, based on weights of evidence, proposed by ILSI. Cry1Ac protein was used as a control *Bt* protein. The history of safe use revealed no convincing hazard reports for *Bt* pesticides and three-domain Cry proteins. The bioinformatics analysis with the primary amino acids sequence of Cry8Ka5 showed no similarity to any known toxic, antinutritional or allergenic proteins. The mode of action of Cry proteins is well understood and their fine specificity is restricted to insects. Cry8Ka5 and Cry1Ac proteins were rapidly degraded in simulated gastric fluid, but were resistant to simulated intestinal fluid and heat treatment. The LD_{50} for Cry8Ka5 and Cry1Ac was >5000 mg/kg body weight when administered by gavage in mice. Thus, no expected relevant risks are associated with the consumption of Cry8Ka5 protein.

1. Introduction

Insect resistant cotton featuring *cry* genes from *Bacillus thuringiensis* (*Bt*) was first planted in 1996, exactly the first year of commercialization of GM crops (James, 2011). Almost a decade later, the *Bt* cotton expressing the Cry1Ac entomotoxin was released for cultivation in Brazil, and today it is possible to find at least 12 varieties of cotton containing *cry* genes. All these varieties express the Cry1Ac toxin alone or fused to Cry1F or Cry2Ab2 (Brazilian National Biosafety Technical Commission (CTNBio), 2012). Nevertheless, these cotton varieties are mainly active against lepidopteran (Oliveira et al., 2011; Brazilian National Biosafety Technical Commission (CTNBio), 2012), whereas the major pest of the Brazilian cotton culture is a coleopteran, the cotton boll weevil (*Anthonomus grandis*) (Silvie and Leroy, 2001).

In this context, Oliveira et al. (2011) applied the DNA shuffling technique coupled to the selection of proteins by phage display to generate a combinatorial variant library of the coleopteracidal gene *cry8Ka1*, isolated from the *Bt* Brazilian strain S811. Variants with improved activity against *A. grandis* were selected. Among the most effective variants, the Cry8Ka5 mutant protein which was three times more potent against *A. grandis* than the original protein (Cry8Ka1) stands out. The *cry8Ka5* gene was proposed to be incorporated into cotton plants to confer protection against *A. grandis* attack, and the first Cry8Ka5 cotton plants have just been obtained. Besides, this mutant gene was suggested to be used in stacked traits for developing other relevant GM commodities for the Brazilian agribusiness. Thus, the gathering of early food safety information on Cry8Ka5 protein could avoid the use of an unsafe protein in those overexpensive activities of producing transgenic plants, providing

Abbreviations: ADFS, Allergen Database for Food Safety; *Bt*, *Bacillus thuringiensis*; CTNBio, Brazilian National Technical Commission on Biosafety; HOSU, history of safe use; ILSI, International Life Sciences Institute; SDAP, Structural Database of Allergenic Proteins; SGF, simulated gastric fluid; SIF, Simulated Intestinal Fluid.

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evidence that this mutant entomotoxin is a harmless biotechnological tool.

The current food safety assessment of biotech proteins has been performed essentially following the WHO and FAO guidelines compiled in the Codex Alimentarius document (Codex Alimentarius Commission, 2009). Alternative methods have been proposed. Among them, a two-tiered (Tier I and II) approach based on weights of evidence proposed by the International Life Sciences Institute (Delaney et al., 2008) stands out. Tier I (potential hazard identification) includes an assessment of the biological function or mode of action, and intended application of the protein, history of safe use, comparison of the amino acid sequence of the protein to other proteins, as well as the biochemical and physico-chemical properties of the proteins. Studies outlined in Tier II (hazard characterization) are conducted when the results from Tier I are not sufficient to allow a determination of safety (reasonable certainty of no harm), but for insecticidal proteins it is recommended that an oral acute toxicity test in mice be performed. In fact, these tests are the same as those recommended by Codex Alimentarius Commission (2009), which are adopted by Brazilian National Technical Commission on Biosafety (CTNBio). In fact, the ILSI approach is a more flexible approach because takes into account all data obtained in a holistic way. Ideally, the predictive value of each piece of evidence must be well understood in order to give certain data more "weight" than others during the assessment, thus leading to greater confidence in the overall assessment (Delaney et al., 2008).

In this context, this study aimed to perform the early food safety assessment of the Cry8Ka5 mutant protein following the two-step approach based on weight of evidence by ILSI. For comparison purposes, the Cry1Ac *Bt* protein was used, since there are well-established informations about its food and feed safety.

2. Materials and methods

2.1. History of safe use

A literature review on the history of safe use (HOSU) of Cry8Ka5 protein was performed according to the principles described by Constable et al. (2007). This search was composed by reports for the *Bt* (source of the *cry8Ka* gene) and three-domain Cry proteins. Since there is no HOSU for Cry8Ka5, results for three-domain Cry proteins (isolated or as active component of insecticidal formulations and/or transgenic food/feed) were considered. This extrapolation was made because three-domain Cry proteins share high structural similarities among them, which are connected to their functions and biological activities (Hammond and Koch, 2012; Sanahuja et al., 2011). Thus, the PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) was accessed, using the following combination of keywords: (a) "history of safe use" and "*Bacillus thuringiensis*"; (b) "history of safe use" and "Cry proteins" (c) "food/feed safety" and "*Bacillus thuringiensis*" (d) "food/feed safety" and "Cry proteins", and (e)"risk assessment" and "*Bacillus thuringiensis*".

2.2. Bioinformatics analysis

Firstly, the similarity of full-length Cry8Ka5 and Cry1Ac (control *Bt* protein) amino acid sequences to allergenic, toxic and/or antinutritional proteins was evaluated. The sequences (shown in the Fig. 1) were compared in FASTA format against the non-redundant (nr) NCBI dataset (http://blast.ncbi.nlm.nih.gov/). Performing non-restrictive searches for both entomotoxins are little informative, since the sequences in the Cry protein class are well conserved that generates a huge number of hits with low E-scores and very high % of identity. Additionally, the use of the limit keyword "toxin" in the searches would bring the same problem because Cry are characteristically known to be toxic proteins to insects. Thus, comparisons were run using or not using keyword limits, and observing carefully specific details of alignment (E-scores <0.001 and shared identity >35%). For each sequence tested, the keywords "allergen", "toxin" or "antinutrient" was used independently. These criteria were particularly determined for this work, and was based on the study of Moran et al. (2014) with a calcium-binding protein expressed by *Escherichia coli*. The algorithm used for comparisons was BLASTP 2.2.27+ and the scoring matrix was BLOSUM62. All comparisons were run in November 15–20, 2014.

Another search was carried out to specifically assess the allergenic potential of both proteins. The sequences were compared to allergen sequences deposited in the following databases: (1) Structural Database of Allergenic Proteins (SDAP) (http://fermi.utmb.edu/SDAP/), updated in February 2013; (2) Allergen Database for Food Safety (ADFS) (http://allergen.nihs.go.jsp/ADFS/), updated in February 2014; (3) AllergenOnline version 14 database (http://www.allergenonline.com/), updated in January 2014; (4) Allermatch™ (http://allermatch.org/), updated in August 2012. Cry8Ka5 and Cry1Ac sequences were subjected to FASTA comparisons using as filter E-value cutoff of 0.1 for detection of identity >50% for the complete sequence similarity and >35% in a window of 80 amino acids. All comparisons were performed in November 21–22, 2014.

2.3. Mode of action and specificity

A literature review was conducted on the mode of action and specificity of three-domain Cry proteins following the recommendations of Delaney et al. (2008). The three-domain Cry proteins is the most relevant group of *Bt* proteins concerning economical relevance, and in which the Cry8Ka5 protein is included (Oliveira et al., 2011). The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) was accessed, using the following combination of keywords: (1) "mode of action" and "*Bacillus thuringiensis*"; (2) "mode of action" and "*Bacillus thuringiensis*" and "specificity"; (3) "mode of action" and "Cry proteins"; (4) "mode of action" and "Cry proteins" and "specificity"; (5) "mode of action" and "Cry8 proteins"; (6) "mode of action" and "Cry8Ka5".

