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Toxicology effects of δ-endotoxins and *β*-exotoxins of *Bacillus thuringiensis* in *Wistar* rats

Efeitos toxicológicos de δ-endotoxinas e *β*-exotoxinas de *Bacillus thuringiensis* em ratos *Wistar*

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Abstract

Bacillus thuringiensis is an entomopathogen consisting of toxic proteins, such as the δ-endotoxins specific to insects. However, some subspecies can produce β-exotoxins that are nonspecific and toxic to the vertebrates. So, in this research the toxicology effect of two isolates of *B. thuringiensis* in *Wistar* rats have been assessed. In the *in vivo* assays the animals have been given 3.10^{10}UFC/mL of *B. thuringiensis thuringiensis* and *B. thuringiensis aizawai* and supernatant bacterial suspensions orally. The excrements and the stomachic content of the animals have been collected and analyzed in SDS-PAGE (10%). The analyzed data show protein fragments between 151 kDa and 28 kDa, seeing that the fecal samples show only one track of peptides in the treatments when compared with the control group. The assessments of the stomach of the treated rats carried out under stereomicroscope have not shown alterations when compared with the control. These results indicate that the toxins which can be found in both *B. thuringiensis* species used in these assays can be degraded by the gastrointestinal conditions of the animals and have not presented oral toxic effect to this mammalian species under the conditions which the experiments had been carried out.

Key words: bacterium, toxins, mammalian, toxicity.

Resumo

Bacillus thuringiensis é um entomopatógeno constituído de proteínas tóxicas, como as δ-endotoxinas específicas a insetos. Porém algumas subespécies podem produzir β-exotoxinas inespecíficas e tóxicas aos vertebrados. Sendo assim, nessa pesquisa foram avaliados os efeitos toxicológicos de dois isolados de *B. thuringiensis* em ratos *Wistar.* Nos ensaios *in vivo* os animais foram tratados via oral com suspensões de 3.10^{10}UFC/mL de *B. thuringiensis aizawai* e *B. thuringiensis thuringiensis* e sobrenadante. As fezes e o conteúdo estomacal dos animais foram coletados e analisados em SDS-PAGE (10%). Os dados das análises do conteúdo estomacal mostram fragmentos protéicos entre 151 e 28 kDa, sendo que as amostras fecais mostram apenas um rastro de peptídeos nos tratamentos, quando comparados à testemunha. As avaliações, realizadas sob estereomicroscópio,

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do estômago dos ratos tratados não mostraram alterações quando comparados com a testemunha. Esses resultados indicam que as toxinas, presentes nas duas subespécies de *B. thuringiensis* utilizadas nestes ensaios, podem ser degradadas pelas condições gastrointestinais dos animais e não apresentaram efeito tóxico, via oral, a essa espécie de mamífero, nas condições em que foram realizados os experimentos.

Palavras-chave: bactéria, toxinas, mamífero, toxocidade.

Introduction

Bacillus thuringiensis is an entomopathogen found naturally on the soil, and it is characterized by the production of paraspores inclusion bodies during sporulation, formed by proteins toxic to different insect groups (Höfte and Witheley, 1989). Those proteins are synthesized by *cry* genes, which are divided into classes and subclasses that show insecticidal activity against different pest insects of cultivated plants (Crickmore *et al.* 1998; Schenpf *et al.* 1998; Pinto and Fiuza, 2002).

During the growing process, *B. thuringiensis* strains bring about many toxins, among which δ-endotoxins and β-exotoxins can be found (Höfte and Witheley, 1989). The δ-endotoxin is known for its pathogenicity and specificity against insects, although the β-exotoxin, also known as thuringiensin, is a non-specific thermostable protein, identified as an rRNA synthesis inhibitor (Mackedonski and Hadjilov, 1972), besides impairing the mitotic fuse development, among other effects noticed in *in vitro* assays, with *Alium cepa* (Sharma and Sahu, 1977).

Due to the characteristics of specificity and selectivity of *B. thuringiensis* there are many commercial insecticides with a *B. thuringiensis*-based formula, with a register in the United States of America since 1961 (Siegel, 2001). According to this author the use of those products reaches Brazil, also, mainly for controlling lepidoptera that attack different national economically significant monocultures. Besides, they can be used to control larvae of human disease vectors, such as the diptera.

