



American Journal of
Food Technology

ISSN 1557-4571



Academic
Journals Inc.

www.academicjournals.com

Toxicological Evaluation for Food Applications of Transglutaminase from a Newly Isolated *Bacillus circulans* BL32

¹C.F.V. de Souza, ¹J.G. Venzke, ^{2,3}R.M. Rosa, ^{2,4}J.A.P. Henriques, ⁵E. Dallegrave, ¹S.H. Flôres and ¹M.A.Z. Ayub

¹Food Science and Technology Institute, (ICTA), Brazil

²Department of Biophysics, Federal University of Rio Grande do Sul State, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil

³Biomedicine and Pharmacy Courses and Pos-Graduation Program in Genetic and Applied Toxicology and

⁴Laboratory of Genetic Toxicology, Luterana University of Brazil, Canoas, RS, Brazil

⁵Toxicology Centre of Rio Grande do Sul State, Health State Secrey, Porto Alegre, RS, Brazil

*Corresponding Author: Marco Antônio Záchia Ayub, Food Science and Technology Institute, Federal University of Rio Grande do Sul State, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil
Tel: +55 51 3308 6685 Fax: +55 51 3308 7048*

ABSTRACT

The present study aimed to submit the transglutaminase (TGase) from a *Bacillus circulans* strain isolated from the Amazon environment to *in vivo* and *in vitro* toxicological evaluations in order to assess its safety in food. The *in vivo* assay was assessed using male Wistar rats in a subacute 14 day oral feeding test using a liquid enzyme preparation administered to 150 U kg b.wt. day⁻¹. The evaluation of cytotoxicity, genotoxicity and mutagenic effects of this microbial TGase was carried out using Chinese hamster lung fibroblasts cultured cells. No evidence of short term *in vivo* toxicity was found for the enzymatic preparation in the subacute 14 day oral toxicity study using white Wistar rats models, daily treated with 150 U kg b.wt. day⁻¹ of TGase preparation. Furthermore, there were no statistical differences between the groups for relative weight gain and for hematological and clinical chemistry values. Histopathological examination of liver, kidneys, spleen, thymus, adrenal glands, heart, lungs and brain did not reveal any treatment-related changes. In addition, it was not verified any evidence of cytotoxicity, genotoxicity and mutagenic effects of the simple application of 150 U treatment⁻¹ of this microbial TGase on Chinese hamster lung fibroblasts cultured cells. It can be concluded that no safety concerns were identified in this study for the BcTGase preparation produced under controlled cultivation conditions. Present results suggest that the microbial TGase from *B. circulans* BL32 is safe for food applications and has a potential for its industrial use.

Key words: Microbial transglutaminase, *Bacillus circulans*, toxicity, genotoxic evaluation, food applications

INTRODUCTION

Transglutaminase (TGase; protein-glutamine γ -glutamyltransferase; EC 2.3.2.13) is a family of enzymes that catalyze acyl transfer reactions using peptide-bonded glutamine residues as acyl donors and several primary amines as acceptors. These reactions introduce covalent cross-links between proteins, as well as peptides and various primary amines (Nonaka *et al.*, 1989). In the

absence of amine substrates, TGase catalyses the deamidation of glutamyl residues in which water molecules are used as acyl acceptors. Substances having TGase activity are abundantly distributed in nature. Calcium-dependent TGases are present in many animal and vegetable tissues, as well as in body fluids of vertebrates and are involved in several biological processes (Zhu *et al.*, 1995). TGases have been isolated from animal tissues, plants and from microorganisms (Kuraishi *et al.*, 2001), but only the microbial TGases are industrially important. Microbial TGases are usually calcium independent, have relatively low molecular weight and, being extracellular, easier and cheaper to purify than other sources (Zhu *et al.*, 1995). So far, the TGase obtained from *Streptovorticillium mobaraense* remains the only commercial source of this enzyme, but the industrial application of this microbial TGase has been limited due to its high market price. Moreover, as each industrial application may require specific properties of a biocatalyst, there is still a great interest in finding new TGases that could allow for novel applications (Arrizubieta, 2007).

Concerning the applications of TGases in the food industry, several studies have been carried out for the processing of meat, fish, dairy, wheat and soybean products, in order to improve texture, water-holding capacity, elasticity, nutritional value and appearance (Zhu *et al.*, 1995; Gauche *et al.*, 2009, 2010; Gan *et al.*, 2009; Askin and Kilic, 2009). This enzyme is also extensively used in biochemical research, pharmaceutical, textile and other chemical industries (Bernard *et al.*, 1998; Jülicher *et al.*, 1998; Collighan *et al.*, 2002; Cortez *et al.*, 2004; Hiller and Lorenzen, 2009).