2.4. Proteins production and characterization

The Cry8Ka5 protein was obtained from the expression of the *cry8Ka5* gene in *E. coli* BL21 (DE3) containing the mutant gene inserted into PET101/D TOPO plasmid (Invitrogen) as well described by Oliveira et al. (2011). The Cry1Ac protein was firstly obtained as a protoxin by heterologous expression in *E. coli* JM109 (Promega) transformed with the recombinant plasmid pKK (Amershan) containing the *cry1Ac* gene of the *Bt* var. *kurstaki* HD73 as well described by Farias et al. (2014).

The production of both proteins was monitored through quantification of soluble proteins by the Bradford method (Bradford, 1976), using a curve constructed with bovine serum albumin as standard. Besides, the purity of Cry8Ka5 and Cry1Ac and activation of the last one were monitored by 12.5% SDS-PAGE (Laemmli, 1970).

From the SDS-PAGE profile of Cry8Ka5 and Cry1Ac, the percentage of relative purity of both proteins was calculated using the Image Master 2D platinum (v.7.0, GE Healthcare) software. This methodology has been adopted successfully by our research team, and it is also widely used by chemical companies to show the purity of commercialized proteins.

Before the use in the *in vitro* and *in vivo* tests, batches of the proteins obtained were also checked for their bioactivity against their respective target insects. The Cry8Ka5 protein was tested against *A. grandis* larvae according to the methodology described by Oliveira et al. (2011) and the Cry1Ac against *Spodoptera frugiperda* larvae following the method described by Grossi-de-Sa et al. (2007).

2.5. N-terminal sequence determination

To confirm the identity of the proteins obtained, the N-terminal amino acid sequences of Cry8Ka5 and Cry1Ac were determined on a Shimadzu PPSQ-10 automated protein sequencer (Kyoto, Japan) performing Edman degradation (Edman, 1964). The sequence was determined from protein blotted on polyvinylidene fluoride (PVDF) after Tricine-SDS-PAGE. Phenylthiohydantoin (PTH) amino acids were detected at 269 nm after separation on a reversed phase C18 column (4.6 mm × 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. The sequences obtained were compared with available amino acids sequences at UniProtKB database (http://www.uniprot.org/) using the FASTA3 search program. In addition, these sequences were aligned in the Uniprot platform (http://www.uniprot.org/) to the complete sequences of Cry8Ka5 and Cry1Ac previously determined by our group.

2.6. In vitro *digestibility and thermal stability*

Susceptibility to *in vitro* digestion of the proteins were evaluated by incubating the Cry8Ka5 and Cry1Ac in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) at a concentration 0.25 mg/mL, in a non-sequential manner. The SGF is a mixture of 34 mM NaCl, 0.7% HCl, pH 1.0–2.0 and 3.2 mg/mL of pepsin (Sigma P7012), while the SIF is composed of 50 mM potassium phosphate, pH 7.5 and 10 mg/mL of pancreatin (Sigma P7545), both prepared according to the recommendations of Roesler and Rao (2001). The mixture of each protein in SGF or SIF was incubated at 37 °C, under constant agitation, was removed of aliquots of 200 μL after 0, 15, 30, 60 s, 2, 5, 10, 20 and 30 min. For SGF incubation, each aliquot of the mixtures was transferred to 200 μL of a stop solution containing 2% SDS, 10% glycerol, 6% β-mercaptoethanol, 0.01% bromophenol blue, 200 mM DTT and 500 mM Tris-HCl pH 7.2. For SIF incubation, the same volume of each aliquot was mixed to a stop solution containing 3% SDS, 17% glycerol, 8.5% β-mercaptoethanol, 0.01% bromophenol blue, 170 mM DTT, 6 mM PMSF, 200 mM Tris-HCl pH 7.2. For the 0 time controls, SGF or SIF and test protein were added directly to their respective stop solutions. After being mixed with stop solution, the samples were subjected to heat treatment (100 °C, for 5 min). The tests were run at least in duplicate for each protein in the different fluids. The digestibility of proteins in SGF and SIF was monitored by 12.5% SDS-PAGE.

The thermal stability of the proteins was evaluated by incubating the Cry8Ka5 and Cry1Ac proteins in 1.5 mL of 20 mM Tris-HCl with 5 mM EDTA, at a concentration of 0.25 mg/mL of buffer solution. Proteins were subjected to a temperature of 100 °C for periods of 10, 30 and 60 min, and duplicates for each protein and for each

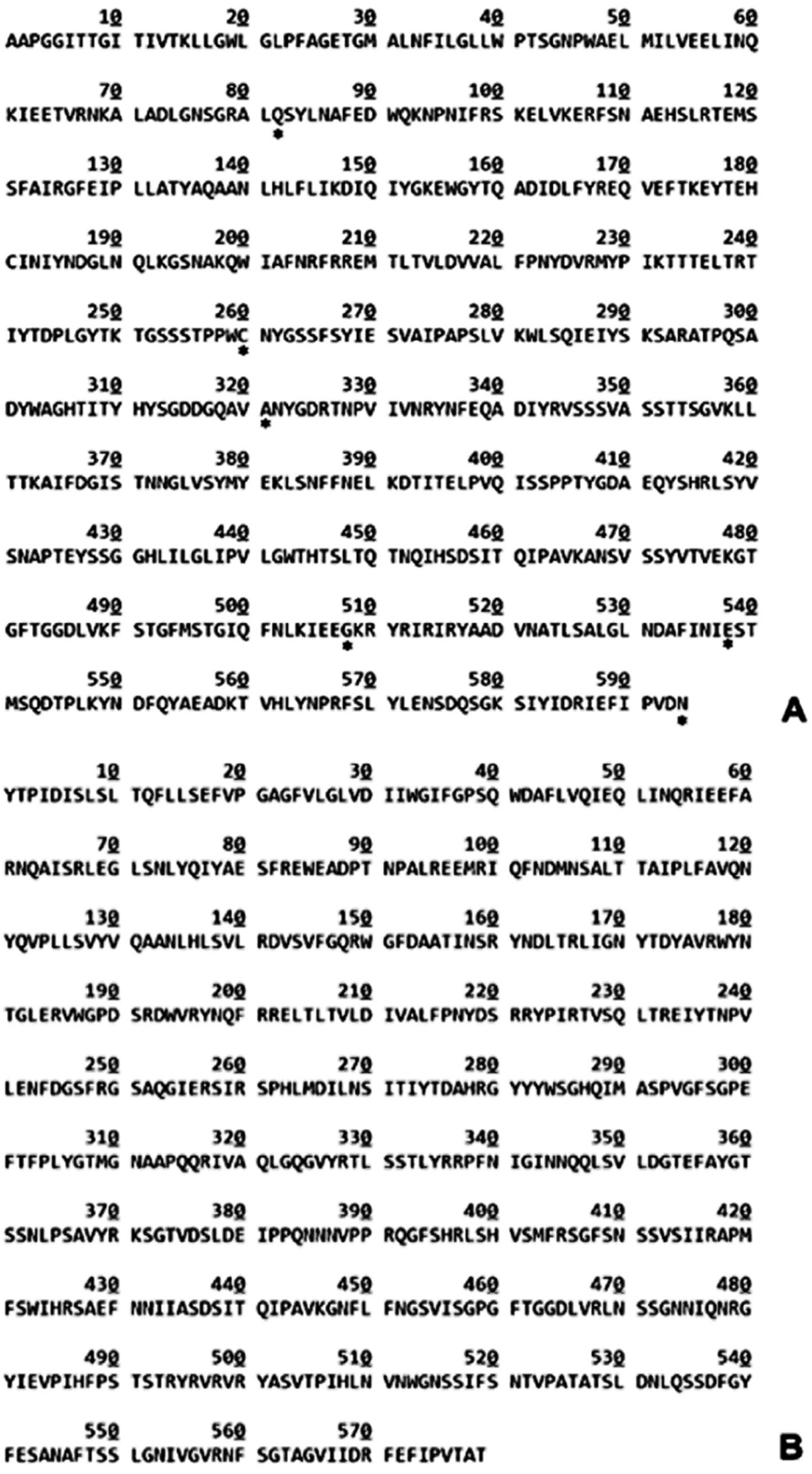

Fig. 1. Amino acids sequences of Cry8Ka5 (A) and Cry1Ac (B) active forms used in the bioinformatics analysis. The amino acids marked with an asterisk in the Cry8Ka5 sequence represent the changes in the sequence of the original protein (Cry8Ka1) as indicated by Oliveira et al. (2011).

time of exposure were performed. The test was stopped by immersing the tubes with the samples on ice and adding sample buffer (50 mM Tris-HCl, 8% sucrose, 2% SDS, 5% 2-mercaptoethanol and 0.02% bromophenol blue). Control samples with no incubation of proteins (kept at 4 °C) were also prepared (Hérouet et al., 2005). After treatments, samples were analyzed for integrity by 12.5% SDS-PAGE.

