



Safety assessment of Cry1Ab/Ac fusion protein

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ABSTRACT

Cry1ab/ac gene was fused by both the *cry1ab* gene (GenBank Accession No. X54939) and the *cry1ac* gene (GenBank Accession No. Y09787), which was widely used in genetically modified (GM) rice, cotton, maize and so on. In order to support the safety assessment of GM food or feed products containing Cry1Ab/Ac protein, sufficient quantities of Cry1Ab/Ac protein were produced in *Escherichia coli* for in vitro evaluation and animal studies. The Cry1Ab/Ac protein does not possess the characteristics associated with food toxins or allergens, i.e., it has no sequence homology with any known allergens or toxins, and no N-glycosylation sites, can be rapidly degraded in gastric and intestinal fluids, and is devoid of adverse effects in mice by gavage at a high dose level of 5 g (Cry1Ab/Ac protein)/kg body weight. In conclusion, there is a reasonable certainty of no harm resulting from the inclusion of the Cry1Ab/Ac protein in human food or animal feed.

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1. Introduction

Insect pests cause the destruction of 20–30% of the world's crops annually (Estruch et al., 1996). GM crops are becoming an increasingly important feature of agricultural landscapes. As one of the most widely grown GM crops worldwide (James, 2004), *Bacillus thuringiensis* (Bt) corns were genetically modified by introducing the Bt gene since 1987 to combat the crop damage. The *Cry1ab/ac* gene in this study originates from the *cry1ab* gene (GenBank Accession No. X54939) and the *cry1ac* gene (GenBank Accession No. Y09787) and its encoded Cry1Ab/Ac protein is high toxic to *Lepidopteran* pests such as *Chilo suppressalis*, *Scirpophaga incertulas*, *Cnaphalocrocis medinalis*.

Vazquez et al. indicated that Cry1Ac was a potent systemic and mucosal immunogen and its protoxin (pCry1Ac) binded to the mucosal surface of the mouse small intestine by immunohistochemical test (Vázquez-Padrón et al., 2000, 1999; Moreno-Fierros et al., 2000). Moreover, this protein induced in situ temporal changes in the electrophysiological properties of the mouse jejunum. The above data indicated a possible interaction in vivo of Cry proteins with the animal bowel, which could induce changes in the physiological status of the intestine (Vázquez-Padrón et al., 2000). But other researches concluded that GMO (Bt-Cry1Ac gene) cottonseed meal had no deleterious effect on growth performance,

blood biochemicals and various carcass characteristics of growing broiler chickens (Elangovan et al., 2006). The GM cottonseed expressing Cry1F, Cry1Ac and PAT proteins had no adverse effects in 90 days of feeding test (Dryzga et al., 2007). Different from Cry1Ac protein, Cry1Ab is testified to be harmless by several studies (Schröder et al., 2007; Onose et al., 2008; Bondzio et al., 2008; Kroghsbo et al., 2008). In this study, Cry1Ab/Ac protein as a new member of the family and the safety assessment of Cry1Ab/Ac protein is necessary for more widely use of the *Cry1ab/ac* genes in rice. Although the separate Cry1Ab and Cry 1Ac are nothing toxic, the changed protein primary structure will lead to the secondary structure change, and then protein digestion characteristics and thermal stability will be changed too. And two fusion proteins may also lead to a new protein associated with the allergens or new glycosylation sites. Further more there are a lot of food allergy we had no idea about whether Cry1Ab and Cry1Ac will be any kind of reaction before this study. The most important thing is that rice is very important to human beings, so safety assessment on Cry1Ab/Ac protein is very necessary.

Because of its low content in rice, Cry1Ab/Ac protein was produced in *Escherichia coli* to obtain sufficient Cry1Ab/Ac protein for in vitro evaluation and animal studies to assess the potential safety of Cry1Ab/Ac protein in human food or animal feed. In order to use this safety package with transgenic crops expressing the Cry1Ab/Ac protein, examples of structural and functional equivalence of the microorganism-produced proteins used in the toxicology tests and the proteins produced in transgenic crops are demonstrated. It is an accepted approach for the safety assessment to compare the novel protein to common food allergens. Searches of the current amino acid sequence databases for homology with

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known allergens and for the presence of N-glycosylation sites provide other points of reference. Since most food allergens resist gastric digestion, the stability of the Cry1Ab/Ac protein under these conditions is also an important consideration. To have a direct assessment of the protein toxicity in mammals, an oral acute study in mice was used for evaluating the safety of the Cry1Ab/Ac protein.

2. Materials and methods

2.1. Protein production and characterization

As the expression level of the Cry1Ab/Ac protein in transgenic rice is extremely low, it is preferable to conduct safety studies with Cry1Ab/Ac protein produced in *E. coli*. The level of Cry1Ab/Ac protein expression was typically $<3 \text{ mg kg}^{-1}$ of rice material (Cry1Ab/Ac protein in rice was tested by Envirologix Cry1Ab/Cry1Ac Kit.), and the plentiful amount of purified protein from rice for animal tests would be technically impractical. Proper characterization of the equivalence between the recombinant protein and rice expressed protein is necessary as a pre-requisite for its use in safety evaluation to support specific transgenic events.