It is known that tissue TGase has been linked to Celiac disease, the intolerance to gluten, caused by a selective lack of T cell tolerance for this protein mediated by a reaction that involves the generation of T cell stimulatory gluten peptides by deamidation of glutamine (Vader *et al.*, 2002). In spite of this fact, literature on the safety of TGase application in food systems is entirely lacking, being our work one of the few ever reported on its toxicological characteristics.

In our research, we have isolated a new TGase producer, a strain of *Bacillus circulans* BL32, which was isolated from the Amazon basin region (Soares *et al.*, 2003; Souza *et al.*, 2006). The enzyme production and purification was extensively studied (Souza *et al.*, 2009a, b) and an understanding of the characteristics of this enzyme is essential in order to allow its use in food systems. Thus, the purpose of this study was to evaluate toxicity and genotoxicity aspects of TGase from *Bacillus circulans* BL32 as short term *in vivo* in a subacute toxicity study using rats as biological models. Furthermore, we determined its cytotoxicity and genotoxicity using the comet assay and the mutagenic effects, employing the micronucleus test, in cultured mammalian cells.

MATERIALS AND METHODS

All the experiments were conducted from March 2008 and October 2009 at The Institute of Food Science and Technology, Federal University of Rio Grande do Sul State, Brazil.

Materials: All chemicals used were of analytical grade and purchased from Merck (Frankfurter, Darmstadt, Germany), unless otherwise mentioned. N-carboxybenzoyl-L-glutaminyglycine (N-CBZ-Gln-Gly), Cytochalasin-B (Cyt-b) and methyl methanesulphonate (MMS) were obtained from Sigma-Aldrich Co. Ltd. (St. Louis, Mo., USA). Q-Sepharose Fast Flow (FF) was from Pharmacia (Uppsala, Sweden). Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, L-glutamine and antibiotics were purchased from Gibco-BRL (Grand Island, N.Y., USA). Low-melting point agarose was obtained from Invitrogen (Carlsbad, C.A., USA).

Microorganism and inocula preparation: A strain of *Bacillus circulans*, coded BL32, which was isolated from the aquatic environment of the Amazon rain forest and described elsewhere (Souza *et al.*, 2006) was used in this study. This strain is kept as lyophilized reference stocks in the Culture Collection of the Microbiology Department of The Federal University of Rio Grande do Sul State, Brazil. Previously to culture, cells were recovered from frozen stocks in glycerol, plated on Mueller-Hinton agar and were kept at 4°C.

Inocula for cultivations were prepared as follows. Erlenmeyer flasks (250 mL) filled with 50 mL of M1 medium, which composition was optimized in a previous work (Souza *et al.*, 2006), containing (in g L⁻¹): glycerol 9.0, sucrose 2.0, peptone 7.0, tryptone 1.0, Na₂HPO₄ 1.0, MgSO₄·7H₂O 1.0 and FeSO₄·7H₂O 0.1, were inoculated with a single colony from a plate and incubated at 30°C in a rotatory shaker at 100 rpm and grown to Optical Density (OD) of 1.0 at 600 nm (approximately 20 h cultivation).

TGase production: Erlenmeyer flasks (1 L) containing 200 mL of M1 medium, pH adjusted to 8.5, were inoculated with 10 mL of inoculum. Cultivations were run at 30°C in a rotatory shaker at 100 rpm. After 8 days of cultivation, cell suspension was centrifuged at 4°C for 20 min at 17,000 g and the supernatant was used to obtain the purified enzyme.

TGase purification: TGase from *B. circulans* BL32 (BcTGase) was precipitated with 60-90% ammonium sulfate (w/v) and after dialysis, was applied to a Q-Sepharose Fast Flow (FF) ion-exchange column pre-equilibrated with 20 mM Tris-HCl buffer, pH 8.0, followed by a linear gradient (0-1 M) of NaCl (Soares *et al.*, 2003). Fractions containing the highest enzymatic activity were pooled, dialyzed against the 20 mM Tris-HCl buffer pH 8.0 and lyophilized. This cleaned fraction of enzyme was then used for further analysis.

Determination of enzyme activity: BcTGase activity was determined by hydroxamate formation with the specific substrate N-CBZ-Gln-Gly, described by Grossowicz *et al.* (1950). A calibration curve was prepared with L-glutamic acid γ -monohydroxamate. One TGase unit (U) was defined as the amount of enzyme that causes the formation of 1 μ mol L-glutamic acid γ -monohydroxamate min⁻¹.

Toxicity study

In vivo assay: BcTGase toxicity was assessed using male Wistar rats in a subacute 14 day oral feeding test using a liquid enzyme preparation. This preparation was comprised of lyophilized purified enzyme, administered to 150 U kg/b.wt./day as described bellow.

