Available online at www.sciencedirect.com
ScienceDirect
Food and Chemical Toxicology 46 (2008) 2184–2189
www.elsevier.com/locate/foodchemtox

Evaluation of subchronic toxicity of dietary administered Cry1Ab protein from *Bacillus thuringiensis* var. *Kurustaki* HD-1 in F344 male rats with chemically induced gastrointestinal impairment

Jun-ichi Onose [a,b], Toshio Imai [a,*], Mai Hasumura [a], Makoto Ueda [a], Yoshihiro Ozeki [c], Masao Hirose [a]

[a] *Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Department of Nutritional Science, Faculty of Applied Bio-science, Tokyo University of Agriculture, 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan*
[c] *Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, Naka-cho 2-24-16, Koganei, Tokyo 184-8588, Japan*

Received 5 May 2007; accepted 19 February 2008

Abstract

Bacillus thuringiensis (Bt) proteins are developed for genetically modified crops and the Bt proteins demonstrate no evidence of toxicity by the oral route in traditional animal models. However, the possible toxicity of Bt proteins under conditions of reduced gastric acid secretion and/or small intestinal damage has not been investigated. In the present study, we therefore evaluated following four F344 rat groups with a purified Bt protein Cry1Ab from *B. thuringiensis* var. *Kurustaki* HD-1. Gastrointestinal impairment (GI) alone and GI + Bt protein fed (GI + Bt) groups were given i.p. injections of famotidine to reduce gastric acid secretion twice a day at 30 mg/kg body weight in weeks 2 and 4. GI and GI + Bt groups were additionally fed diets containing 80 ppm indomethacin for induction of intestinal damage during weeks 1 and 3. Bt alone and GI + Bt groups were also fed diet containing Bt protein Cry1Ab at a concentration of 10 ppm in weeks 2 and 4. A no treatment control group was also included. At the end of week 4, all animals were euthanized under ether anesthesia, blood samples were collected for hematology and serum biochemistry and a complete necropsy was performed. No significant changes indicative of toxicity of the Bt protein Cry1Ab used here were noted with any of the parameters investigated. In conclusion, no significant toxicological effects were detected in this subchronic gastrointestinal impairment rat model.

Keywords: *Bacillus thuringiensis* protein; A gastrointestinal impairment rat model; Subchronic toxicity

1. Introduction

Numerous toxicological studies of genetically modified organism (GMO) foods using laboratory animals have been conducted (Betz et al., 2000; Knudsen and Poulsen, 2007). One possible problem on such *in vivo* studies is how nutritional influence can be eliminated when safety examination of exogenous dietary constituents is performed with whole food exposure (Kuiper et al., 1999; Konig et al., 2004). Therefore, safety assessment based on results of *in vitro* and *in vivo* studies of the pure novel gene products is considered to be appropriate (Betz et al., 2000).

Newly expressed heterologously proteins in GMO foods have been evaluated for safety, including allergenicity, using *in vitro* digestibility studies with stimulated gastric juice as one of the recommended many studies (Astwood et al., 1996). Ingested proteins in the stomach with high gastric digestibility do not reach intestinal mucosa, where absorption and/or sensitization can occur, although this

* Corresponding author. Tel.: +81 3 3700 9818; fax: +81 3 3700 1425.
E-mail address: imai@nihs.go.jp (T. Imai).

0278-6915/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fct.2008.02.015

will depend on sufficient amounts of the responsible enzymes. Conventionally ingested proteins, such as ovalbumin and a certain type of wheat protein were actually known to sometimes escape hydrolysis in healthy states and reach to intestines, and they have functional/immune activities (MacFarlane et al., 2003; Van Niel et al., 2003). There is therefore concern about incomplete digestion in the stomach with gastric achlorhydria due to gastrointestinal impairment or antacid use. In addition, concomitant intestinal damage with inflammatory bowel disease (IBD) and upper gastrointestinal disorders known to be adverse effects of non-steroidal anti-inflammatory and anti-cancer treatment (Sartori et al., 2000; Seager and Hawkey, 2001) might influence absorption. In fact, the intestinal mucosa of IBD patients shows increased permeability to ovalbumin (Soderholm et al., 1999) and absorption of viable bacteria and bacterial products (Linskens et al., 2001). However, the possible toxicity of GMO products under conditions of reduced gastric acid secretion and/or small intestinal damage has not been investigated.

Bacillus thuringiensis (Bt) products are the most widely used biopesticide in the world. Bt is a gram-positive spore-forming bacterium, and various strains of Bt produce different insecticidal protein toxins in parasporal crystals, which have been used as insecticides and which have been engineered into plants for crop protection (Carozzi et al., 1991). More recently, a *cry* gene encoding the insecticidal proteins in these Bt products has been cloned (Schnepf and Whiteley, 1981), introduced, and expressed in genetically modified plants (Vaeck et al., 1987; Konig et al., 2004) to provide protection against insect damage. Bt proteins demonstrate no evidence of toxicity by the oral route in traditional animal models (Thomas and Ellar, 1983; Betz et al., 2000). In addition, Bt proteins have been shown to be rapidly degraded *in vitro* using simulated gastric fluids (Betz et al., 2000). On the other hand, trace amounts of the Bt protein Cry1Ab have been demonstrated in the gastrointestinal tract but this was not absorbed in animals fed GM corn Bt11 (Chowdhury et al., 2003a,b), and this might raise a concern about the digestibility of Cry1Ab *in vivo*. Therefore, toxicological studies using animals under conditions of reduced gastric acid secretion and/or small intestinal damage should provide useful evidences for confirmation of the safety.