2.1.1. Protein purification from transgenic rice leaves

The Cry1Ab/Ac protein was purified from frozen rice leaves ground in a mortar prechilled with liquefied nitrogen. Extracts were prepared by mixing ground leaves with extraction buffer in a ratio of 1:10. The extracts were centrifuged at 12,000 rpm for 10 min and further clarified by filtration through a nitrocellulose filter. The extracted Cry1Ab/Ac proteins were further purified by affinity chromatography using purified rabbit anti-Cry1Ab/Ac antibodies. Anti-Cry1Ab/Ac antibody affinity column was made using a kit (Pierce, product number 44894) according to the manufacturer's instructions. Extracts were allowed to freely flow through the column until saturation and then proteins were eluted from the antibody columns according to the manufacturer's instructions.

2.1.2. Expression and purification of Cry1Ab/Ac protein from *E. coli*

PET-30a(+)-Cry1ab/ac-rcp-BL21 (DE3) (Laboratory of Food Safety, CAU, Beijing, China) was used to highly express Cry1Ab/Ac protein. Cry1ab/ac-rcp (GenBank Accession No. EU816953) referred to the modified Cry1ab/ac gene in order to be expressed in *E. coli* and both of them encoded the same amino acid sequence. The Cry1Ab/Ac fusion protein was expressed as follows: 6 mL of an overnight grown culture was added to a 200 mL medium, and incubated with vigorous shaking at 37 °C. At $\text{OD}_{600} = 0.6\text{--}1.0$ of the culture, protein expression was induced with 1 mM IPTG at 37 °C for 4 h. Cells were collected by centrifugation and resuspended in 20 mL Buffer A (50 mM tris-HCl, 50 mM NaCl, 0.5 mM EDTA, 2% DOC, pH 8.0), and sonicated at 4 °C with 30 cycles, each cycle consisting of 10 s on and 20 s off times. The above step was repeated for twice (Marshak et al., 2000). The pellets were resuspended in 40 mL Buffer B (20 mM sodium phosphate containing 8 M urea, pH 8.0), stirred at room temperature for 20 h and the lysate was subjected to centrifugation and the supernatant was purified by His Trap™ FF crude column (Amersham Biosciences, Uppsala, Sweden). The purified protein was refolded by 0.4 M L-arginine in a linear 8–0 M urea gradient refolding buffer followed by removal of the His-Tag with enterokinase (Sigma, USA) for 16 h at 25 °C.

2.1.3. Structural and functional equivalence assay

The purified Cry1Ab/Ac protein was identified by SDS-PAGE, Western Blot, glycoprotein assay and MALDI-TOF-MS. The proteins from rice and prokaryotic cells were run on 10% SDS-PAGE, transferred onto a cellulose acetate film (Millipore Co., Bedford, Mass., USA), which incubated 2 h at room temperature in 5% skimmed milk, and then incubated 2 h at room temperature with anti-Cry1Ab/Ac rabbit polyclonal IgG, washed with TBS six times, in addition incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Wang Baomin's patent, the patent number is ZL200510130058.9), washed with TBS six times, at last developed with BCIP-NBT solution (Lu et al., 2007). Glycoprotein assay was carried out by using glycoprotein staining kit (PIERCE, Rckford) after SDS-PAGE according to the manufacturer's instructions. CBBG-stained protein bands were excised and digested in gel (Xia et al., 2005; Fernandez et al., 1998). All mass spectra of MALDI-TOF-MS were obtained on an AXIMA-CFR plus MALDI-TOF-MS (SHZMADZU Corporation, Japan). PMF obtained was compared with theoretical Cry1Ab/Ac searched through the Peptide Mass database (<http://www.expasy.ch/tools/peptide-mass.html>).

2.2. Amino acid sequence homology and potential site of glycosylation searches

2.2.1. Amino acid sequence homology search

An in silico search was conducted to identify any protein known to present potential allergenicity concerns possessing a 35% amino acid identity with the Cry1Ab/Ac proteins, on a window of 80 amino acids. To a certain extent, this evaluation may be extrapolated to potential toxicity concerns. The complete amino acid

sequence of the Cry1Ab/Ac proteins was compared with all protein sequences present in six large reference public databases: NR, Refseq_Protein, SwissProt, Pat, PDB and Env_nr. The algorithm used was BLASTP 2.2.18+ and the scoring matrix BLO-SUM62. With the same bioinformatics program (see the section above), a study was carried out by comparing the Cry1Ab/Ac protein, subdivided into eight amino acid blocks, with epitopes of all probable allergens present in the Allergen database (Hérouet et al., 2005).

2.2.2. N-glycosylation search

The full lengths of the Cry1Ab/Ac protein were examined for any N-glycosylation sites by NetNGlyc 1.0 Server (<http://cn.expasy.org>). This study was also complemented by an in vitro assay.