2.7. Animals and housing conditions

Fifteen conventional female mice, three weeks old, of Biocen-UFC outbred stock originally from Unib:SW (Swiss) (Unicamp, 1965, São Paulo, Brazil) mouse stock were acquired from the Central Animal Facility of Federal University of Ceará (UFC, Fortaleza, Brazil). The animals were housed at the Department of Biology, at the same University, with temperature (23.0 ± 2.0 °C), photoperiod (12 h of light/12 h of dark) and humidity (45–55%) controlled. The mice were kept in adequate numbers in polypropylene cages with pine shavings as substrate and water and feed (Biobase, Bio-Tec, São Paulo, Brazil) *ad libitum* until they reach the approximate weight of 20 g.

All protocols with animals adopted in this work were submitted for approval by the Ethics Committee on Animal Research of the Federal University of Ceará, which adopts the principles of the Brazilian College of Animal Experimentation (COBEA) and follows the Law n° 11.794 of 8 October 2008 ("Lei Arouca") that regulates the use of animals in the scientific research in Brazil.

2.8. Acute oral toxicity assay

An acute oral toxicity study (single dose) of Cry8Ka5 and Cry1Ac was performed following the general principles of the OECD guideline 425, limit test (OECD, 2001). Two modifications in the cited method were done, such as the inclusion of a control group (vehicle), and performing blood biochemistry, hematology, histopathology and relative wet weights analysis of organs in all animals at the end of the trial. These can increase our certainty about potential deleterious effects observed, and provide evidences to indicate further hypothesis-based studies.

In detail, female mice (n = 5/per group) of Biocen-UFC outbred stock 5 weeks of age and weighing 18–22 g were used. Separately, the Cry proteins were suspended in distilled water and administered in a single dose of 5000 mg/kg of body weight by gavage to each mouse. One group of mice administered only with the vehicle, distilled water, was also performed. All mice were observed within the first hours after administration of the proteins and then twice daily for 14 days. The possible intervention of the proteins on the natural behavior of mice was observed, as well as evidences of toxicity from the verification of the following symptoms/characteristics: piloerection, anesthesia, motor activity, vocal tremor, touch response, balance, presence of writhing, tremors, ptosis and presence of misshapen or dark feces. The weight of all mice was recorded on days 1, 3, 7, 10 and 14. On day 14, the animals were slightly sedated with halothane (Zeneca, São Paulo, Brazil) and exsanguinated via retro-orbital sinus for determination of hematological and serum biochemical parameters. Then the animals were sacrificed by cervical dislocation and dissected for observation (monitored by a pathologist) of the anatomical and morphological condition of the vital organs, and then carefully weighed to obtain the wet weight. Finally, the organs were immersed in 70% ethyl alcohol for 24 h and then transferred to 10% formalin until the realization of the histological routine.

2.9. Hematology and serum biochemistry

A part of the blood collected at the end of the acute oral toxicity were kept in heparinized tubes for determination of hematological parameters and another part was collected to obtain serum for the determination of biochemical parameters. For hematological analysis an aliquot of 15 μL of blood of each animal was applied into a veterinary hematology analyzer (Sysmex, model 100iV Poch – Diff, Kobe, Japan). The analyzed parameters were: number of white blood cells (leukocytes), number of red blood cells (RBCs), hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, % lymphocytes of the total white cell, % of neutrophils, basophils and monocytes, red cell distribution width measured as coefficient of variation (RDW-CV) and red cell distribution width measured as standard deviation (RDW-SD). From serum samples of the animals the following biochemical parameters were analyzed: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, urea, total protein, albumin, total cholesterol, triglycerides and creatinine. Serological analyzes were performed using spectrometric kits (Bioclin, Quibasa, Belo Horizonte, Brazil) for each parameter, following the manufacturer's instructions.

2.10. Organs wet weight and pathology

At the end of the experiment of acute toxicity, the animals were carefully dissected and the organs/tissues were checked for their appearance (color, presence of stains and/or anatomical irregularities) by a histopathologist, and then weighed in a precision balance (0.0001 g). Subsequently, the organs were fixed with 70% ethanol for 24 h, and then with 10% formalin. The organs/tissues dissected were: brain, thymus, heart, lungs, liver, spleen, pancreas, stomach, small intestine (duodenum, jejunum and ileum, but the only part that continued for histopathology was duodenum), large intestine, kidneys, bladder, oviduct + uterus and ovaries.

All organs/tissues fixed were processed at various stages of dehydration with ethanol in increasing concentrations (from 70 until 100%), diaphanization with xylene, and then embedded in paraffin until solidification. Later, cuts were made in a microtome and then stained with hematoxylin and eosin. The blades of each structure were examined by a histopathologist, using a light microscope.

2.11. Statistical analysis

Statistical comparisons were designed to determine if differences in the response variables described above (body weight, hematology, serum biochemical parameters and relative organ wet weights) between groups were attributable to Cry8Ka5 protein compared to the control groups, Cry1Ac and water, separately. The homogeneity of variance was analyzed by simple analysis of variance (One-way ANOVA) with the GraphPad Prism 6.0 software (San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

3. Results and discussion

3.1. History of safe use

The first step of the Cry8Ka5 food safety assessment was the search in the scientific literature on its HOSU. As there is no HOSU for this novel protein, results for *Bt* as a source of the *cryK8a* gene and three-domain Cry proteins were considered. The literature has accumulated several positive reports about the safe use of *Bt*-based products in agriculture for over 50 years (Hammond and Koch, 2012; Kumar et al., 2008; Sanahuja et al., 2011; Yu et al., 2011). In addition, the *Bt* is not considered as a source of allergens (Federici and Siegel, 2008). With respect to the proteins structurally/functionally related to Cry8Ka5 (three-domain Cry proteins), for over 15 years, varieties of corn, cotton and other GM crops expressing Cry proteins are planted without any reported adverse effect from the consumption of these GMOs or their derivatives in human beings and animals (Hammond and Koch, 2012). In addition, a great number of food safety tests, especially *in vivo* studies with laboratory rodents, has been carried out with isolated Cry proteins or incorporated in GM plants. In general, a tendency for the safety of these proteins to mammals was observed (Cao et al., 2010; Juberg et al., 2009; Liu et al., 2012; Xu et al., 2009).

3.2. Bioinformatics analysis

The *in silico* analysis of the amino acid full-length sequences of Cry8Ka5 and Cry1Ac revealed no relevant similarity to sequences of known toxic, antinutritional and/or allergenic proteins deposited in the nr NCBI database. Without the use of any keyword to limit the search or when the keyword "toxin" was used, Cry8Ka5 sequence showed to be similar to other *Bt* Cry proteins and to *B. cereus* proteins, which are not considered harmful to humans and animals. Likewise, the control *Bt* protein, Cry1Ac, showed to be similar to various other deposited Cry proteins, as well as to Cry1Ac modified forms. When the keywords "allergen" and "antinutrient" were employed, the number of hits decreased substantially and without revealing any significant findings (E-score > 0.001 and identity <35%).

To assess specifically the allergenic potential of the Cry8Ka5 and Cry1Ac proteins, their full-length sequences were compared to allergen sequences deposited in four databases. Comparisons were performed for detection of identity >50% for the complete sequence and for >35% in a window of 80 amino acids.