We here conducted a subchronic toxicity study of Bt protein Cry1Ab in animals with chemically induced gastric and intestinal disorders that may influence digestion and absorption in the alimentary canal. Intermittent treatments with famotidine, a selective histamine H2 receptor antagonist (Miwa et al., 1984), to reduce gastric acid secretion, and indomethacin, a non-steroidal anti-inflammatory drug, for induction of enteritis were applied for this purpose. Indomethacin is widely used in rat models for the study of either acute or chronic small intestinal inflammation (Krieglstein et al., 2001) and reported to increase in intestinal permeability to polyethylene glycol (Krugliak et al., 1990).

2. Materials and methods

2.1. Chemicals and formulations

Indomethacin and famotidine were purchased from Wako Pure Chemical Industries, Osaka, Japan. A crystallized Bt protein, Cry1Ab was obtained as follows; *B. thuringiensis* var. *kurustaki* HD-1 harboring *cry1Ab* gene (kindly gifted from Dr. Ryoichi Sato, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology) was inoculated into Luria–Bertani (LB) liquid medium containing 0.1% glucose of 80 mL in 500 mL flasks and grown at 28 °C for 20 days with vigorous shaking until all cells were lysed. Cultured medium of three flasks was combined to 240 mL in total, and crystals and spores were harvested by centrifugation (15,000*g*, 10 min, 4 °C) then washed twice in cold distilled water by centrifugation. Crystals and spores were purified by a two-phase system using polyethylene glycol 6000 (Goodman et al., 1967). After the separation, the viscous crystal-rich phase was diluted with twice volume of distilled water and centrifuged (15000*g*, 30 min, 4 °C). The sediment was resuspended in 50 mM NaCl and then centrifuged using a swing rotor (2000*g*, 15 min, 4 °C). Remaining spores and debris onto the crystal pellet or suspended in the supernatant fluids were removed with a pipette. The crystals were washed five times by centrifugation in the same manner. The purified crystals were suspended in 10 mL of 1% SDS containing 0.1 M 2-mercaptoethanol, then incubated at 4 °C for 5 min. After the removal of debris by centrifugation (15,000*g*, 10 min, 25 °C), Bt protein Cry1Ab solution dissolved the crystals were put into visking tubes and dialyzed into 1 L of 50 mM HEPES-KOH (pH 7.0) containing 10% Triton X-100 for 5 h, followed by into 1 L of 50 mM HEPES-KOH (pH 7.0) containing 0.5% Triton X-100 overnight, into 10 mM HEPES-KOH (pH 7.0) for 5 h and then finally into 1 L of 10 mM $NaHCO_3$ for 5 h subsequently. The solution was recovered into a plastic tube and frozen into liquid nitrogen, then freeze-dried. The purified Bt protein Cry1Ab powders of 78.9 mg was obtained. A small part of the powder was dissolved into SDS-PAGE sample buffer and analyzed by SDS-PAGE. After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 and analyzed using gel scanner. The purity of Bt protein Cry1Ab in this powder was be estimated to be ca. 93% by the band image analysis. The preparations were repeated five times to prepare enough amounts of Bt protein Cry1Ab for the present experiment.

Indomethacin and Bt protein Cry1Ab were mixed into powdered basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) in a mortar and plastic bags at concentration of 80 and 10 ppm, respectively. Homogeneity of the Bt protein Cry1Ab formulation was confirmed with Western blot analysis using anti mouse Bt protein polyclonal antibodies. Basal diet containing indomethacin or Bt protein Cry1Ab were prepared weekly, because their stabilities in the diet were not confirmed. Famotidine was dissolved with saline just before the administration.

2.2. Animals

A total of 32 male F344 rats were purchased at 6 weeks of age from Charles River Japan (Kanagawa, Japan) and housed 4 rats per polycarbonate cage with white wood chips (Sankyo Laboratory Service, Tokyo, Japan) for bedding under standard conditions (room temperature, 24 ± 1 °C; relative humidity, 55 ± 5%; ventilation frequency, 18 times/h; 12 h light and dark cycle) and allowed free access to a water and basal diet (CRF-1). Because of a small amount of Bt protein Cry1Ab obtained for dietary administration to rats as described above, only males were used in the present study. In a previous literature, orally administration of 8400 mg/kg body weight/day of Bt microbial pesticide from *B. thuringiensis* var. *kurustaki* for 90 days was reported not to induce any toxic effects in both male and female rats, indicating no obvious gender differences even at high dose levels.

2.3. Experimental design

The experimental design is shown in Fig. 1. The rats were divided into four groups. In our preliminary study, pH of gastric juice of untreated