



Safety assessment of Cry1C protein from genetically modified rice according to the national standards of PR China for a new food resource

Sishuo Cao^{a,1}, Xiaoyun He^{b,1}, Wentao Xu^{a,b,*}, Wenjun Ran^a, Lixing Liang^a, YunBo Luo^a, Yanfang Yuan^a, Nan Zhang^b, Xin Zhou^b, Kunlun Huang^{a,b,**}

^a Laboratory of Food Safety and Molecular Biology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, PR China

^b The Supervision, Inspection and Testing Center of Genetically Modified Organisms, Ministry of Agriculture, Beijing 100083, PR China

ARTICLE INFO

Article history:

Received 12 April 2010

Available online 27 August 2010

Keywords:

Cry1C protein

Oral acute study

Safety assessment

Micronucleus assay

Sperm shape abnormality test

Ames test

GMO

ABSTRACT

The Cry1C protein produced in *Escherichia coli* was used for *in vitro* evaluation and animal studies to support the safety assessment of GM food or feed products containing the Cry1C protein. The Cry1C protein does not have any sequence homology with known allergens or toxins. Although the Cry1C protein was heat stable it was rapidly degraded *in vitro* with simulated gastric or intestinal fluids. It did not cause adverse effects in mice as administered by gavage at a high level dosage of 5 g (Cry1C protein)/kg body weight. The mutagenicity of this protein was evaluated according to the national standards of People's Republic of China (PR China) for a new food resource. In mutagenic tests, the Cry1C protein caused <4 micronucleated cells per 1000 cells, <16 sperm abnormalities per 1000 cells and was not associated with any increased mutations in the Ames test. Taken together, these data indicate that the Cry1C protein is not a potential allergen or toxin.

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1. Introduction

Beginning in 1987, *Bacillus thuringiensis* (Bt) corn was genetically modified by introducing the Bt gene to combat crop damage (Barton et al., 1987; Vaeck et al., 1987; Fischhoff et al., 1987). The Bt genes in crops have been developing at a very fast pace to become one of the most widely grown GM crops worldwide (James, 2009). By 2009, the global area planted with commercial transgenic crops had reached 134 million hectares, of which Bt crops accounted for 15%, or about 20.7 million acres (James, 2009). These crops have been beneficial to the growers and the environment because they greatly reduce the use of chemical insecticides (Ferré and Van Rie, 2002).

Although a large number of Bt toxins are known, only a small fraction of these are currently used in developing transgenic crops to control *lepidopteran* pests. The most commonly used Bt genes in transgenic crops, including rice, are Cry1Ab, Cry1Ac and the fusion gene Cry1Ac/Cry1Ab (Tang et al., 2006). However,

there is concern that the widespread adoption of genetically modified crops could lead to the development of resistant insect populations (Frutos et al., 1999). Indeed, it has already been shown that some insects have developed resistance to Bt proteins under laboratory and greenhouse conditions (Kain et al., 2004). The results of binding tests of midgut brush border membrane vesicles indicated that the Cry1Aa, Cry1Ab, and Cry1Ac toxins share a common binding site (Karim and Dean, 2000); thus, a mutant that is able to overcome one of these Cry1A toxins may be resistant to the other Cry1A toxins. Consequently, identifying Cry toxins with modes of action that differ from those induced by the Cry1Aa, Cry1Ab, and Cry1Ac toxins should be explored. The Cry1C toxin is effective against a variety of *lepidopteran* pests, including rice stemborers. Cry1C binds with weak affinity to the Cry1Ab and Cry1Ac binding sites in the striped stem borer and yellow stem borer, which means that Cry1C does not share a common binding site with the Cry1A toxins (Alcantara et al., 2004). Strizhov et al. (1996) produced transgenic plants expressing a modified Cry1Ca gene in alfalfa and tobacco that showed enhanced resistance to the Egyptian cotton leafworm (*Spodoptera littoralis*) and the beet armyworm. Cao et al. (1999) transformed broccoli with a modified Cry1C gene, and the resultant transgenic plants were highly resistant to the diamond back moth (*Plutella xylostella*). Therefore, it appears that the Cry1C toxin can be a potential alternative to the Cry1A toxin, and it can also be combined with other Cry1A genes to develop two-toxin Bt crops (Tang et al., 2006).

* Corresponding author at: Laboratory of Food Safety and Molecular Biology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, PR China. Fax: +86 10 6273 7786.

** Corresponding author at: Laboratory of Food Safety and Molecular Biology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, PR China. Fax: +86 10 6273 7786.

E-mail addresses: cauxwt@yahoo.cn (W. Xu), hkl009@163.com (K. Huang).

¹ These two authors contributed equally.

The objective of this study was to conduct a safety assessment of the Cry1C protein. Because of its low content in rice, the protein was produced in *Escherichia coli* to obtain sufficient levels for the *in vitro* evaluation and animal studies, so that its potential safety in human food or animal feed could be properly assessed. Examples of the structural and functional equivalences of the microorganism-produced proteins and the proteins produced in the transgenic crop were demonstrated to support its safety for widespread use. One approach of the safety assessment is to compare the novel protein to known food allergens. The Cry1C amino acid sequence was compared to protein databases of known allergens and toxins. Because most food allergens resist gastric and intestinal digestion, the stability of the Cry1C protein to *in vitro* simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) was conducted. To assess protein toxicity in mammals, an acute oral study in mice and *in vitro* mutational assays were performed, according to the national standards of PR China for a new food resource.