Search for similarity in primary amino acid sequences of Cry8Ka5 and Cry1Ac proteins in different databases of allergenic proteins showed no identity (>50%) with any allergens. This includes well-known allergen groups such as storage proteins (e.g. β-lactoglobulin B from cow's milk, ovalbumin from egg, β-conglycinin from soybean, *Ara h* 1 and *Ara h* 2 from peanut), plant lectins (e.g. peanut lectin and soybean lectin), contractile proteins (e.g. tropomyosin from shrimp) and enzymes (e.g. lysozyme from egg, papain from papaya and actinidin from kiwi fruit). The same result was observed for

Cry1Ac by Randhawa et al. (2011). The relevance of this result relies on the fact that proteins that share over 50–70% identity throughout their sequence with an allergen is likely to show cross reaction or to share the same epitopes for IgE (Aalberse, 2000).

Searching a window of 80 amino acids, being taken into account alignments with identity greater than 35% between the sequences deposited and the Cry8Ka5 and Cry1Ac sequences, no similarities were detected. The same results were observed for the Cry1C (Cao et al., 2010) and Cry1Ab/Ac (Xu et al., 2009) proteins. This is one of the most important tests for investigation of allergenic potential of a protein using bioinformatics tools. Mishra et al. (2012) investigated the allergenic potential of novel candidate proteins for the development of transgenic plants being detected with high percentage of identity (37.5 to 97.5%) between the sequences of six of these molecules with known allergens. Moreover, the authors evaluated the importance of these findings, subjecting one of these proteins, a chitinase from *Trichoderma viride*, to *in vitro* cross-reactivity test using sera from food allergic patient, which confirmed significant IgE binding.

Searches for 8, 7 and 6 contiguous identical amino acid sequences in allergen databases have been conducted to identify peptides that may represent linear IgE-binding epitopes (Ladics et al., 2011). However, these comparisons have been successively discouraged since numerous reports have described their moderate (at 8 amino acids) to high (6 and 7 amino acids) false positive predictive value (Goodman and Tetteh, 2011; Goodman et al., 2008). Primarily, the *in silico* approach for prediction of allergenicity aims to verify whether there is sufficient evidence to perform serum IgE-binding tests. Thus, for the Cry8Ka5 or Cry1Ac proteins the comparisons using the full-length sequences in a window of 80 amino acids were sufficient to refuse the necessity for additional investigation.

3.3. Mode of action and specificity

Studies on the mode of action of three-domain Cry proteins have been very intense, and more than 2000 results in the scientific literature were found. In general, there is a consensus on how they give their effects on target organisms. A general view can be found at Sanahuja et al. (2011), and is shortly described here. After ingestion, Cry proteins are activated in proper environmental conditions of the target insect midgut by specific proteases. Each activated toxin binds to specific receptors on the brush border membranes, and this, in turn, results in oligomerization of the Cry toxin monomers. The toxin complex translocates into the cellular membrane of the gastrointestinal tract epithelial cells and forms pores. This stops the movement of solutes across the intestinal epithelium and causes an influx of water that culminates in the death of the insect. With respect to Cry8 proteins, that includes Cry8Ka5, it is known that they follow the general principles established for Cry proteins.

As to the specificity of three-domain Cry proteins, it is known that they are selectively toxic for certain insect classes (or even insect species) (Soberón et al., 2010). In the case of Cry8 proteins, they are active mainly against coleopteran insects (Bravo et al., 1998). In this sense, being selectively active against certain insect classes also means possession of little or no effect against human beings and other non-target animals. In this context, the proper conditions of the insect midgut and the presence of specific receptors are crucial points of the mechanism of action of Cry toxins to determine their specificities. In the case of coleopteran-active Cry proteins (it comprises Cry8 proteins), the protoxin must be solubilized in neutral to slightly acidic pH, and then must be cleaved (activated) by cysteine proteases. The Cry toxin binds to specific receptors located on the microvilli membrane of the midgut epithelium columnar cells. Several protein receptors for Cry proteins have been described, but four are the best characterized: cadherin-like protein [CADR], glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP) and 270 kDa glycoconjugate (Soberón et al., 2010). Beyond the binding of Cry proteins to the aforementioned receptors, oligomerization of the Cry toxin monomers must also occur to cause toxicity to the target insects. For Cry8Ka5, some protein receptors were identified in the coleopteran insect, *A. grandis* (Nakasu et al., 2010). In a hypothetical situation of Cry8 protein intake by humans, they would not find the optimal conditions for solubilization and activation in the gastrointestinal tract. Furthermore, it is very well-known that there are no high affinity Cry protein-binding receptors in mammals (Hammond and Koch, 2012).

3.4. Recombinant proteins production and characterization

SDS-PAGE profiles of Cry8Ka5 and Cry1Ac recombinant proteins, as well as some steps of the expression and purification process, are shown in Fig. 2. Both entomotoxins presented the expected molecular mass (near to 70.0 kDa). From the SDS-PAGE profiles it was possible to estimate the relative purity of proteins using an adequate software. Either for Cry8Ka5 or Cry1Ac the different batches of expression and purification presented relative purity which ranged from 75% up to 95%.

The obtained Cry8Ka5 and Cry1Ac proteins had their bioactivity checked against the target insects. This can contribute to confirm the identity of the proteins that are under tests as well as to assert the correct expression and processing of those in the *E. coli* system. The batches of Cry8Ka5 and Cry1Ac used in the *in vitro* and *in vivo* tests in the present study showed to be active against *A. grandis* and *S. frugiperda* larvae, respectively, in the expected magnitude.

The first twenty (AAPGGITTGITIVTKLLGWL) and twenty-three (YTPIDISLSLTQFLLSEFVPGAG) amino acids from the N-terminal side of the active forms of Cry8Ka5 and Cry1Ac, respectively, were identical to the sequences previously obtained by our team as shown in Fig. 1.

3.5. In vitro *digestibility and thermal stability*

As shown in Fig. 3, Cry8Ka5 (A) and Cry1Ac (B) proteins were highly susceptible to SGF digestion, being degraded in only 2 min of incubation. Both proteins were resistant to SIF digestion (at least for the maximum time of incubation tested, 30 min) as shown in Fig. 4. Although the relationship between resistance to pepsin digestion and allergenicity is not absolute, data on digestibility taken together with other protein features (e.g. high amino acid sequence homology to allergens) have been considered appropriate to evaluate the allergenic potential of a novel protein (Delaney et al., 2008; Codex Alimentarius Commission, 2009; Foster et al., 2013). Other Cry proteins evaluated, such as Cry1C (Cao et al., 2010) and Cry1Ab/Ac (Xu et al., 2009), were also quite susceptible to the action of pepsin and resistant to digestion in simulated intestinal fluid throughout the duration of the test (30 min). The resistance of the Cry1Ac protein to pancreatin degradation has already been reported; however, when this protein was subjected to sequential digestion, the peptides that arrived in the simulated intestinal fluid could be completely digested (US EPA, 2010). This fact has great relevance, since proteins are efficiently digested in the stomach, reaching the intestine as simple amino acids or, at most, as peptides of low molecular mass.

Regarding the thermal stability of Cry8Ka5 and Cry1Ac, both recombinant proteins were resistant to the hardest heat treatment tested (100 °C for 1 h) (Fig. 5). Usually, the thermal stability of a protein has been associated to the presence of allergenic potential. Xu et al. (2009) showed that Cry1Ab/Ac protein was also resistant to heat treatment under the same conditions. The importance of this finding is difficult to interpret and, in addition, this test has been severely criticized on its actual usefulness in the context of a food safety study, its implementation in later tests being discouraged (Privalle et al., 2011).