Animal tests: Adult, 90 days old (200-280 g), male Wistar rats, obtained from the Central Animal House, Pelotas Federal University, Rio Grande do Sul, Brazil, were used in this study. The rats were randomly assigned into 2 groups, each consisting of 10 individuals. The animals were individually housed in stainless steel metabolic cages designed for the separate collection of faeces and urine. Cages were located in a room with a 12 h light/dark cycle, at a temperature of 21±2°C, fitted with an appropriate ventilation system. Food and water were given *ad libitum* during the experiment. The control group received distilled water, by daily oral gavage (a volume of 10 mL kg⁻¹ b.wt. day⁻¹). The experimental group (BcTGase group) received the liquid enzyme preparation, by daily oral gavage, at dose of 150 U kg b.wt. day⁻¹. The animals were observed

daily for clinical signs. Feed consumption and body weights were measured daily throughout the study period. At the end of 14th day, final body weights of individual animals were recorded. The animals were anesthetized with a combination of 5 mg kg⁻¹ xylazine (2% xylazine chloride) and 90 mg kg⁻¹ ketamine (5% ketamine chloride), injected intramuscularly (Allen *et al.*, 1998) and after abdominal incision, blood samples were collected of the cava vein from each rat. Haematology and serum clinical chemistry were carried out for all animals from each group using standard analytical methods at the Laboratory of Veterinary Clinical Analysis (Faculdade de Veterinária, UFRGS, Porto Alegre, Brazil). Haematology parameters included: Red Blood Cell Count (RBC), White Blood Cell (WBC) count, haemoglobin concentration (Hb), haematocrit concentration (Hct), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin Concentration (MCHC) and differential leukocyte count. Clinical chemistry parameters included: total protein concentration, creatinine concentration (CRE) and alanine aminotransferase activity (ALT). Under anaesthesia, the diaphragm was incised to kill the animal. After sacrifices, liver, kidneys, spleen, thymus, adrenal glands, heart, lungs and brain were carefully dissected and immediately weighed. The organ weight was related to body weight. This parameter was expressed as relative systemic organ weight.

Histological processing: All the animals in the BcTGase and control groups were taken for histopathological analysis. The internal organs (liver, kidneys, spleen, thymus, adrenal glands, heart, lungs and brain) from each rat were dissected. Representative fragments of each organ were fixed in 10% formalin, embedded in paraffin in order to obtain serial sections of approximately 4 µm, fixed on poly-lysine-coated glass slides and de-paraffinised. The sections were stained with haematoxylin-eosin (H and E) and inspected on an Olympus AX70 Routine microscope adapted with a Nikon E 4500 camera (Zeiss) for any morphological changes in the tissues due to the consumption of BcTGase preparation. Photographs of representative organs from each group of rats were taken at Laboratory of Veterinary Pathology (Faculdade de Veterinária, UFRGS, Porto Alegre, Brazil). All stained slides and paraffin blocks were archived.

Evaluation of cytotoxicity, genotoxicity and mutagenic effects in mammalian cultured cells

Cell culture and treatment: V79 cells (Chinese hamster lung fibroblasts) were cultured under standard conditions in DMEM supplemented with 10% heat-inactivated-FBS, 0.2 mg mL⁻¹ L-glutamine, 100 IU mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. Cells were kept in tissue-culture flasks at 37°C in a humidified atmosphere containing 5% CO₂ in air and were harvested by treatment with 0.15% trypsin-0.08% EDTA in Phosphate-Buffered Saline (PBS) solution. Cells (3×10⁶ cells) were seeded in 5 mL complete medium in a 25 cm² flask and grown for 2 days up to 60-70% of confluence before treatment with the test substance. BcTGase was added to FBS-free medium to 150 U treatment⁻¹ while the control group was exposed to solvent.

Cytotoxicity evaluation by colony-forming ability (Clonal survival): Exponentially-growing V79 cells were treated according to the experimental protocol. Thereafter, they were trypsinized and 500 cells/60 mm dish were seeded in triplicates to determine colony-forming ability. After cell attachment (4 h), an aliquot of BcTGase solution was added to cells to a final concentration of 150 U treatment⁻¹. After 5 days of incubation, colonies were fixed with ethanol, stained with Giemsa, counted and their survival calculated as a percentage relative to control treatment.