2.3. Heat stability study

The Cry1Ab/Ac protein was dissolved in 20 mM tris-HCl and 5 mM EDTA buffer at a concentration of 0.1 mg mL^{-1} in 1.5 mL microcentrifuge tubes. They were tested for stability at temperature of 100 °C for periods of 10, 30, and 60 min. The assay was terminated by placing the sample tubes on ice, and adding SDS-sample buffer (50 mM tris-Cl, 8% sucrose, 2% SDS, with 5% 2-mercaptoethanol, and 0.02% bromophenol blue). Control samples of a 0 min incubation of the protein (kept at 4 °C) were also prepared (Hérouet et al., 2005). The resulting proteins were analyzed by SDS-PAGE and Western Blot.

2.4. In vitro digestion of Cry1Ab/Ac protein

The concentrations of the Cry1Ab/Ac protein in simulated gastric fluid (SGF) or simulated intestinal fluid (SIF) (Sigma, USA) were 0.25 mg mL^{-1} or 0.1 mg mL^{-1} , respectively. The mixture was incubated at 37 °C and the samples were taken out at intervals of 0 s, 15 s, 30 s, 60 s, 2 min, 5 min, 10 min, 20 min, and 30 min. According to the results of SDS-PAGE and Western Blot, the stability of the protein to be digested in SGF/SIF was observed. The experiments were also done for positive control soybean trypsin inhibitor (STI) and negative control bovine serum albumin (BSA) or α -casein for SGF or SIF, respectively, as the method above (Lu et al., 2007).

2.5. Acute toxicity experiment of Cry1Ab/Ac protein in KM mice

2.5.1. Husbandry and dosing

Female and male Kun-Ming strain (KM) mice approximately 6 weeks old, weighing 18–22 g, were obtained from Experimental Animal Center of Peking University (Beijing, China). Following 5 day acclimatization they were divided randomly into three groups, 12 mice for each group (six males and six females per treatment). Then they were kept in stainless steel wire cages (two/cage) at 21–23 °C, relative humidity 40–60%, air change 15 times h^{-1} , and electric light from 09.00 to 21.00 animal rooms.

The Cry1Ab/Ac protein was dissolved in redistilled water and according to the 96.4% purity, the concentration of Cry1Ab/Ac protein was adjusted to 250 mg mL^{-1} . Each mouse received 20 mL kg^{-1} protein (5000 mg kg^{-1} Cry1Ab/Ac protein for each mouse) by gavage at the first day. BSA was used as negative control and water as the blank control. The mice were given basal diet and tap water and observed for 14 days for any signs of morbidity or mortality. During the experimental period all animals were inspected twice daily (Cage side). On day 15 at completion, all the animals were anaesthetized by carbon dioxide inhalation and killed by exsanguinations for gross and histopathological examination (Chinese Standard GB15193.13-2003).

2.5.2. Blood biochemistry and haematology assay

The animals in 2.5.1 were fasted overnight to minimize the fluctuation in the parameters measured. Blood samples were taken from the eyeball veniplex and stabilized by heparin. Following biochemical parameters were measured: urea (BUN), alanine aminotransferase (ALT), sodium, potassium, cholesterol, protein, albumin, creatinine and glucose. All analyses on blood plasma were performed on an RA-1000 autoanalyzer (Technicon, Tarrytown, NY, USA).

Blood samples for haematology characteristics were stabilized by using EDTA. The following characteristics were assessed using a HEMAVET 950FS (Drew Scientific, USA): white blood cells (WBC), erythrocyte count (RBC), haemoglobin concentration (HC), hematocrit (HCT), mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and platelet count (PLT). The differential count distinguishes neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E) and basophils (B).

2.5.3. Organ weights, gross necropsy and histopathology

The anatomy staffs were divided into three groups to respectively dissect the groups of water, BSA and Cry1Ab/Ac at the same time and females were dissected before males. A thorough necropsy was performed and the following organs were sequentially excised: brain, thymus, heart, lungs, liver, spleen, stomach, duodenum, adrenals, kidneys, ovaries and testes. Brain, heart, liver, spleen, lungs, kidneys, thymus, adrenals, ovaries and testes were immediately weighed and stomach, duodenum, kidneys and liver were immediately fixed in 4% buffered formaldehyde for

histological processing. Tissue samples were embedded in paraffin and sections, 4–6 µm thick, were then stained with standard hematoxylin–eosin for light microscopy.

2.5.4. Statistical analysis

Statistical comparisons were designed to determine whether the differences in the aforementioned response variables between groups were attributable to the Cry1Ab/Ac protein compared to control groups. Data obtained from the Cry1Ab/Ac protein groups were compared with the values from the water control and BSA control groups sexes separately. Homogeneity variance was analyzed by one-way analysis of variance (ANOVA) with a statistical software Statistical Product and Service Solutions (SPSS) v12.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$ and then a step-down analysis was conducted by least squares differences (LSD).