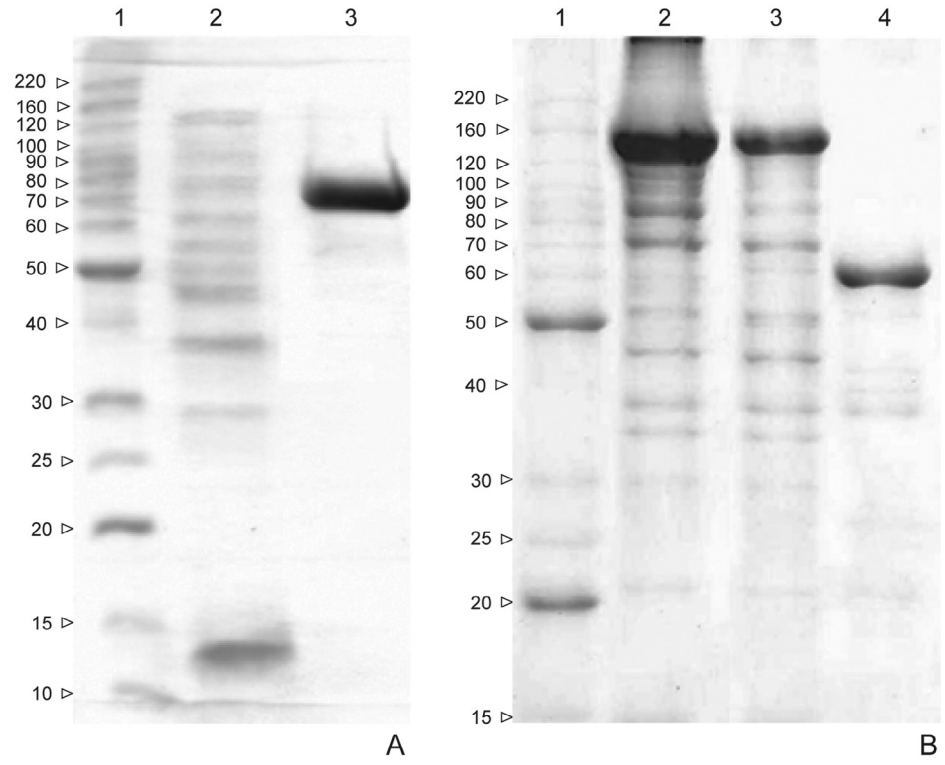

Fig. 2. (A) 12.5% SDS-PAGE profiles of the heterologous expression products of Cry8Ka5 protein. Lane 1: molecular mass marker (BenchMark Protein Ladder, Invitrogen); Lane 2: Peak not retained on the Ni-NTA chromatography resin; Lane 3: Peak retained on the Ni-NTA chromatography resin eluted with 20 mM imidazole. (B) 12.5% SDS-PAGE profiles of the heterologous expression products of the Cry1Ac protein. Lane 1: molecular mass marker; Lane 2: Cry1Ac protoxin not dialyzed; Lane 3: Cry1Ac protoxin dialyzed; Lane 4: Cry1Ac toxin after trypsinization and dialysis.

3.6. Acute oral toxicity assay

At the end of the Tier I, it can be concluded that Cry8Ka5 has no potential hazard to human beings. However, performing an acute oral toxicity test (single dose) in mice is mandatory for some regulatory agencies such as the Brazilian National Biosafety Technical Commission (CTNBio) for *Bt* microbial pesticides or Cry proteins that end up in food and feed crops. The rationale for this recommendation is based on the fact that Cry proteins act through an acute mode of action to kill insect pests. In addition, the use of mouse as a model for such testing have been considered adequate because it is susceptible to the toxic effects of several known mammalian protein toxins (Delaney et al., 2008). In the framework of the two-tiered approach, this testing could be characterized as a part of the Tier II. In view of this, groups of female mice were subjected to a single dose (5000 mg/kg body weight) of Cry8Ka5 or Cry1Ac (control *Bt* protein), and a third group received only distilled water. After 14 days of observation, the animals of all groups did not show any apparent symptoms of poisoning or abnormal behavior. In addition, body weight was measured periodically during the observation period (days 1, 3, 7, 10 and 14). These data were plotted on a graph as shown in Fig. 6. The distilled water group, on average, showed a slight tendency to gain more body weight than animals in the other groups. However, this difference was not significant ($p > 0.05$) for any of the measurements (days). This result reflects generally the absence of antinutritional effects of the administered protein, since it is known that some classes of proteins (protease inhibitors, lectins, etc.) interfere with the digestion and absorption of dietary proteins (Carvalho et al., 2011). Xu et al. (2009) and Cao et al. (2010) found the same result in a study with Cry1Ab/Ac and Cry1C, respectively, both at a dose of 5000 mg/kg body weight.

At the end of the 14 days of observation, the animals were sacrificed and their internal organs dissected for measuring the relative wet weight as shown in Table 1. No significant differences between the organs' relative wet weight of the Cry8Ka5 group and those from the control groups (Cry1Ac and water groups) were observed. Some normal variations among all experimental groups were detected. When animals were administered with Cry1Ab/Ac, bovine serum albumin or only the vehicle (water), relative increase or decrease

Table 1
Relative organ wet weights (g wet tissue/g body weight, %) of female mice (n = 5/group) administered orally with a single dose (5000 mg/kg of body weight) of Cry8Ka5 and Cry1Ac (control *Bt* protein) recombinant proteins, and with distilled water (the vehicle).

Organs	Groups		
	Cry8Ka5	Cry1Ac	Distilled water
Brain	2.58 ± 0.28	2.47 ± 0.23	2.46 ± 0.14
Thymus	0.47 ± 0.10	0.45 ± 0.05	0.41 ± 0.10
Heart	0.89 ± 0.11	0.84 ± 0.08	0.90 ± 0.12
Lungs	0.93 ± 0.09	0.88 ± 0.09	0.89 ± 0.11
Spleen	0.38 ± 0.04	0.40 ± 0.07	0.39 ± 0.09
Stomach	1.25 ± 0.10	1.06 ± 0.19	1.17 ± 0.20
Duodenum	2.97 ± 0.61	2.73 ± 0.15	2.92 ± 0.28
Jejunum	2.56 ± 0.47	2.20 ± 0.34	2.64 ± 0.35
Ileum	1.87 ± 0.11	1.75 ± 0.24	1.81 ± 0.29
Large intestine	3.85 ± 0.35	3.34 ± 0.47	3.15 ± 0.27
Pancreas	0.90 ± 0.29	0.83 ± 0.20	0.69 ± 0.15
Liver	7.53 ± 0.53	7.17 ± 0.55	7.29 ± 0.57
Kidneys	2.04 ± 0.13	2.03 ± 0.13	2.06 ± 0.13
Bladder	0.12 ± 0.01	0.11 ± 0.03	0.13 ± 0.02
Ovaries	0.05 ± 0.01	0.05 ± 0.02	0.03 ± 0.01
Oviduct + uterus	0.64 ± 0.16	0.59 ± 0.15	0.57 ± 0.10

Values are means ± standard deviation (n = 5); $p > 0.05$ (One-way ANOVA) for Cry8Ka5 group compared with the water group, Cry8Ka5 group compared to Cry1Ac and Cry1Ac compared to water.