2. Materials and methods

2.1. Protein production and characterization

PET-30a(+)-Cry1C-rcp-BL21 (DE3) (Laboratory of Food safety, CAU, Beijing, China) was used to highly express the Cry1C protein. The Cry1C fusion protein was expressed and identified as Xu et al. (2009). The protein was expressed in *E. coli* after incubation at 37 °C and a 4-h induction. The bacteria were fragmented by ultrasound and then purified using a His Trap™ FF crude column (Amersham Biosciences, Uppsala, Sweden). After purification and refolding, the protein was identified by western blot and LC–MS/MS.

2.2. Amino acid sequence homology search

The bioinformatics search for allergen sequence matches was conducted according to Goodman et al. (2007). We searched for 80 amino acid alignments, looking for identities greater than 35% on AllergenOnline (<http://www.allergenonline.org/>, version 9.0). We also searched for eight exact amino acid matches on the same web site. A sequence similarity search was conducted of the amino acid sequence of the Cry1C protein using BLASTP 2.2.20 with an E score cutoff of 1.0, according to Delaney et al. (2008a). A total of 8996,415 sequences were searched. Sequence annotations of proteins with similarity to the Cry1C protein sequence were inspected manually to identify known allergens or toxic proteins.

2.3. Heat stability study

The Cry1C 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. The stability was assessed at a 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 subjected to no incubation (kept at 4 °C) were also prepared (Hérouet et al., 2005). The resulting proteins were analyzed by SDS–PAGE and western blot (Anti-Cry1C rabbit polyclonal antibody was used in western blot, 1:10000).

2.4. *In vitro* digestion of Cry1C protein

In vitro digestion of Cry1C protein was conducted according to the Ministry of Agriculture of PR China No.869 Bulletin 2-2007. The concentrations of Cry1C protein in simulated gastric fluid

(SGF) and simulated intestinal fluid (SIF) (Sigma, USA) were 0.25 mg mL^{−1} and 0.1 mg mL^{−1}, respectively. The ratio of the Cry1C protein (5 g L^{−1} for SGF, 2 g L^{−1} for SIF) and enzyme was 1:19. The mixture was incubated at 37 °C, and the samples were taken out at intervals of 0, 15, 30, 60 s, 2, 5, 10, 20, and 30 min. The SDS–PAGE and western blot (Anti-Cry1C rabbit polyclonal antibody was used in western blot, 1:10000) enabled the examination of the stability of the protein that was to be digested in SGF/SIF. The experiments were also performed for the positive control STI (soybean trypsin inhibitor) and negative control BSA (bovine serum albumin) or α -casein for SGF and SIF, using the method above (Lu et al., 2007).

2.5. Acute toxicity experiment of Cry1C protein in ICR mice

2.5.1. Husbandry and dosing

Approximately 6-week-old female and male ICR (Institute of Cancer Research) mice, weighing 18–22 g, were obtained from the Experimental Animal Center of Peking University (Beijing, China). Following a 5-day acclimatization, they were divided randomly into three groups, with 12 mice for each group (6 males and 6 females per treatment). The mice were kept in stainless steel wire cages (two/cage) at 21–23 °C and a relative humidity of 40–60%, with an air change 15 times h^{−1} and electric light from 09.00 to 21.00 in animal rooms. Food and water were supplied to the mice from the public supply, which was available *ad libitum*.

The Cry1C protein was dissolved in redistilled water, and the concentration of Cry1C protein was adjusted to 250 mg mL^{−1} to produce a solution with 95.8% purity. Each mouse received 20 mL kg^{−1} protein (5000 mg kg^{−1} Cry1C protein for each mouse) by gavage on the first day. BSA was used as the protein control and water as the blank control. The mice were given basal diets 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 examinations (Chinese standard GB15193.13-2003).

2.5.2. Blood biochemistry and hematology assay

The animals in Section 2.5.1 were fasted overnight to minimize any potential fluctuations in the parameters measured. Blood samples were taken from the eyeball veniplex and stabilized by heparin. The following biochemical parameters were measured: total protein (TP), albumin (ALB), glucose (GLU), blood urea nitrogen (BUN), triglycerides (TRIG), total cholesterol (CHOL), creatinine (CREA), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALKP). All analyses on blood plasma were performed on an RA-1000 autoanalyzer (Technicon, Tarrytown, NY, USA). Blood samples used to assess hematology characteristics were stabilized with EDTA. The following characteristics were measured using a HEMA-VET 950FS (Drew Scientific, USA): hemoglobin (HGB), white blood cells (WBC), red blood cell counts (RBC), blood platelet counts (PLT), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and mean platelet volume (MPV).

2.5.3. Organ weights, gross necropsy and histopathology

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. These organs were immediately weighed, and the stomach, duodenum, kidneys and liver were immediately fixed in 4% buffered formaldehyde for histological processing. Tissue samples were embedded in paraffin, and 4- to 6- μ m thick sections were then stained with standard hematoxylin–eosin for light microscopy.