3. Results

3.1. Structural and functional equivalence of Cry1Ab/Ac protein derived from bacteria and rice

E. coli BL21 (DE3) containing the *Cry1ab/ac-rcp* gene was used to produce Cry1Ab/Ac protein instead of extracting it directly from genetically modified rice. Tests for evaluating equivalence between the *E. coli*- and the rice-derived forms were carried out and the results demonstrated that the recombinant protein produced from *E. coli* was an appropriate substitute for the extracted protein directly from genetically modified rice. The result was shown in Fig. 1. SDS–PAGE and Western Blot proved that *E. coli*- and the rice-derived proteins had the same molecular weight and epitope accessibility, and both of them had no N-glycosylation sites. Eight peptides (range 1000–3000 Da) were identified 20.2% with Cry1Ab/Ac protein sequence by searching Peptide Mass database (<http://www.expasy.ch/tools/peptide-mass.html>) and they were as follows: WYNTGLER, APMFSWIHR, WGFDAATINSR, QGFSLRLSHVSMFR, LEGLSNLYQIYAESFR, SGTVDLSLEIPP-QNNVPPR, LSHVSMFRSGFSNSSVSIIR and NFSGTAGVIIDRFEFIPV-TATLEAE, which peptide masses were 1039.8, 1145.3, 1238.5, 1670.1, 1902.3, 2150.8, 2213.5 and 2699.4, respectively.

3.2. No homology with known toxins or allergens

The recent results of the *in silico* analysis showed no evidence for any similarity between the Cry1Ab/Ac protein and any known toxic or allergenic proteins, i.e., less than 35% identity with known toxins or allergens across a length of 80 amino acids and no continuous eight amino acids identity with known allergens.

3.3. No N-glycosylation sites

The search for potential N-glycosylation sites indicated four such sites on the Cry1Ab/Ac protein. *In vitro* assays were employed

to verify the above result. Fig. 1C showed that there was no N-glycosylation site on the Cry1Ab/Ac protein actually.

3.4. Heat stability

On SDS–PAGE, the Cry1Ab/Ac protein was clearly visible at about 67 kDa at time zero (Fig. 2A). When treated at 100 °C for 60 min, Cry1Ab/Ac protein still remained detectable by SDS–PAGE, i.e., no protein degradation (Fig. 2A). Therefore, the Cry1Ab/Ac protein were not degraded or modified in a way that would affect their migration in SDS–PAGE at 100 °C for 60 min. In addition, their immunoreactivity was still detectable (Fig. 2B). The same recognition of the heat-treated and the native proteins by anti-Cry1Ab/Ac antibodies indicated that the conformational changes associated with denaturation did not affect the epitope accessibility. Interestingly, this means that the epitope homology search, which showed no similarities with known allergenic epitopes, has a major weight of evidence in this safety assessment.

3.5. Rapid degradation in digestive fluids

The digestibility of Cry1Ab/Ac protein was confirmed by using SGF and SIF. CBBG-stained gel and Western Blot of the protein samples (Figs. 3A, B, 4A and B) indicated that high molecular weight proteins were completely digested within 15 s, which consistent with the *silico* analysis (PeptideMass). The reference proteins of the SGF/SIF assay, α -casein or BSA and STI, were rapidly and slowly digested, respectively, which is in agreement with published data (data not shown).

3.6. Acute toxicity experiment of Cry1Ab/Ac protein in KM mice

In the acute toxicity experiment, control mice treated with 5000 mg kg^{−1} BSA (negative controls) or water at 20 mL kg^{−1} (blank controls) showed no visible signs of systemic toxicity. In this study, there was no mortality or toxic effect in mice after acute oral administration of the Cry1Ab/Ac protein at 5000 mg kg^{−1} body weight.

3.6.1. Clinical observations and body weight

During the course of the experiment, no treatment-related signs of adverse effects in clinical appearance of the animals were observed. Both of the body weights of male and female mice in Cry1Ab/Ac groups were comparable with control groups at the beginning and the end of the experiment (Table 1).

3.6.2. Blood biochemistry and haematology

Results for blood biochemistry and haematology for males and females, measured at study termination (day 15), are listed in Tables 2 and 3, respectively. There were no statistically significant

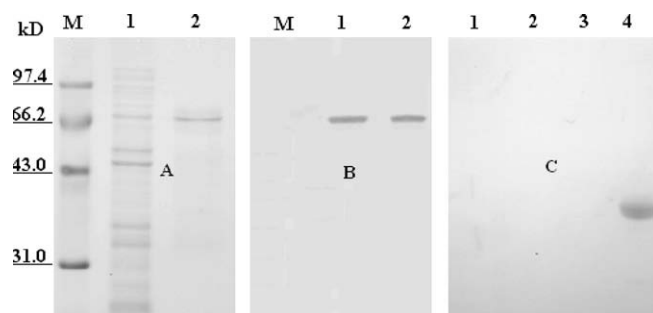


Fig. 1. Structural and functional equivalence of Cry1Ab/Ac protein derived from *E. coli* and rice. Lane 1, rice-derived protein; Lane 2, bacteria-derived protein; Lane 3, negative control; Lane 4, positive control. (A) SDS–PAGE; (B) Western Blot; (C) glycoprotein assay.