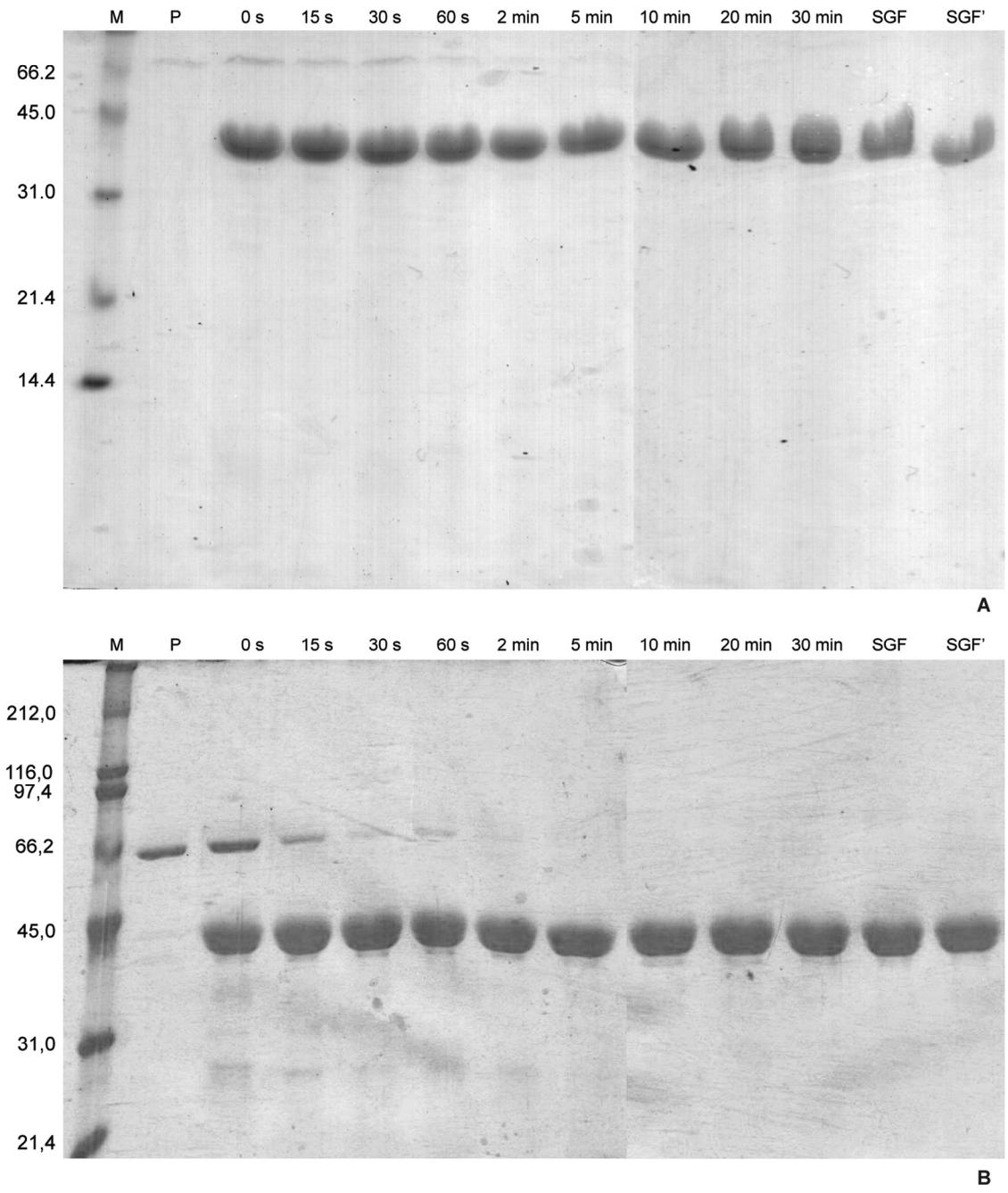

Fig. 3. Digestion in simulated gastric fluid (SGF) of Cry8Ka5 (A) and Cry1Ac (B) proteins, both at a concentration of 0.25 mg/mL. M, molecular mass markers; Cry8Ka5 (A) and Cry1Ac (B) (both close to 66.2 kDa marker) without SGF; Cry8Ka5 (A) and Cry1Ac (B) incubated in SGF for 0, 15, 30, 60 s, 2, 5, 10, 20 and 30 min; SGF, SGF without incubation; SGF′, SGF incubated for 30 min.

of some vital organs were found for all groups (Xu et al., 2009). This is an example that results must be interpreted in an integrative approach, taking into account all the results obtained in the context of a food safety study.

Blood was collected from the animals of each group at the end of the trial period, and led to reading on a hematology analyzer. The values of the measured parameters are shown in Table 2. Among all parameters analyzed, only the number of leukocytes of the Cry8Ka5 ($5.28 \pm 0.41 \times 10^3/\mu L$) and Cry1Ac ($7.02 \pm 0.61 \times 10^3/\mu L$) groups called attention, which were significantly lower ($p < 0.05$) than the water group ($8.38 \pm 0.87 \times 10^3/\mu L$), but that were not different ($p > 0.05$) from each other. The platelet counting of Cry8Ka5 group ($507.00 \pm 47.02 \times 10^3/\mu L$) was also significantly lower ($p < 0.05$) than the water group (689, $25 \pm 39.70 \times 10^3/\mu L$), but was similar ($p > 0.05$) to the Cry1Ac group ($620.50 \pm 52.56 \times 10^3/\mu L$). In addition, the lymphocyte counting had fewer cells ($p < 0.05$) for the Cry1Ac group ($4.18 \pm 0.43 \times 10^3/\mu L$) than for the water group ($6.30 \pm 0.63 \times 10^3/\mu L$), but Cry8Ka5 group ($3.50 \pm 0.18 \times 10^3/\mu L$) was not different ($p > 0.05$) from the others. Finally, the RDW-CV values of Cry1Ac group ($17.48 \pm 0.77\%$) was significantly greater ($p < 0.05$) than the water group ($16.20 \pm 0.66\%$), but both were not different from the Cry8Ka5 group (17.52 ± 0.55 %).

Probably, the most relevant finding to the Cry8Ka5 group was the lower number of platelets when compared to the control group, water. Low platelet count is related to decreased production or increased destruction of these cells. A reduction in output would be

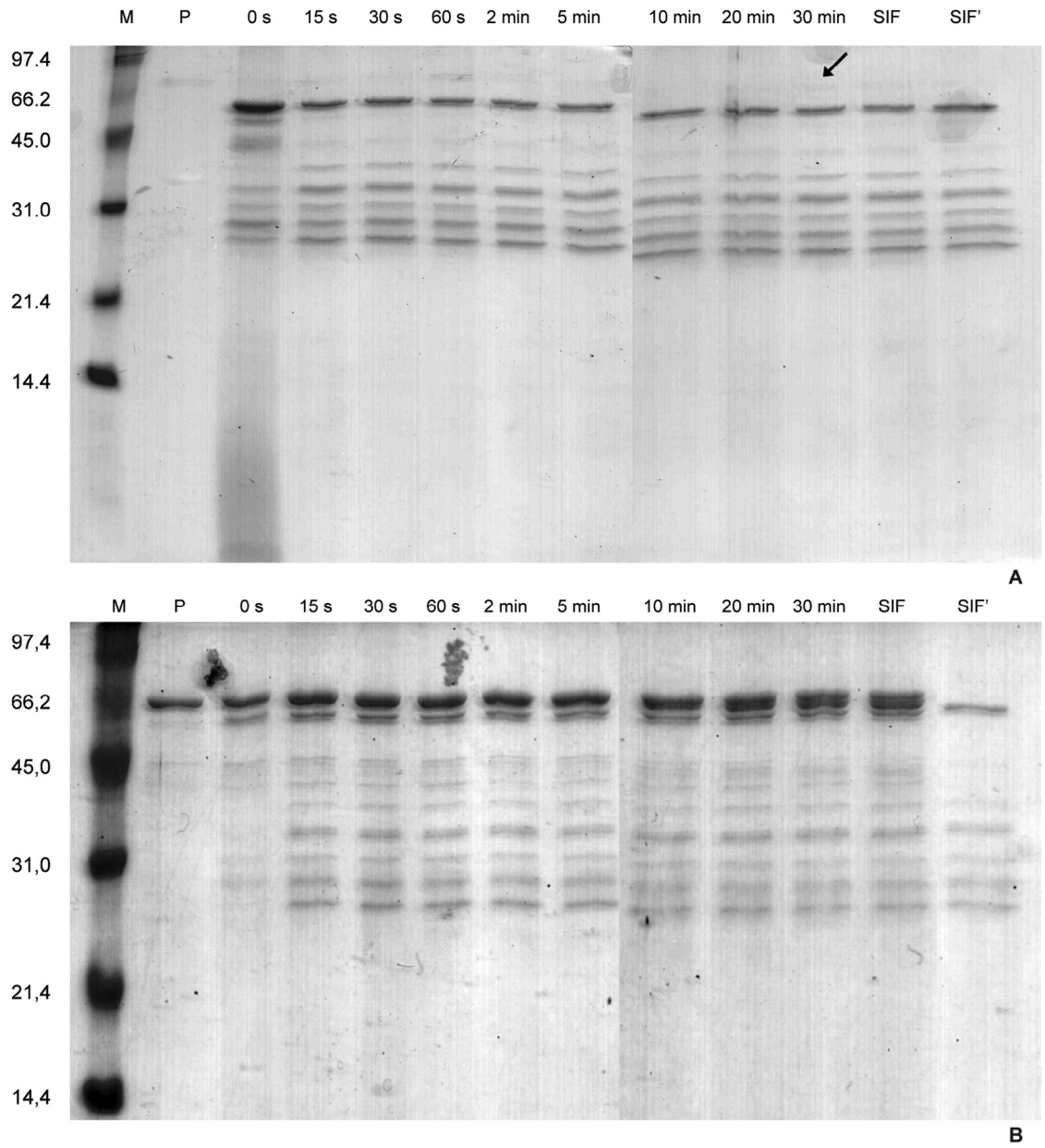

Fig. 4. Digestion in simulated intestinal fluid (SIF) of Cry8Ka5 (A) and Cry1Ac (B) proteins, both at a concentration of 0.25 mg/mL. M, molecular mass markers; P, Cry8Ka5 (A) and Cry1Ac (B) (both close to 66.2 kDa marker) without SIF; Cry8Ka5 (A) and Cry1Ac (B) incubated in SIF for 0, 15, 30, 60 s, 2, 5, 10, 20 and 30 min; SIF, SIF without incubation; SIF′, SIF incubated for 30 min. The black arrow is indicating the Cry8Ka5 remaining.

related to injuries or changes in the megakaryocytes which produce platelets, while the increased destruction occurs from an accelerated or immune activation of platelets recognition (Hedrich, 2012). At this time, it is difficult to assert that this decrease (non-severe) in platelet counting for Cry8Ka5 group is related to one of the situations mentioned. In addition, this fact does not appear to have great importance since the number of platelets in this group did not differ from another reference group, Cry1Ac.