2.6. Micronucleus test

The micronucleus test was conducted according to GB 15193.5-2003. ICR mice were obtained and kept until they reached the age of 7–12 weeks before being used. On test days 1 and 3, four groups of male mice (5 mice/group) were exposed through oral gavage to 0, 125, 625 or 1250 $\mu\text{g kg}^{-1}$ b.w. of Cry1C protein (Tang et al., 2006). Cyclophosphamide was used at a dose of 40 mg kg^{-1} as the positive control. Mice from each group were killed after the second gavage by cervical dislocation, their sternums were removed and their bone-marrow fluid was extruded. The bone-marrow fluid was mixed with bovine serum and smeared onto the slides, then air dried. After fixing them with methanol for 5–10 min, the bone-marrow cells were stained with Giemsa for 10–15 min, rinsed with distilled water and air dried. For each suspension, 1000 chromatophilic red cells were examined.

2.7. Sperm shape abnormality test

Sperm abnormalities in the mice experiment were assessed according to GB 15193.7-2003. ICR mice were obtained and kept until they reached an age of 6–8 weeks before being used. Four groups of male mice (5 mice/group) were exposed through oral gavage to 0, 125, 625 or 1250 $\mu\text{g kg}^{-1}$ b.w. of Cry1C protein (Tang et al., 2006). Cyclophosphamide was used at a dose of 40 mg kg^{-1} as the positive control. Each animal was given a daily intragastric administration of the Cry1C protein or cyclophosphamide for five days. Mice from each group were killed on test day 40 by cervical dislocation, and their cauda epididymides were removed. The sperm suspensions were prepared by mincing the cauda in 2 ml of physiological saline, pipetting the resulting suspension and filtering it through an 80 μm stainless steel mesh to remove tissue fragments. A fraction of each suspension was then mixed with methanol for 5 min, allowed to air dry, stained with eosin for 1 h and then rinsed with distilled water and air dried. For each suspension, 1000 sperm were examined at a 400-fold magnification with blue-green filters.

2.8. Ames test

An *in vitro* evaluation of the mutagenic potential of the Cry1C protein in bacteria (Ames test) was performed with and without an exogenous metabolic activating system (S9) (GB 15193.4-2003). The S9 metabolic fraction and the *Salmonella typhimurium* strains TA97, TA98, TA100, and TA102 were obtained from the Chinese Center for Disease Control and Prevention (China CDC). The sensitivity of the *S. typhimurium* strains was verified using the following positive controls with the S9 mix: 2,4,7-trinitrofluorenone (2,4,7-TNF-one) (0.5 $\mu\text{g/plate}$) with strain TA97 and strain TA98, NaN_3 (2.5 $\mu\text{g/plate}$) with strain TA100 and mitomycin C (MMC) (1.0 $\mu\text{g/plate}$) with strain TA102. For the positive controls without the S9 mix, the following were used: 2-acetylaminofluorene (2-AAF) (20 $\mu\text{g/plate}$) with strain TA97 and strain TA98, benzo(a)pyrene (50 $\mu\text{g/plate}$) with strain TA100 and 1,8-dihydroxyanthraquinone (1.0 $\mu\text{g/plate}$) with strain TA102. Sterile water was used as a negative control. The Cry1C protein was dissolved in sterile water at concentrations of 50, 250, 1250, 2500, and 5000 $\mu\text{g/plate}$. A positive result was defined as a reproducible, dose-related increase in the number of his $\pm$ revertants, with the increase at least doubling.

2.9. Statistical analysis

Statistical comparisons were designed to determine whether the differences in the aforementioned response variables between groups were attributable to the Cry1C protein as compared to con-

trol groups. Data obtained from the male and female Cry1C protein groups were compared separately using the values from the water control and BSA control groups. Homogeneity of variance was analyzed by one-way analysis of variance (ANOVA) with the statistical software program Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p < 0.05$, and a step-down analysis was conducted using least squares differences (LSD).

3. Results

3.1. Structural and functional equivalence of Cry1C protein derived from bacteria and rice

E. coli BL21 (DE3) containing the Cry1C-rcp (GenBank HM107006) gene (Laboratory of Food safety, CAU, Beijing, China) was used to produce Cry1C protein instead of extracting it directly from genetically modified rice. Tests for evaluating the 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 protein extracted directly from the genetically modified rice. *E. coli* and the rice-derived proteins had the same epitope accessibility, which was proven by western blot (Fig. 1). LC-MS/MS revealed that 37 peptides (*E. coli*-Cry1C) displayed 63.33% identification with the Cry1C protein sequence (data not shown).

3.2. No homology with known toxins or allergens

The *in silico* analysis showed no evidence of any similarity between the Cry1C protein and any known toxic or allergenic proteins in the NCBI database. Using AllergenOnline, there were no matches of >35% identity over a window of 80 amino acids or any evidence of the protein having 8 or greater continuous identical amino acid matches with known allergens.

3.3. Heat stability

On SDS-PAGE, the Cry1C protein was clearly visible at about 67 kDa at time zero (Fig. 2A). When incubated at 100 °C for 60 min, the Cry1C protein persisted and was still detectable (Fig. 2A). Therefore, it was not degraded or modified in a way that would affect its migration on SDS-PAGE after exposure to 100 °C for 60 min. In addition, its immunoreactivity was still detectable (Fig. 2B).

3.4. Rapid degradation in digestive fluids

The digestibility of the Cry1C protein was confirmed using SGF and SIF. CBBG-stained gels and western blots of the protein sam-



Fig. 1. Western blot for rice protein and *E. coli* protein. Lane 1, rice protein; Lane 2, *E. coli* protein.