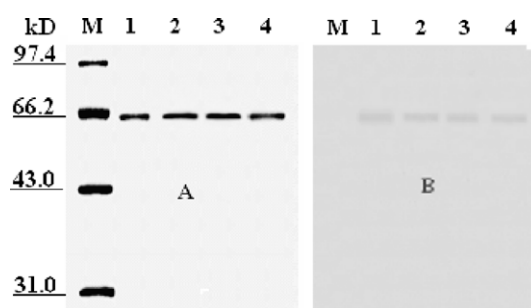


Fig. 2. Heat stability assay. Lanes 1–4, the Cry1Ab/Ac protein heated at 100 °C for 0, 10, 30 and 60 min. (A) SDS–PAGE; (B) Western Blot.

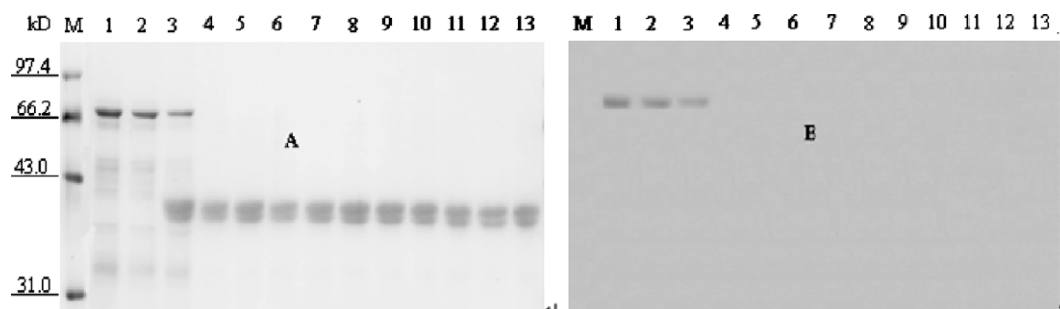


Fig. 3. SGF digestion test. Lane 1, the Cry1Ab/Ac protein without degradation fluid and incubation. Lane 2, the Cry1Ab/Ac protein without degradation fluid but incubation. Lanes 3–11, the Cry1Ab/Ac protein digested for 0 s, 15 s, 30 s, 60 s, 2 min, 5 min, 10 min, 20 min, and 30 min; Lane 12, the degradation fluid without incubation; Lane 13, the degradation fluid with incubation. (A) SDS–PAGE for SGF digestion; (B) Western Blot for SGF digestion.

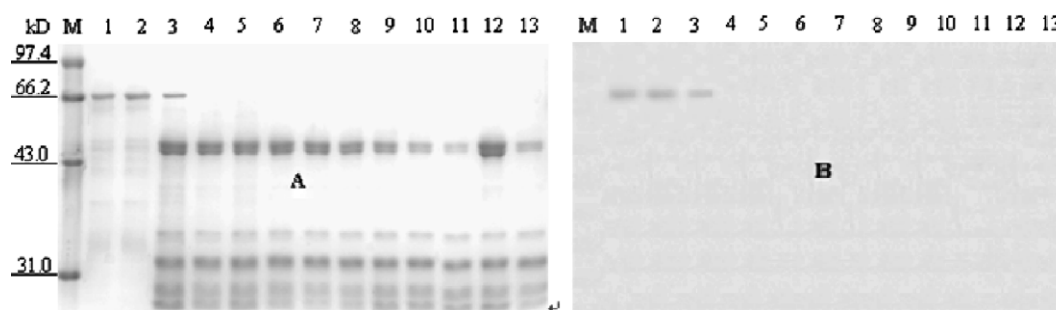


Fig. 4. SIF digestion test. Lane 1, the Cry1Ab/Ac protein without degradation fluid and incubation. Lane 2, the Cry1Ab/Ac protein without degradation fluid but incubation. Lanes 3–11, the Cry1Ab/Ac protein digested for 0 s, 15 s, 30 s, 60 s, 2 min, 5 min, 10 min, 20 min, and 30 min; Lane 12, the degradation fluid without incubation; Lane 13, the degradation fluid with incubation. (A) SDS–PAGE for SIF digestion; (B) Western Blot for SIF digestion.

Table 1

Organ/body weight mean values $\pm$ SD.