Analysis were carried out to determine some serum parameters of the animals from all groups, and the results are presented in Table 3. Differences were detected between the groups of mice only for the values of total protein, AST and urea. The measurement of total protein in the serum of the Cry1Ac group (6.49 ± 0.64 g/dL) showed a significant difference ($p < 0.05$) when compared to the average value of the water group (6.75 ± 1.00 g/dL), but none of the groups were different ($p > 0.05$) from the Cry8Ka5 group (6.49 ± 0.64 g/dL). AST values of Cry8Ka5 (248.50 ± 10.92 U/L) and Cry1Ac (244.67 ± 5.36 U/L) groups were both significantly higher ($p < 0.05$) than the water group (230.33 ± 2.25 U/L). As for measurement of serum urea, Cry8Ka5 group (62.68 ± 5.33 mg/dL) showed a greater value ($p < 0.05$) than that presented by water group (48.33 ± 3.79 mg/dL), but it was not different ($p < 0.05$) from the Cry1Ac group (44.28 ± 4.50 mg/dL).

High AST levels are primarily correlated to possible liver disease or injuries to the heart or skeletal muscles (Duncan and Prasse, 1982). Likewise, increased level of urea in Cry8Ka5 group is difficult to explain, since this result suggests deterioration of the renal function. On the other hand, the level of creatinine which is also a good indicator of kidney health showed no difference among groups (Duncan and Prasse, 1982). It is likely that these increased levels of AST and urea have nothing to do with permanent injuries in the livers and kidneys, respectively, of the animals, since 14 days after

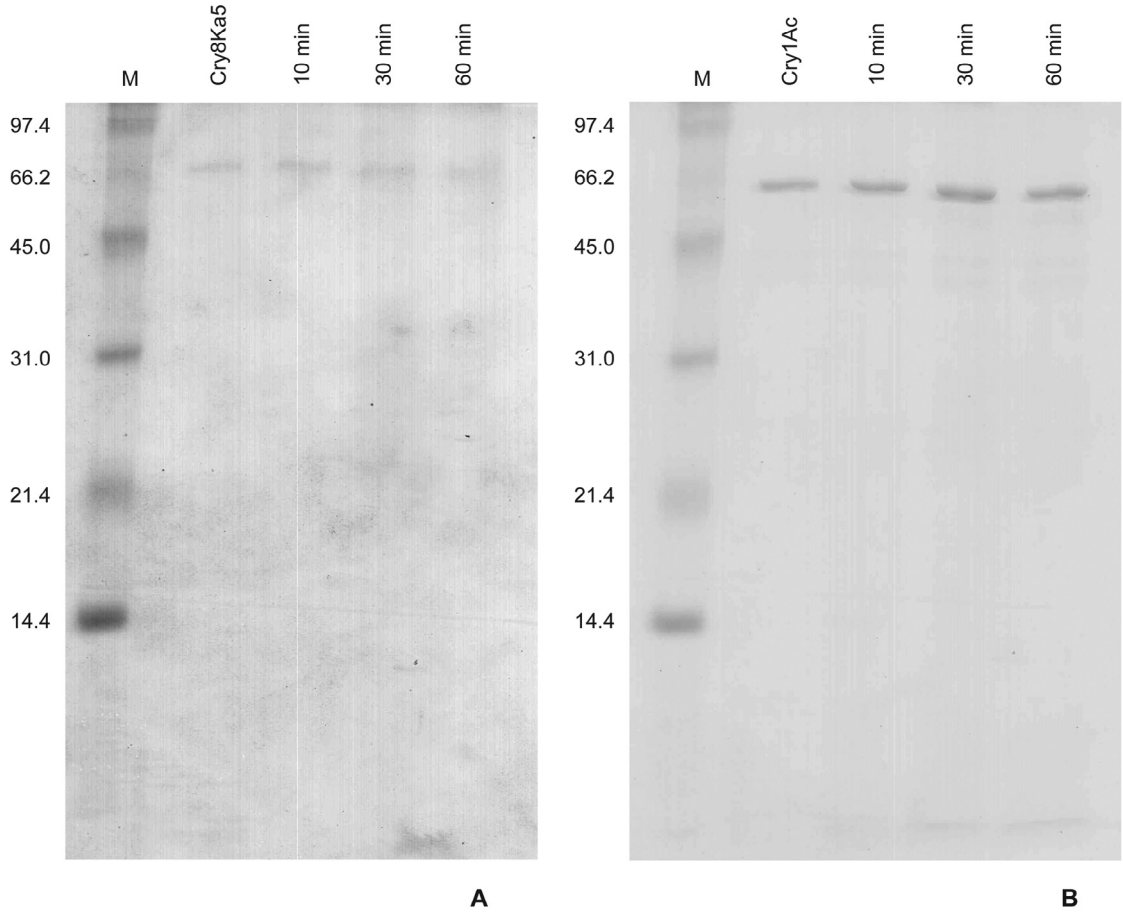

Fig. 5. Thermal stability of Cry8Ka5 (A) and Cry1Ac (B) proteins, both at a concentration of 0.25 mg/mL. M, molecular mass markers; Cry8Ka5 (A) and Cry1Ac (both close to 66.2 kDa marker) (B) at 4 °C; Cry8Ka5 (A) and Cry1Ac (B) subjected to 100 °C for 10, 30 and 60 min.

the acute exposure no histopathological changes were detected in these organs.

Despite the significant differences, in statistical terms, the changes detected in hematology and serum biochemistry were not so great. It is more likely that these changes were due to a protein overload in the animals, or even due to punctual variations within groups composed by outbred animals (Hedrich, 2012). It is noteworthy that no significant difference ($p > 0.05$) between the Cry8Ka5 and Cry1Ac (protein control) groups were detected, either in hematological or serum biochemical parameters. In a very similar way, oral acute studies with Cry1Ab/Ac (Xu et al., 2009) and Cry1C (Cao et al., 2010), both at 5000 mg/kg, using protein and vehicle controls also detected punctual differences among all groups, but unassociated

Table 2
Hematology of female mice (n = 5/group) administered orally with a single dose (5000 mg/kg of body weight) of Cry8Ka5 and Cry1Ac (control *Bt* protein), and with distilled water (the vehicle).