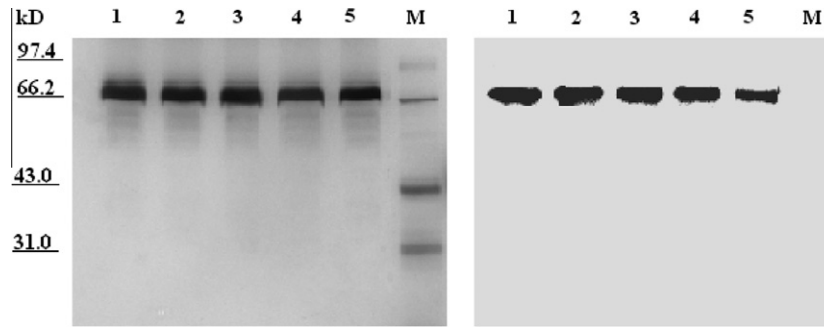


Fig. 2. Heat stability assay. Lanes 1, 2, 3, 4, 5: Cry1C protein dissolved in 20 mM Tris–HCl and 5 mM EDTA buffer at a concentration of 0.1 mg mL⁻¹ and heated to 100 °C for 0, 10, 20, 30, 60 min, respectively. (A) SDS–PAGE; (B) Western blot.

ples (Fig. 3A and B; Fig. 4A and B) indicated that high molecular weight proteins were completely digested within 15 s. The reference proteins of the SGF/SIF assay, α -casein and STI, were rapidly and slowly digested, respectively, which is in agreement with published data (Xu et al., 2009).

3.5. Acute toxicity experiment of Cry1C protein in ICR mice

In the acute toxicity experiment, control mice treated with 5000 mg kg⁻¹ BSA (protein controls) or 20 mL kg⁻¹ water (blank controls) showed no visible signs of systemic toxicity. In this study, there was no mortality nor any toxic effects observed in mice after acute oral administration of the Cry1C protein at 5000 mg kg⁻¹ body weight.

3.5.1. Clinical observations and body weight

During the course of the experiment, no treatment-related adverse effects in the clinical appearance of the animals were

observed. The body weights of male and female mice in the Cry1C groups were comparable with the control groups at the beginning, day 7 and the end of the experiment (19–21 g for females and 20–22 g for males).

3.5.2. Blood biochemistry and hematology

Results of the blood biochemistry and hematology assessment for males and females measured at study termination (day 15) are listed in Table 2 and 3, respectively. There were no statistically significant differences between male and female Cry1C protein groups and the controls groups for most of the parameters measured ($p < 0.05$ for the experimental group compared to the blank control group or to the protein control group but not for both). HGB, RBC, HCT, MCH2 and MPV in males showed statistically significant differences as compared to the water group. These results were not considered to be biologically significant because the BSA group (with the exception of MPV) also showed statistically significant differences as compared to the water group, and the

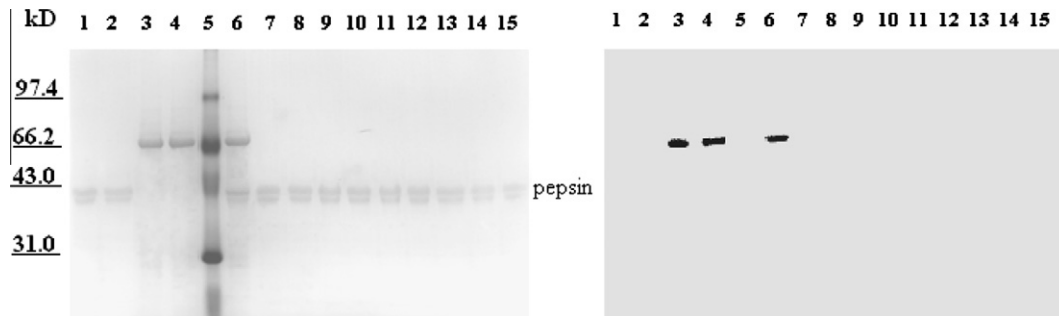


Fig. 3. SGF digestion test. Lane 1, the degradation fluid without incubation; Lane 2, the degradation fluid with incubation; Lane 3, the Cry1C protein without degradation fluid and incubation; Lane 4, the Cry1C protein without degradation fluid but with incubation; Lane 5, low molecular weight protein marker. Lanes 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: Cry1C protein digested for 0, 15, 30, 60 s, 2, 5, 10, 20, 30 and 60 min, respectively. (A) SDS–PAGE; (B) Western blot.

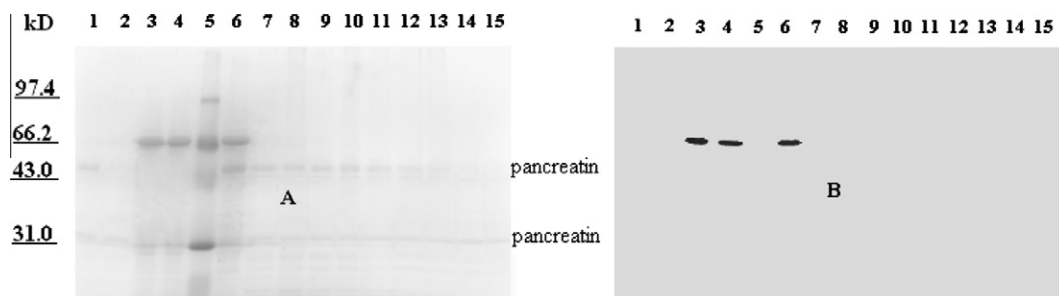


Fig. 4. SIF digestion test. Lane 1, the degradation fluid without incubation; Lane 2, the degradation fluid with incubation; Lane 3, the Cry1C protein without degradation fluid and incubation; Lane 4, the Cry1C protein without degradation fluid but with incubation; Lane 5, low molecular weight protein marker; Lanes 6, 7, 8, 9, 10, 11, 12, 13, 14, 15: Cry1C protein digested for 0, 15, 30, 60 s, 2, 5, 10, 20, 30 and 60 min, respectively. (A) SDS–PAGE; (B) Western blot.