Groups	Female			Male		
	Water	BSA	Cry1Ab/Ac	Water	BSA	Cry1Ab/Ac
Final body weight	19.65 $\pm$ 0.55	19.65 $\pm$ 1.70	20.25 $\pm$ 1.16	25.47 $\pm$ 1.37	26.57 $\pm$ 2.20	26.78 $\pm$ 0.66
Brain	1.99 $\pm$ 0.14	2.07 $\pm$ 0.20	2.08 $\pm$ 0.21	1.66 $\pm$ 0.05	1.58 $\pm$ 0.17	1.67 $\pm$ 0.07
Heart	0.51 $\pm$ 0.05	0.48 $\pm$ 0.04	0.54 $\pm$ 0.06	0.51 $\pm$ 0.08	0.46 $\pm$ 0.04	0.53 $\pm$ 0.07
Livers	4.97 $\pm$ 0.33	5.23 $\pm$ 0.41	5.47 $\pm$ 0.12 ^a	5.40 $\pm$ 0.86	5.30 $\pm$ 0.52	5.67 $\pm$ 0.21
Spleen	0.43 $\pm$ 0.06	0.48 $\pm$ 0.07	0.50 $\pm$ 0.09	0.44 $\pm$ 0.07	0.48 $\pm$ 0.06	0.46 $\pm$ 0.08
Lungs	0.77 $\pm$ 0.05	0.65 $\pm$ 0.05 ^c	0.69 $\pm$ 0.05 ^a	0.65 $\pm$ 0.04	0.63 $\pm$ 0.08	0.64 $\pm$ 0.02
Kidneys	1.21 $\pm$ 0.16	1.24 $\pm$ 0.07	1.21 $\pm$ 0.10	1.53 $\pm$ 0.09	1.29 $\pm$ 0.13 ^c	1.46 $\pm$ 0.11 ^b
Thymus	0.33 $\pm$ 0.08	0.36 $\pm$ 0.06	0.45 $\pm$ 0.10 ^a	0.27 $\pm$ 0.06	0.30 $\pm$ 0.07	0.31 $\pm$ 0.08
Adrenals	0.04 $\pm$ 0.02	0.02 $\pm$ 0.01	0.04 $\pm$ 0.01	0.02 $\pm$ 0.02	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01
Testes/ovaries	0.11 $\pm$ 0.04	0.07 $\pm$ 0.04	0.09 $\pm$ 0.03	0.67 $\pm$ 0.08	0.59 $\pm$ 0.08	0.59 $\pm$ 0.08

^a $p < 0.05$ experimental group compared to blank control group.

^b $p < 0.05$ experimental group compared to negative control group.

^c $p < 0.05$ negative control group compared to blank control group.

difference between male and female Cry1Ab/Ac protein groups and the controls groups for most of the parameters measured ($p < 0.05$ experimental group compared to blank control group or negative control group but not for both for only three parameters). PLT showed statistically significant differences in males of BSA group but females are statistically different in water group. And for males BSA group showed statistically different from water. But all the results obtained in this study were comparable with Schröder et al. (2007) and He et al. (2008)). So the significant fines are only chance events. The statistically difference on MCV is only for males with BSA group and the differences are less than 10%. And there is statistically different between water group and BSA group for females too. So the differences have no practical significance. The normal range for E and B is 0.0–0.2. Some mice had those kinds of cells but others didn't have them, which could affect the result very significantly. And the statistically difference was just for females of

BSA group, furthermore there are statistically differences between water group and BSA group too, so the deference could not be related with Cry1Ab/Ac protein.

3.6.3. Organ weights, gross necropsy and histopathology

There were no group-related alterations or statistically identified difference in organ weights of male and female mice given Cry1Ab/Ac protein versus the control groups (Table 1). All observations were considered to be the result of spontaneous alterations, unassociated with exposure to the Cry1Ab/Ac protein (only five of the experimental group compared to blank control group or negative control group $p < 0.05$ but not for both). Although organ/body weights of liver were larger in both Cry males and females, the histopathology and alanine aminotransferase (ALT) for liver had no differences and the mice liver in Cry1Ab/Ac protein had no problem. Female lungs were statistically different from water group

Table 2Haematology mean values $\pm$ SD.