Parameters	Groups		
	Cry8Ka5	Cry1Ac	Distilled water
Leucocytes (10^3/μL)	5.28 ± 0.41[b]	7.02 ± 0.61[b]	8.38 ± 0.87
Erythrocytes (10^{12}/μL)	8.34 ± 0.48	8.54 ± 0.41	8.45 ± 0.43
Hemoglobin (g/dL)	13.72 ± 0.58	13.96 ± 0.95	13.72 ± 0.58
Hematocrit (%)	35.66 ± 1.86	36.58 ± 2.25	37.06 ± 0.36
MCV (fL)	42.76 ± 1.41	42.84 ± 1.89	42.58 ± 3.92
MCH (ρg)	16.46 ± 0.49	16.34 ± 0.60	16.24 ± 0.28
MCHC (g/dL)	38.50 ± 0.55	38.14 ± 0.64	37.02 ± 1.63
Platelets (10^3/μL)	507.00 ± 47.02[a]	620.50 ± 52.56	689.25 ± 39.70
Linfocytes (10^3/μL)	3.50 ± 0.18	4.18 ± 0.43[b]	6.30 ± 0.63
Neutrophils, basophils and monocytes (10^3/μL)	1.93 ± 0.20	2.65 ± 0.11	2.63 ± 0.66
RDW-SD[#] (fL)	28.84 ± 1.07	29.76 ± 1.51	27.70 ± 1.09
RDW-CV[†] (%)	17.52 ± 0.55	17.48 ± 0.77[b]	16.20 ± 0.66

Values are means ± standard deviation (n = 5).
$p > 0.05$ (one-way ANOVA) for Cry8Ka5 group compared to the group Cry1Ac.
[a] $p < 0.05$ (one-way ANOVA) for Cry8Ka5 group compared to the distilled water group.
[b] $p < 0.05$ (one-way ANOVA) for Cry1Ac group compared to the distilled water group.

Table 3
Serum biochemistry of female mice (n = 5/group) administered orally with a single dose (5000 mg/kg of body weight) of Cry8Ka5 and Cry1Ac (control *Bt* protein), and with distilled water (the vehicle).

Parameters	Groups		
	Cry8Ka5	Cry1Ac	Distilled water
Total protein (g/dL)	6.49 ± 0.64	6.75 ± 1.00[b]	5.39 ± 0.37
Albumin (g/dL)	3.53 ± 0.19	3.57 ± 0.24	3.56 ± 0.33
Alkaline phosphatase (U/L)	94.00 ± 7.38	77.43 ± 6.95	88.57 ± 8.81
AST (U/mL)	248.50 ± 10.92[a]	244.67 ± 5.36[b]	230.33 ± 2.25
ALT (U/mL)	157.41 ± 7.48	156.94 ± 3.98	156.47 ± 4.30
Urea (mg/dL)	62.68 ± 5.33[a]	44.28 ± 4.50	48.33 ± 3.79
Creatinine (mg/dL)	0.46 ± 0.05	0.43 ± 0.11	0.44 ± 0.05
Cholesterol (mg/dL)	119.33 ± 19.98	117.53 ± 11.20	107.22 ± 22.09
Triglycerides (mg/dL)	210.65 ± 77.16	154.17 ± 10.30	180.56 ± 30.45

Values are means ± SD (n = 5).
$p > 0.05$ (ANOVA) for Cry8Ka5 group compared to the Cry1Ac group.
[a] $p < 0.05$ (ANOVA) for Cry8Ka5 group compared to the distilled water group.
[b] $p < 0.05$ (ANOVA) for Cry1Ac group compared to the distilled water group.

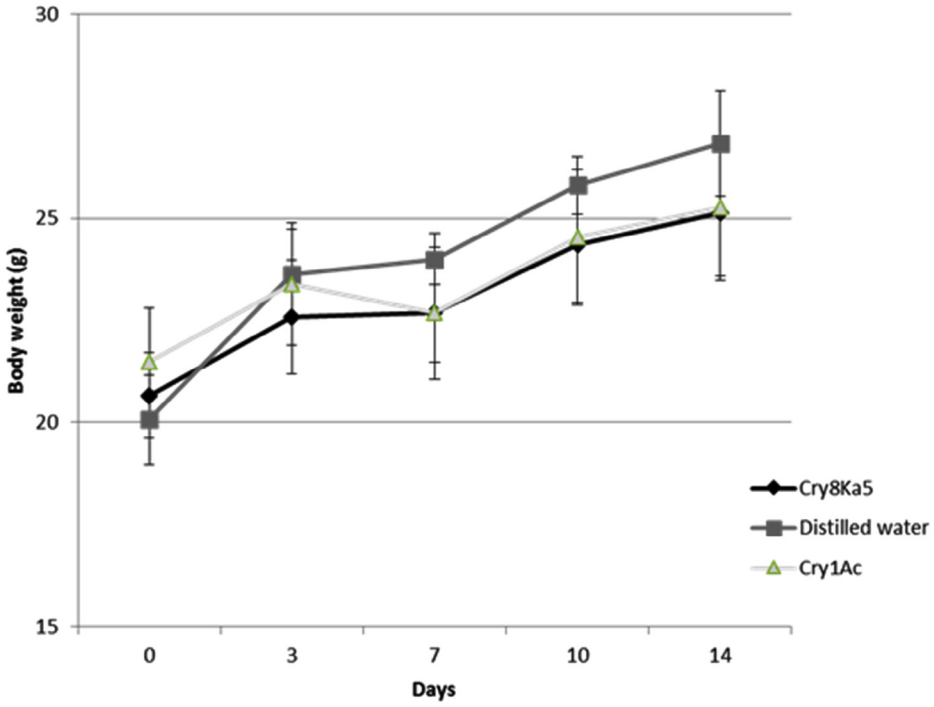

Fig. 6. Body weight evolution of female mice (n = 5/group) administered orally with a single dose (5000 mg/kg of body weight) of Cry8Ka5 and Cry1Ac (control *Bt* protein) recombinant proteins, and with distilled water (the vehicle) for 14 days. Values are means ± standard deviations for each day of weighing, with all standard deviations ≤5%. Measurements of body weights showed no significant difference ($p > 0.05$, One-way ANOVA) between groups.

exclusively with the exposure to the test proteins. They considered these differences as the result of spontaneous alterations.

During the dissection of the animals of all experimental groups, no macroscopic changes were observed. Subsequently, the dissected organs were conducted for histopathological analysis. No histopathological observations related to a specific group were observed. The few spontaneous findings observed can be classified from minimum to mild (in terms of severity), being randomly distributed among all groups and frequently observed in the water group mice.

Thus, we can attest that the LD_{50} of Cry8Ka5 protein is >5000 mg/kg body weight of mice, which belongs to slightly practically non-toxic grade to mice via the oral route. For Cry1Ac, used as a control *Bt* protein, its safety in the tested conditions was confirmed and its LD_{50} value has increased from >4200 mg/kg, as previously reported by Betz et al. (2000), to >5000 mg/kg. Many other Cry food safety studies had similar LD_{50} results, for example, Cry1Ab > 4000 mg/kg (Betz et al., 2000), Cry1Ab/Ac > 5000 mg/kg (Xu et al., 2009), Cry1C > 5000 mg/kg, (Cao et al., 2010) and Cry3A > 5,220 mg/kg (Betz et al., 2000).

4. Conclusions

The two-tiered, weight-of-evidence approach to protein hazard assessment proposed by ILSI provided significant information on the food safety of Cry8Ka5, a mutant protein from *Bt*, candidate to be used for developing transgenic plants resistant to coleopteran attack. The Tier I testing showed for Cry8Ka5: a history of safe use (based on data for *Bt* and three-domain Cry proteins) with no convincing hazard reports; no amino acid sequence homology with antinutrients, toxins or allergens; and that its putative mode of action and specificity are strictly related to the insect biology. Besides, Cry8Ka5 is resistant to pancreatin digestion and heat treatment, but it is rapidly degraded by pepsin as also shown for Cry1Ac. The Tier II testing (acute oral toxicity test in mice at 5000 mg/kg body weight) revealed no mortality, no signs of intoxication/behavior change and no alterations on body weight gain or in the organs relative wet weight of the animals from Cry8Ka5 group. Significant changes in some hematological and serum biochemical parameters were detected between the Cry8Ka5 and distilled water groups, but no difference were evidenced between the Cry groups. The LD_{50} for Cry8Ka5 was >5000 mg/kg, which was the same found for Cry1Ac. If taken together and in comparison with Cry1Ac, the results indicate that there are no expected relevant risks associated with the consumption of Cry8Ka5 protein.

This early food safety assessment of Cry8Ka5 mutant entomotoxin contributed sufficient evidence to support the use of this protein for developing *Bt* plants. Indeed, it is noteworthy that the results presented here were based in a Cry8Ka5 sample expressed by *E. coli*. These do not exempt this protein from a further structural and functional characterization in the framework of the activities for commercial release of a transgenic plant expressing this protein.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

Transparency document

The Transparency document associated with this article can be found in the online version.

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