differences did not occur in females. For treated females, the MCH and HCT values were different from the BSA group but were comparable to the water group. The mean of the TP and ALB concentrations in the females from the Cry1C group were lower ($p < 0.05$) than the concentrations observed in the BSA group. This statistical difference was not considered adverse or related to the Cry1C protein because the value in males was higher than that observed from both the BSA and water groups. The means of GLU, UREA, TG and GOT concentrations in females were statistically significant as compared to the water group. These differences were not considered to be biologically significant because they were comparable with the BSA group, and such differences were not observed in males. The statistical difference in ALP was only observed in males as compared to the water group and was not found in females. All of the results obtained in this study were comparable

with published studies (Schröder et al., 2007; He et al., 2008), and the differences observed in the blood biochemistry and hematology endpoints were within the historical control range for our laboratory (Xu et al., 2009).

3.5.3. Organ weights, gross necropsy and histopathology

There were no group-related alterations or statistically identified differences in the organ/body weights of male and female mice given the Cry1C protein as compared to the control groups (Table 1). All observations were considered to be the result of spontaneous alterations, unassociated with exposure to the Cry1C protein (only three organ weights from the experimental group were compared to either the blank control group or protein control group $p < 0.05$). Liver weights were higher in females but lower in males as compared to the control groups. Kidney weights were

Table 1
Organ/body weight mean values $\pm$ SD.

Groups	Female			Male		
	Water	BSA	Cry1C	Water	BSA	Cry1C
Brain	20.739 $\pm$ 0.820	19.996 $\pm$ 1.441	20.355 $\pm$ 1.524	18.101 $\pm$ 1.789	17.531 $\pm$ 0.837	18.148 $\pm$ 0.535
Heart	5.162 $\pm$ 0.269	4.985 $\pm$ 0.611	4.910 $\pm$ 0.295	5.328 $\pm$ 0.193	5.255 $\pm$ 0.218	5.448 $\pm$ 0.857
Livers	37.370 $\pm$ 1.679	34.973 $\pm$ 1.962	41.298 $\pm$ 3.903 ^{a,b}	41.907 $\pm$ 2.389	41.818 $\pm$ 2.472 ^c	40.502 $\pm$ 0.499 ^a
Spleen	4.240 $\pm$ 0.395	3.445 $\pm$ 0.330 ^c	3.798 $\pm$ 0.356	4.505 $\pm$ 1.154	3.069 $\pm$ 0.250	3.138 $\pm$ 0.147
Lungs	6.574 $\pm$ 0.681	6.182 $\pm$ 0.627	6.346 $\pm$ 1.139	5.781 $\pm$ 0.459	5.427 $\pm$ 0.691	5.689 $\pm$ 0.397
Kidneys	11.814 $\pm$ 0.740	10.717 $\pm$ 0.866 ^c	12.230 $\pm$ 0.282 ^b	14.663 $\pm$ 2.280	14.840 $\pm$ 1.090	14.016 $\pm$ 0.608
Thymus	1.937 $\pm$ 0.321	1.373 $\pm$ 0.247 ^c	1.689 $\pm$ 0.385	1.267 $\pm$ 0.318	1.192 $\pm$ 0.245	1.250 $\pm$ 0.145
Adrenals	0.296 $\pm$ 0.123	0.369 $\pm$ 0.066	0.375 $\pm$ 0.097	0.262 $\pm$ 0.123	0.175 $\pm$ 0.108	0.207 $\pm$ 0.096
Testes/Ovaries	1.048 $\pm$ 0.277	0.845 $\pm$ 0.329	0.969 $\pm$ 0.361	7.119 $\pm$ 0.653	7.043 $\pm$ 0.407	6.695 $\pm$ 0.944

^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 2
Blood biochemistry mean values $\pm$ SD.