Technology development has not only brought formulated bioinsecticides, but DNA microbial sequences as well, which have been included in plants to promote resistance to insects. The Environmental Protection Agency (EPA) in the United States is responsible for the register of genetically modified plants, where they are assessed for their impact on natural enemies or non-target organisms, the frequency on which their pollen fertilizes other plants, by focusing the study on the probable toxicity and the allergenic ability of the new proteins in plants, which can change their nutrient composition and defense mechanisms (Schelton *et al.*, 2002).

This being so, this work aimed at the analysis of the toxicological effect of proteins of two *B. thuringiensis* subspecies on the digestive system of *Wistar* mice in *in vivo* laboratory assays.

Material and methods

Male adult Wistar strain mice, 80 and 100 days old, from the biotery of the Universidade do Vale do Rio do Sinos were used. The animals were kept in acrylic boxes in an acclimated room (21 C and a 12-hour photophase), and fed with Purina® mice food and distilled water.

The *B. thuringiensis aizawai* isolate was obtained from the commercial product Xentari® (*Hokko do Brasil Indústria Química e Agropecuária Ltda.),* and the *B. thuringiensis thuringiensis* isolate was correlated with H1 serovar provided by the *International Entomopathogenic Bacillus Centre,* Pasteur Institute, Paris.

Bacterial growth was carried out following De-Barjac and Lecadet's description (1976). Then, the material was centrifuged, and the supernatant was separated from the bacterial *pellet*. Bacterial cells quantification was performed in Neubauer's Chamber and optical microscopy. The concentration was adapted to 3.10^{10} UFC/mL, with the β-exotoxins supernatant.

The mice were individually kept in the acrylic boxes, divided into five treatments, amounting to 30 animals. In treatment 1 (T_1) 400 µL of the *B. thuringiensis aizawai* suspension were applied, at a concentration of 3.10^{10} UFC/mL; in treatment 2 (T_2) 400µL of the *B. thuringiensis thuringiensis* suspension were applied, at a concentration of 3.10^{10} UFC/mL; in treatments 3 and 4 (T_3 e T_4) 1000µL of *B. thuringiensis aizawai* and *B. thuringiensis thuringiensis* supernatant were applied, and in treatment 5 (T_5), which corresponds to control, distilled water instead of the products was applied.

Treatments were performed by gavages at different times: 0, 12, and 24 hours. Each animal's total feces collection, according to the treatment, was made 12, 24, and 36 hours after their application (HAT). The animals were killed 24 and 36 HAT, and their stomach contents removed for analysis in SDS-PAGE 10% (Laemmli, 1970), to detect toxic proteic fragments of *B. thuringiensis*. The mice's stomachs were assessed through stereomicroscope (40X), according to a method adapted from de Marroni *et al.* (1994).

Results

Data regarding the proteins molecular weight from *B. thuringiensis thuringiensis* and *B. thuringiensis aizawai* isolates are shown in

Table 1. As to β-exotoxin, found in the supernatant, it has a molecular weight of 0.7 kDa, according to Gohar and Perchart's data (2001).

The stomach macroscopic assessments of the mice treated with *B. thuringiensis* suspension and supernatant do not show anomalies due to the treatments if compared to the control. There are no reference data about the action of *B. thuringiensis* on mammals' stomachs, *in vivo* assessments; although Oates and Hakkinem (1998) report that a 35% concentration ethanol produces mucous and sub-mucous membranes hyperemia, besides edema and bleeding. But there are no distinct changes in the stomach mucous membrane in mice under the reported conditions.

Data in Table 2 show assessments in SDS-PAGE 10% in samples of the stomach contents of the sacrificed mice 24 and 36 HAT.

In both treatments the noticed fragments show that the protein does not keep itself intact as it goes through the stomach, thus undergoing digestion and being changed into smaller than 140 kDa peptides, which correspond to the δ-endotoxins found in the isolates used in this study (Bravo *et al.* 1998). Regarding data about the stomach content of the 24 and 36 HAT killed mice it was noticed that the peptide digestion process persisted according to the material shown in Table 2. The difference in the molecular weight of the proteic fragments between 24 and 36 HAT decreased, and it can be connected to a supplemental dose given to the animals in treatments corresponding to the 36 HAT.

Data about the fecal content of the mice treated with the isolates suspension and supernatant were similar to the witness's thus showing a protein trace which can point to the resolution of the peptides' digestion process, found in the respective treatments.