Groups	Female			Male		
	Water	BSA	Cry1Ab/Ac	Water	BSA	Cry1Ab/Ac
WBC ($10^9/L$)	4.15 $\pm$ 1.11	4.61 $\pm$ 0.92	4.50 $\pm$ 1.65	3.69 $\pm$ 1.86	5.16 $\pm$ 1.60	3.97 $\pm$ 0.74
RBC ($10^{12}/L$)	8.40 $\pm$ 1.34	8.28 $\pm$ 0.44	8.45 $\pm$ 0.21	7.29 $\pm$ 1.60	8.34 $\pm$ 1.12	8.23 $\pm$ 0.26
HC (g/dL)	13.37 $\pm$ 1.34	13.58 $\pm$ 0.73	13.68 $\pm$ 0.57	13.48 $\pm$ 0.88	14.03 $\pm$ 1.26	13.22 $\pm$ 0.31
HCT (%)	39.95 $\pm$ 6.34	42.90 $\pm$ 2.93	40.79 $\pm$ 2.32	30.93 $\pm$ 17.18	44.43 $\pm$ 6.54 ^c	39.55 $\pm$ 2.21
MCV (fL)	47.63 $\pm$ 2.96	51.88 $\pm$ 3.43 ^c	48.35 $\pm$ 2.49	51.32 $\pm$ 3.87	53.18 $\pm$ 2.90	48.12 $\pm$ 2.99 ^b
MCH (pg)	15.45 $\pm$ 0.38	15.92 $\pm$ 0.41 ^c	15.77 $\pm$ 0.24	15.97 $\pm$ 0.54	16.00 $\pm$ 0.38	15.60 $\pm$ 0.41
MCHC (g/L)	306 $\pm$ 72	271 $\pm$ 16	287 $\pm$ 13	282 $\pm$ 37	277 $\pm$ 41	284 $\pm$ 12
PLT ($10^9/L$)	718 $\pm$ 107	836 $\pm$ 210	934 $\pm$ 67 ^a	1025 $\pm$ 132	758 $\pm$ 119 ^c	1030 $\pm$ 136 ^b
N ($10^9/L$)	0.77 $\pm$ 0.19	1.18 $\pm$ 0.42	1.27 $\pm$ 0.72	0.91 $\pm$ 0.60	1.20 $\pm$ 0.37	1.22 $\pm$ 0.25
L ($10^9/L$)	3.14 $\pm$ 0.93	3.12 $\pm$ 0.87	2.96 $\pm$ 1.11	2.49 $\pm$ 1.08	3.61 $\pm$ 1.18	2.55 $\pm$ 0.49
M ($10^9/L$)	0.22 $\pm$ 0.08	0.23 $\pm$ 0.04	0.24 $\pm$ 0.08	0.19 $\pm$ 0.17	0.27 $\pm$ 0.06	0.15 $\pm$ 0.03
E ($10^9/L$)	0.01 $\pm$ 0.01	0.06 $\pm$ 0.06 ^c	0.01 $\pm$ 0.01 ^b	0.08 $\pm$ 0.08	0.06 $\pm$ 0.06	0.04 $\pm$ 0.04
B ($10^9/L$)	0.00 $\pm$ 0.01	0.02 $\pm$ 0.02 ^c	0.00 $\pm$ 0.00 ^b	0.02 $\pm$ 0.03	0.02 $\pm$ 0.03	0.01 $\pm$ 0.01

^a $p < 0.05$ experimental group compared to blank control group.^b $p < 0.05$ experimental group compared to negative control group.^c $p < 0.05$ negative control group compared to blank control group.**Table 3**Blood biochemistry mean values $\pm$ SD.

Groups	Female			Male		
	Water	BSA	Cry1Ab/Ac	Water	BSA	Cry1Ab/Ac
BUN (mmol/L)	5.15 $\pm$ 0.71	5.09 $\pm$ 0.71	5.12 $\pm$ 0.72	4.75 $\pm$ 0.40	4.59 $\pm$ 1.04	4.93 $\pm$ 0.70
ALT (U/L)	45.87 $\pm$ 7.37	48.03 $\pm$ 6.71	45.25 $\pm$ 6.61	45.69 $\pm$ 5.81	43.23 $\pm$ 7.52	45.29 $\pm$ 5.98
Sodium (mmol/L)	141.07 $\pm$ 4.27	139.91 $\pm$ 4.04	138.14 $\pm$ 4.56	138.63 $\pm$ 8.44	140.58 $\pm$ 7.78	141.81 $\pm$ 4.12
Potassium (mmol/L)	4.26 $\pm$ 0.59	4.61 $\pm$ 0.91	4.29 $\pm$ 0.66	4.01 $\pm$ 0.67	4.44 $\pm$ 0.70	4.55 $\pm$ 0.93
Cholesterol (mmol/L)	1.30 $\pm$ 0.13	1.31 $\pm$ 0.11	1.30 $\pm$ 0.13	1.46 $\pm$ 0.05	1.61 $\pm$ 0.23	1.55 $\pm$ 0.11
Protein (g/L)	44.70 $\pm$ 3.92	45.75 $\pm$ 3.28	42.77 $\pm$ 3.19	41.40 $\pm$ 5.10	44.56 $\pm$ 3.97	41.90 $\pm$ 4.80
Albumin (g/L)	36.26 $\pm$ 6.64	36.43 $\pm$ 6.65	36.44 $\pm$ 6.64	34.00 $\pm$ 4.42	32.33 $\pm$ 0.55	30.50 $\pm$ 7.84
Creatinine (mmol/L)	51.83 $\pm$ 1.44	50.87 $\pm$ 0.65	51.16 $\pm$ 2.52	48.46 $\pm$ 1.44	47.97 $\pm$ 3.19	48.78 $\pm$ 1.79
Glucose (mmol/L)	7.27 $\pm$ 0.46	7.35 $\pm$ 0.50	7.66 $\pm$ 0.74	8.98 $\pm$ 0.40	8.97 $\pm$ 0.40	9.47 $\pm$ 0.55

but the data were between water group and BSA group and the difference could not be found in males group. So the difference was not treatment related. Statistically difference on kidneys was only for males and there was statistically different between water group and BSA group. Further more no difference was found for females, so the differences found had nothing to do with Cry protein.