Groups	Female			Male		
	Water	BSA	Water	BSA	Water	BSA
TP (g/l)	61.73 $\pm$ 2.27	62.96 $\pm$ 3.30	58.54 $\pm$ 2.83 ^b	56.13 $\pm$ 2.50	61.86 $\pm$ 2.77 ^c	62.62 $\pm$ 2.87 ^a
ALB (g/l)	35.03 $\pm$ 0.79	36.41 $\pm$ 1.53	34.57 $\pm$ 1.18 ^b	30.01 $\pm$ 1.03	33.59 $\pm$ 1.15 ^c	34.72 $\pm$ 1.27 ^a
GLU (mmol/l)	2.72 $\pm$ 0.52	3.32 $\pm$ 1.12	3.77 $\pm$ 0.66 ^a	3.12 $\pm$ 0.93	3.25 $\pm$ 0.79	3.20 $\pm$ 0.60 ^a
UREA (mmol/l)	2.42 $\pm$ 0.28	2.64 $\pm$ 0.40	3.05 $\pm$ 0.53 ^a	2.62 $\pm$ 0.43	2.57 $\pm$ 0.40	2.54 $\pm$ 0.28
TRIG (mmol/l)	1.14 $\pm$ 0.17	1.08 $\pm$ 0.26	0.87 $\pm$ 0.14 ^a	1.01 $\pm$ 0.23	1.28 $\pm$ 0.12 ^c	1.10 $\pm$ 0.07
CHOL (mmol/l)	1.87 $\pm$ 0.15	1.88 $\pm$ 0.47	1.70 $\pm$ 0.10	2.36 $\pm$ 0.13	2.41 $\pm$ 0.25	2.29 $\pm$ 0.28
CREA (umol/l)	130.13 $\pm$ 3.91	131.74 $\pm$ 8.00	127.99 $\pm$ 3.53	123.40 $\pm$ 3.92	123.40 $\pm$ 3.88	125.79 $\pm$ 3.64
LDH (U/L)	1136.3 $\pm$ 159.1	1030.0 $\pm$ 446.6	886.8 $\pm$ 187.5	1246.0 $\pm$ 301.4	958.0 $\pm$ 315.3	1208.2 $\pm$ 504.9
ALT (U/L)	91.50 $\pm$ 30.53	173.00 $\pm$ 242.97	85.17 $\pm$ 21.40	49.33 $\pm$ 14.49	77.17 $\pm$ 43.18	130.17 $\pm$ 125.64
AST (U/L)	309.50 $\pm$ 26.77	378.33 $\pm$ 112.00	480.17 $\pm$ 142.43 ^a	308.83 $\pm$ 112.11	390.50 $\pm$ 170.59	359.33 $\pm$ 150.09
ALKP (U/L)	110.50 $\pm$ 57.18	130.83 $\pm$ 17.30	146.33 $\pm$ 7.69	124.17 $\pm$ 14.36	151.00 $\pm$ 18.45 ^c	150.17 $\pm$ 17.75 ^a

^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
Haematology mean values $\pm$ SD.

Groups	Female			Male		
	Water	BSA	Water	BSA	Water	BSA
HGB (g/L)	140.17 $\pm$ 2.48	142.33 $\pm$ 4.32	142.50 $\pm$ 1.87	132.17 $\pm$ 7.68	143.83 $\pm$ 1.33 ^c	146.00 $\pm$ 4.05 ^a
WBC (10^9 /L)	1.99 $\pm$ 0.44	2.22 $\pm$ 0.34	2.46 $\pm$ 0.36	3.10 $\pm$ 0.77	2.48 $\pm$ 0.49	2.21 $\pm$ 0.47
RBC (10^{12} /L)	9.13 $\pm$ 0.11	9.33 $\pm$ 0.23	9.20 $\pm$ 0.18	8.77 $\pm$ 0.45	9.40 $\pm$ 0.13 ^c	9.47 $\pm$ 0.18 ^a
PLT (10^9 /L)	656.50 $\pm$ 70.65	681.33 $\pm$ 64.19	706.83 $\pm$ 159.40	818.50 $\pm$ 54.99	836.00 $\pm$ 70.63	864.17 $\pm$ 75.76
HCT (10^9 /L)	41.63 $\pm$ 0.55	45.13 $\pm$ 0.80 ^c	42.08 $\pm$ 0.62 ^b	40.90 $\pm$ 1.90	42.82 $\pm$ 0.42 ^c	43.37 $\pm$ 1.26 ^a
MCV (fl)	45.62 $\pm$ 0.65	45.13 $\pm$ 0.80	45.75 $\pm$ 0.29	46.72 $\pm$ 2.36	45.55 $\pm$ 0.34	45.80 $\pm$ 1.16
MCH (pg)	15.35 $\pm$ 0.18	15.27 $\pm$ 0.20	15.48 $\pm$ 0.12 ^b	15.35 $\pm$ 0.19	15.30 $\pm$ 0.13	15.43 $\pm$ 0.29
MCHC (g/L)	336.67 $\pm$ 3.88	338.00 $\pm$ 3.79	338.67 $\pm$ 1.37	323.00 $\pm$ 5.06	335.67 $\pm$ 1.37 ^c	336.67 $\pm$ 2.73 ^a
MPV (fl)	7.52 $\pm$ 0.17	7.58 $\pm$ 0.40	7.38 $\pm$ 0.12	7.77 $\pm$ 0.36	7.53 $\pm$ 0.27	7.37 $\pm$ 0.31 ^a

^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.

higher in females as compared to the BSA group, but no differences could be found in males.

No group-related histopathologic observations were found. The few spontaneous findings observed were generally minimal to slight/mild in severity, were randomly distributed among all groups and were the type commonly observed in control rats of this age and strain (data not shown).

3.6. Micronucleus test

Table 4 shows the incidence of micronucleated polychromatic erythrocytes (MPE) per 5000 chromatophilic red cells. The Cry1C protein did not increase MPE at any of the dose levels used when compared to vehicle controls for each sex. MPE levels did not exceed 3% in the Cry1C group. The positive control cyclophosphamide produced a significant increase in MPE ($26.6 \pm 3.4\%$ for males and $26.6 \pm 3.4\%$ for females).

3.7. Sperm shape abnormality test

The effects of the Cry1C protein on the incidence of sperm abnormalities in mice are shown in Table 5. The frequencies of abnormalities in the high, middle and low dose Cry1C groups were $1.50 \pm 0.5\%$, $1.24 \pm 0.6\%$ and $1.38 \pm 0.4\%$. The data showed no significant differences between the groups or the negative control, whose frequency of abnormality was $1.28 \pm 0.5\%$. In the positive control group, $4.86 \pm 1.2\%$ showed sperm abnormalities, which was significantly higher than that observed in the negative control group.