Discussion

Studies by Sebesta *et al.* (1981) and Vankova *et al.* (1974) exhibited data that show that the thuringiensin is more toxic if given intraperitoneally than orally, since through the latter β-exotoxin undergoes degradation due to the digestive system phosphatases. As to *B. thuringiensis thuringiensis* isolates, a review by McClintock *et al.* (1995) states that this subspecies produce the β-exotoxin, which, if orally administered, shows a 170-mg-kg^{1} LD_{50}, in mice. The same authors state that β- exotoxin is slightly or highly toxic, depending on the tested species and the administration route. One of the reported examples has to do with low toxicity of that toxin, dermally administered in mice, otherwise highly toxic to rabbits, via dermal route. In that case it is possible to conclude that in this study, when given orally and in the used dose (3.10^{10} UFC/mL), *B. thuringiensis thuringiensis* and *B. thuringiensis aizawai* isolates did not show peptides corresponding to the β-exotoxin. If they were present at a non-detected concentration, under these assays conditions, they were non-toxic to *Wistar* mice.

Data by Hernández *et al.* (2003) show that out of 25 *B. thuringiensis thuringiensis* isolates assessed in his study, 21 presented the β-exotoxin, and out of 33 isolates, 79% are toxin synthesized, which contradicts the 0% production for *B. thuringiensis aizawai*. Despite that, the presence of such toxin was not stressed in this study through 10% polyacrilamid gel.

As to δ-endotoxins, Vilas-Bôas *et al.* (1998) say that *B. thuringiensis* spores have their germination inhibited in a less than 5.0 pH, seeing that this factor, together with the presence of indigenous microorganisms, are decisive to that bacterium survival and viability. This study results suggest that the acid pH (pH 3.15 ± 0.1) (Vidal *et al.,* 2004) of the mice's stomachs has possibly been the main factor for the absence of gastric changes.

Other researches also report that Cry proteins show an anti-tumor action against the Yoshida sarcoma in mice, and they stress the immunological reaction of the sheep's blood (Vasquez-Padrón *et al.,* 2000). Some

Table 1. Protein profile of *Bacillus thuringiensis* isolates and of the food used in the *Wistar* mice treatments.

Products	Preparation	Protein profile (kDa)*
B. thuringiensis aizawai	Suspension	150 a 34
	Supernatant	Ø
B. thuringiensis thuring-iensis	Suspension	164 a 24
	Supernatant	Ø
Purina® rat food	Suspension	200 a 32

*= protein molecular weight in Kilo Daltons; Ø= no bands

Table 2. Protein profile of the stomach contents of *Wistar* mice treated with suspension and supernatant of *Bacillus thuringiensis aizawai* and *Bacillus thuringiensis thuringiensis*.

Treatment	Protein profile (kDa)*	
	24 HAT**	36 HAT
T_1*	58 a 53	71 a 51
T_2	83 a 34	151 a 34
T_3	52	87 a 28
T_4	51 a 34	84 a 36

*Treatments: (T_1): suspension to *B. thuringiensis aizawai*; (T_2) suspension to *B. thuringiensis thuringiensis*; (T_3) supernatant to *B. thuringiensis aizawai*; (T_4) supernatant to *B. thuringiensis thuringiensis*. *= protein molecular weight in Kilo Daltons; ** hours after application.

data of *in vitro* assays which use the Cry proteins intraperitoneally in *Balb/c* mice by these authors show the rodent's quick immunological reaction, thus displaying a great local production of IgG antibodies, followed by IgM and IgA, which points to those animals' effective defense mechanism. Other element related to these assays was the existence of still unknown mechanisms that either eliminate or inhibit the effect of *B. thuringiensis* toxins on non-target organisms, such as the mammals.

The recent growth of toxicological researches with *B. thuringiensis* isolates shows the scientists' growing concern about the impact of bioinsecticides formulated with this bacterium, and also with their application for obtaining transgenic plants resistant against insects. As such, Betz *et al.* (2000) report that the *B. thuringiensis* Cry proteins are toxic when in direct contact, and also that the non-target animals' exposure is extremely low. As to transgenic, the same author points out that these proteins are expressed in low concentrations in plant tissues, of which Azevedo and Araújo's data (2003) show the absence of toxic, mutagenic, teratogenic or clastogenic effects.

It was noticed that adult *Wistar* mice, either treated and non-treated with *B. thuringiensis* isolates, show no different results if compared regarding the stomach content and the total feces analysis. This evidences that toxins found in *B. thuringiensis thuringiensis* and *B. thuringiensis aizawai* subspecies, used in these assays, do not show toxic effect, when orally administered against this rodents species, and under these experimental conditions. This fact can be related to the mammals' natural defense mechanism, such as gastrointestinal conditions and the effective reaction to those fragments, this way inhibiting their toxicity.

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