For histopathology assay, a female mouse of the control group showed gastric mucosa partial loss, gastric wall cells and secretion of mucus enhancement. The phenomenon was more likely caused by stomach gavage. Mechanical stimulation and mild damage induced increase mucus secretion of cells wall to coverage the gastric mucosal surface to protect the body from injury. And one of the Cry1Ab/Ac group male mouse represented mild renal glomerular permeability, with a small amount of the tubular type. There may be many reasons for that phenomenon not rule out the possibility of impact by the protein, but 1/12 cases of mild changes cannot be determined with the experimental lesions relating with the Cry1Ab/Ac protein.

4. Discussion

Microbial Bt products have been used as insecticides for more than 40 years without documented evidence of adverse effects. Additionally, a number of toxicological studies with insecticidal Cry proteins have not identified safety concerns with Bt proteins (McClintock et al., 1995; EPA, 1998). But as genetically modified (GM) crops are becoming an increasingly important feature of agricultural landscapes. Several international organizations have developed guidelines to investigate safety of foods or feeds obtained from genetically modified plants to humans and livestock

(WHO, 1991, 1995; OECD, 1993, 1996, 1997; FAO, 1996; FAO/WHO, 2000; ILSI, 1996).

The safety of plants containing novel proteins such as the Cry1Ab/Ac protein should be addressed in two complementary steps: (1) an initial characterization and safety assessment of the protein itself and (2) the characterization and safety evaluation of the transgenic plant. This article focuses on the first step. This initial determination of the toxicological profile of the proteins is independent of the genetic modification and can be extended to any plant variety as one element of the safety assessment of any plant that contains and expresses the same protein. The safety of the Cry1Ab/Ac protein is evaluated based on an assessment of published information and on scientific studies conducted with these proteins. The safety evaluation is consistent with several recommendations provided by Authorities and International Organizations including the FAO/WHO, Codex Alimentarius and OECD.

One of the key endpoints for assessing the safety of a novel protein is its amino acid sequence similarity with proteins that could have potential safety concerns, i.e., known toxins or allergens. For this reason, an amino acid sequence comparison was performed as part of the current food safety decision tree strategy as recommended by the FAO/WHO (2001) and the Codex Alimentarius (2003). Immunoglobulin E (IgE) cross-reactivity between a newly expressed protein and a known allergen should be considered a possibility when there is more than 35% identity in a segment of 80 or more amino acids (Codex Alimentarius, 2003; FAO/WHO, 2001; Hileman et al., 2002). It is reasonable to assume that only matches of eight contiguous and identical amino acids have some relevance for identifying potential allergens. The recent results of the *in silico* analysis showed no evidence for any similarity be-

tween the Cry1Ab/Ac protein and any known toxic or allergenic proteins, i.e., less than 35% identity with known toxins or allergens across a length of 80 amino acids and no continuous eight amino acids identity with known allergens. Theoretically the Cry1Ab/Ac protein is safe but the silico analysis had to be verified by in vitro assays.

Many protein allergens are glycosylated, raising the possibility that the glycosyl groups may contribute to their allergenicity (Jenkins et al., 1996). This is potentially relevant when considering the allergenicity of novel proteins for which glycosylation patterns may differ substantially from their native counterparts. The search for potential N-glycosylation sites found four such sites on the Cry1Ab/Ac protein but in vitro assays proved that there was no N-glycosylation site on the Cry1Ab/Ac protein actually.

With the exception of pollen protein, all allergenic proteins have high stability to digestive enzymes. Therefore this stability can be used as an indicator of the potential of a protein being an allergen (Astwood, 1996). It has been reported that proteins that are rapidly hydrolyzed to peptides and single amino acids smaller than 3.5 kDa by pepsin can be considered less likely to be allergenic (Fu et al., 2002). And if protein allergens are to reach and pass through the intestine mucosa to elicit an allergenic response, they must be able to resist peptic and tryptic digestion and the acid conditions of the digestive system. The Cry1Ab/Ac protein was degraded rapidly by digestive enzymes as assessed by both SDS-PAGE and Western Blot. These results suggest, but do not prove, that the protein has no allergenic activity, since the correlation between resistance to proteolysis and allergenic activity is not absolute (Fu et al., 2002).

The recombinant protein was used in this paper to assess the safety of Cry1Ab/Ac protein. The result obtained from structural and functional equivalence assay explain that the recombinant protein produced from *E. coli* was an appropriate substitute for the extracted protein directly from genetically modified rice. Heat stability study showed the same recognition of the heat-treated and the native proteins by anti-Cry1Ab/Ac antibodies indicated that the conformational changes associated with denaturation did not affect the epitope accessibility which means that the epitope homology search, which showed no similarities with known allergenic epitopes, has a major weight of evidence in this safety assessment. To have a direct assessment of the protein toxicity in mammals, an oral acute study in mice is used for evaluating the safety of the Cry1Ab/Ac protein. In this study, the mouse acute oral LD50 was >5000 mg kg⁻¹ (body weight), which belongs to practically non-toxic grade. The results are coincidence with Delaney et al. (2008) which showed the results from acute mouse toxicology studies with proteins in GM crops were: Cry1Ab > 4000 (oral) and Cry1Ac > 4200 (oral). These results indicated that the Cry1Ab/Ac protein has no harm to human beings and animal.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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