3.8. Ames test

The revertants with or without metabolic activation in each group are listed in Table 6. The mutant frequencies of the negative controls were in the range of the historical controls, and the positive controls induced significant increases in the mutant frequencies, verifying the sensitivity of the strains used. No increases in the mutant frequencies were observed for the four tester strains after treatment with 50–5000 $\mu\text{g}/\text{plate}$ of Cry1C protein, either with or without metabolic activation.

4. Discussion

The Codex Alimentarius Commission has suggested a conceptual framework for the conduct of safety assessments for allergenicity (Codex, 2009). The amino acid sequence homology with, or structural similarity to allergenic proteins, the resistance of novel proteins to digestion in a simulated gastric fluid and case by case basis serological identity of the protein of interest with proven human allergens should be considered (Codex, 2009). Identification of allergic potential is a key issue whenever new proteins are brought into contact with humans, either through food or other modes of

Table 5

Results of sperm abnormality test of mice mean values $\pm$ SD.

Groups	Dose (ug/kg)	Sperms examined	Abnormal sperms	Frequencies of abnormality (%)
Cry1C	1250	5×1000	75	1.50 ± 0.5
Cry1C	625	5×1000	62	1.24 ± 0.6
Cry1C	125	5×1000	69	1.38 ± 0.4
Negative Control	0	5×1000	64	1.28 ± 0.5
CP	40	5×1000	243	$4.86 \pm 1.2^*$

Compared with control group: $*p < 0.05$.

exposure. Bioinformatics is an important tool for managing allergen data, analysis of the properties of allergens, assessment of protein allergenicity and analysis of potential allergic cross-reactivity (Zhang et al., 2006; Thomas et al., 2009). Sequence searches are performed to evaluate the sequential and structural similarities between the query sequences and known allergens to evaluate the possibility that one or more of the query sequences may share one or more common IgE binding epitopes with an allergen. If matched, there is a possibility that the query sequence might elicit an allergic response in those with existing allergies and a serum screening study with relevant subjects should be conducted. Epitopes that are bound by IgE may be short sequential segments of amino acids. Proteins sharing high percent identities by local alignment programs such as FASTA or BLASTP are highly likely to cause cross-reactions (Goodman et al., 2005, 2008). The Cry1C protein had no homology with known toxins or allergens in NCBI and allergens in AllergenOnline.

As the Cry1C protein was expressed in rice, it would be boiled before eaten. Indeed, in addition to potential loss of the Cry1C protein in the boiled water that reduces the exposure level, it was interesting to discern whether it would be degraded by heat. Although heat stability and protein allergenicity is not strictly correlated, fragmenting the amino acid structure is certainly consistent with a decrease in the allergenicity potential. We assessed whether the Cry1C protein would be completely degraded, leave some fragments or be heat stable. We found that the Cry1C protein is heat stable. Importantly, this does not suggest that the protein is allergenic. In fact, the weight of evidence indicates that the Cry1C protein lacks allergenic potential.

Food allergens are considered to be able to exert systemic allergic reactions only after they have been absorbed through the intestinal mucosa. In addition, the resistance of a protein toward digestion in the human gastrointestinal tract is considered in a weight of evidence assessment. Scientifically, when the protein is digested in the SGF (and no adverse effects are observed in the acute oral study in rodents), there is no utility to run an SIF study. We performed this experiment, since the Ministry of Agriculture of PR China No.869 Bulletin 2-2007 requires the SIF test. And the SIF test can consolidate the Cry1C safety conclusion in a certain extent. The Cry1C protein was digested in both SGF and SIF within 15 s.

Table 4

Permillage of micronucleated polychromatic erythrocytes in 5×1000 polychromatic erythrocytes and the ratio between polychromatic and normochromatic erythrocytes in bone-marrow cells mean values $\pm$ SD.

Groups	Dose (ug/kg)	Males			Females		
		Chromatophilic red cells	MPE	Permillage (%)	Chromatophilic red cells	MPE	Permillage (%)
Cry1C	125	5×1000	13	2.6 ± 1.0	5×1000	11	2.2 ± 1.1
Cry1C	625	5×1000	11	2.2 ± 1.3	5×1000	14	2.8 ± 2.0
Cry1C	1250	5×1000	15	3.0 ± 1.5	5×1000	6	1.2 ± 1.0
Negative control	0	5×1000	8	1.6 ± 1.2	5×1000	7	1.4 ± 1.1
CP	40	5×1000	135	$27.0 \pm 2.9^*$	5×1000	133	$26.6 \pm 3.4^*$

Compared with control group: $*p < 0.01$.

Table 6Ames test: summary of revertants with or without metabolic activation mean values $\pm$ SD.

Groups	(mg/plate)	TA97		TA98		TA100		TA102	
		–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
Cry1C	0.05	125 $\pm$ 8	123 $\pm$ 10	35 $\pm$ 1	33 $\pm$ 3	134 $\pm$ 11	136 $\pm$ 18	269 $\pm$ 8	273 $\pm$ 30
	0.25	119 $\pm$ 12	122 $\pm$ 5	32 $\pm$ 3	34 $\pm$ 2	133 $\pm$ 12	134 $\pm$ 8	264 $\pm$ 21	269 $\pm$ 14
	1.25	121 $\pm$ 8	118 $\pm$ 14	34 $\pm$ 3	36 $\pm$ 3	123 $\pm$ 9	125 $\pm$ 10	267 $\pm$ 41	272 $\pm$ 52
	2.5	105 $\pm$ 7	123 $\pm$ 7	33 $\pm$ 2	33 $\pm$ 4	119 $\pm$ 23	122 $\pm$ 7	265 $\pm$ 20	268 $\pm$ 23
	5	114 $\pm$ 14	123 $\pm$ 24	34 $\pm$ 5	35 $\pm$ 7	139 $\pm$ 28	136 $\pm$ 11	263 $\pm$ 31	275 $\pm$ 24
Negative control		137 $\pm$ 6	103 $\pm$ 3	35 $\pm$ 5	33 $\pm$ 3	145 $\pm$ 41	156 $\pm$ 38	262 $\pm$ 23	294 $\pm$ 40
Positive control		3190 $\pm$ 251 ⁽¹⁾	1254 $\pm$ 291 ⁽⁴⁾	3291 $\pm$ 301 ⁽¹⁾	1060 $\pm$ 189 ⁽⁴⁾	2902 $\pm$ 206 ⁽²⁾	2312 $\pm$ 188 ⁽⁵⁾	2408 $\pm$ 312 ⁽³⁾	1092 $\pm$ 182 ⁽⁶⁾

(1) 2,4,7-TNF-one (0.5 μ g/plate); (2) NaN₃ (2.5 μ g/plate); (3) MMC (1.0 μ g/plate); (4) 2-AAF (20 μ g/plate); (5) Benzo(a)pyrene (1.0 μ g/plate); (6) 1,8-dihydroxyanthraquinone (50 μ g/plate).

Based on the experimental results, the Cry1C protein is rapidly digested.

Acute toxicity testing of transgenic proteins is performed in rodents (usually mice) to assess potential mammalian toxicity following a single exposure to high concentrations of the protein. Acute toxicology studies are required by the EPA to assess the hazards of protein-based pesticides, which are the active components of microbial pesticides that are Plant-Incorporated Protectants (PIPs; US EPA, 2000). To date, no tested transgenic proteins used in GM crops have demonstrated adverse effects in acute toxicity studies, even at extremely high doses of Cry1Ab >4000 (oral), Cry1Ac >4200 (oral), Cry2A >3000 (oral), Cry3A >5200 (oral), Cry3Bb1 >3850 (oral), Cry1F >600 (oral) and Cry9C >3760 (oral) (Delaney et al., 2008b). In this study, there was no mortality or toxic effect observed in mice after acute oral administration of the Cry1C protein at 5 g/kg body weight, which is considered to be the limit dose (Chinese standard GB15193.13-2003).

As the Cry1C protein is rapidly degraded, its absorption is negligible. Nevertheless, we have evaluated the genotoxic potential of the Cry1C protein for consolidating the Cry1C safety conclusion in case of impairment of physiological digestive conditions, in accord with the recommendations of PR China. National standard of PR China requires the micronucleus test, sperm shape abnormality test and Ames test for new food sources. Wang et al. (2004) proved that Cry1Ab rice could not cause any increase in the incidence of MPE in male or female mice at any of the dose levels. In this study, the Cry1C protein showed no adverse effects on mice chromophilic red cells, which coincides with the study performed by Wang et al. (2004). Sperm abnormalities of Cry1Ac cotton were evaluated to provide a scientific basis for transgenic crop safety (Zhang et al., 2000). Sperm abnormalities have long been associated with male infertility and sterility in most species. These abnormalities vary from morphological defects that are evident upon clinical examination to those that are more subtly defective. In general, sperm structure can play a substantial role in both fertilization and pregnancy outcome (Chauhan et al., 2000; Chenoweth, 2005). The Cry1C protein had no significant effect on the incidence of sperm abnormality in mice. These results are concordant with Zhang et al. (2000). In another study, herbicide-tolerant gene (*bar*) transgenic rice was not found to be mutagenic using the *S. typhimurium* assay (Wang et al., 2000). Fifty-fifty thousand μ g/plate of Cry1C protein did not increase the revertants in the *S. typhimurium* strains TA97, TA98, TA100 and TA102, and the mean number of revertants in the Cry1C protein groups had no mutagenic effect in this study, which is concordant with Wang et al. (2000). In summary, the Cry1C protein was proven to have no mutagenicity potential.

5. Conclusion

The Cry1C protein had no sequence homology with any known allergens or toxins by bioinformatics analysis. Although the Cry1C protein was heat stable it was rapidly digested in SGF and SIF

(according to Chinese standard). The protein caused no adverse effects in mice by gavage at a high dose level of 5 g (Cry1C protein)/kg body weight (according to Chinese standard). In the mutagenic test and sperm abnormalities study, the Cry1C protein group results were similar to those obtained in the control group. Furthermore, the Cry1C protein caused no increased mutation in the Ames test (according to Chinese standard). Taken together, these data indicate that the Cry1C protein is not a potential allergen or toxin.

Acknowledgments

This work was funded by the Genetically Modified Organisms Breeding Major Projects of PR China (2008ZX08011-005 and 2009ZX08011-001B), and we express our gratitude to the Ministry of Science and Technology and the Ministry of Agriculture of PR China for their financial support.

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