Genotoxic evaluation using comet assay: Alkaline comet assay was performed as described by Singh *et al.* (1988) with minor modifications (Collins, 2004; Hartmann and Speit, 1997). V79 cells were incubated with BcTGase for 3 h in FBS-free medium. After treatment, cells were washed with ice-cold PBS, trypsinized and resuspended in complete medium. Then, 20 μL of cell suspension (3×10^6 cells mL^{-1}) were mixed with 0.75% low-melting point agarose and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose and allowed to set at 4°C for 5 min. Slides were incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for at least 1 h to remove cell proteins, leaving DNA as nucleoids. After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with fresh buffer (300 mM NaOH, 1 mM EDTA, pH 13.0) for 20 min at 4°C to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min at 25 V (300 mA). All above steps were conducted under yellow light or in the dark in order to prevent additional DNA damage. Slides were then neutralized (0.4 M Tris, pH 7.5), washed with bi-distilled water and stained using a silver staining protocol. After drying overnight at room temperature, gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed for each test substance. When selecting cells, the edges and cells around air bubbles were avoided (Collins, 2004). Cells were visually scored according to tail length into five classes: (1) class 0: undamaged, without a tail; (2) class 1: with a tail shorter than the diameter of the head (nucleus); (3) class 2: with a tail length 1-2x the diameter of the head; (4) class 3: with a tail longer than 2x the diameter of the head and (5) class 4: comets with no heads. A value (damage index) was assigned to each comet according to its class. International guidelines and recommendations for the comet assay consider that visual scoring of comets is a well-validated evaluation method, as it is highly correlated with computer-based image analysis. The damage index is based on the length of migration and on the amount of DNA in the tail and it is considered a sensitive DNA measurement. The other parameter, damage frequency (DF), although considered in the analysis, was only used as complementary DNA damage parameter. Damage index ranged from 0 (completely undamaged: 100 cells \times 0) to 400 (with maximum damage: 100 cells \times 4) (Collins, 2004; Hartmann and Speit, 1997; Tice *et al.*, 2000; Burlinson *et al.*, 2007). The damage frequency (%) was calculated based on the number of cells with tails versus those without tails. The vehicle was used as negative control.

Clastogenic evaluation using micronucleus test: The micronucleus assay in binucleated cells was performed according to Bonacker *et al.* (2004), with modifications by Fenech (2000). V79 cells were incubated with BcTGase for 3 h in FBS-free medium. Cultures were then twice washed with medium and Cyt-B was added to a final concentration of 2 $\mu\text{g mL}^{-1}$. Cultures were harvested 21 h after Cyt-B addition. Cells were separated from the bottle by trypsinization and the cell suspension was centrifuged at 1,000 g for 5 min. Cells were resuspended in 0.075 M KCl and maintained at 4°C for 3 min (mild hypotonic treatment). They were then centrifuged and a methanol/acetic acid (3:1) solution was slowly added. This fixation step was twice repeated and cells were finally resuspended in a small volume of methanol/acetic acid, dropped on clean slides and stained with 10% Giemsa (pH 6.8) for 3-4 min. Slides were mounted and codified prior to microscopic analysis. The vehicle was used as negative control. Micronuclei were counted in 2,000 binucleated cells (BNC) with well-preserved cytoplasm. The identification of micronuclei was carried out according to Fenech (2000).

Statistical analysis: All experiments were independently performed in triplicates and the results are expressed as Mean±SE. Statistical analysis of the data was carried out by Student's t-test and by one-way analysis of variance (ANOVA), followed by Tukey's test. Differences were considered to be statistically significant when $p < 0.05$.

RESULTS AND DISCUSSION

According to Bernard *et al.* (1998), enzymes are generally considered to be of low toxic potential, but microbial enzymes obtained in bioprocesses are preparations containing not only the desired protein but also other metabolites (Pariza and Johnson, 2001). Therefore, it would be very important to evaluate the toxicological aspects of these preparations before their use by the food industry (Pariza and Foster, 1983; Kessler *et al.*, 1992). In the present study we evaluated safety of microbial TGase produced by *B. circulans* BL32. This is one the few reports on TGase safety for food uses.

In vivo assay: No losses were observed among rats of the control or test groups during the 14 day study. All animals were normal in appearance throughout the test and no clinical signs related to the treatment with BcTGase (test group) were observed. Harbak and Thygesen (2002) and Ciofalo *et al.* (2003) reported, respectively, the safety evaluation of a xylanase expressed in *Bacillus subtilis* and of a phytase expressed in *Schizosaccharomyces pombe*. The authors of both works also observed no toxicity of these enzymes in the acute oral toxicity study, since none the rats showed any clinical abnormal signs and all survived treatment and the 14-day observation period.

The total weight gain (Mean±SE) was 50.27 ± 3.06 and 48.98 ± 3.71 g for the control and test groups, respectively ($p = 0.125$, one-way ANOVA). The relative weight gain of the rats treated with distilled water and BcTGase are illustrated in Fig. 1. According to Jahn and Günzel (1997), in all toxicity studies, clinical observations, body weights and body weight changes can provide useful indicators of the general health status of the animal. Therefore, interpretation of toxic effects of enzymatic preparations should always consider the presence or absence of body weight alterations. Depression in body weight or reduction in weight gain may reflect a variety of responses, including treatment-induced anorexia or systemic toxicity. Body weight gain should also be considered as a

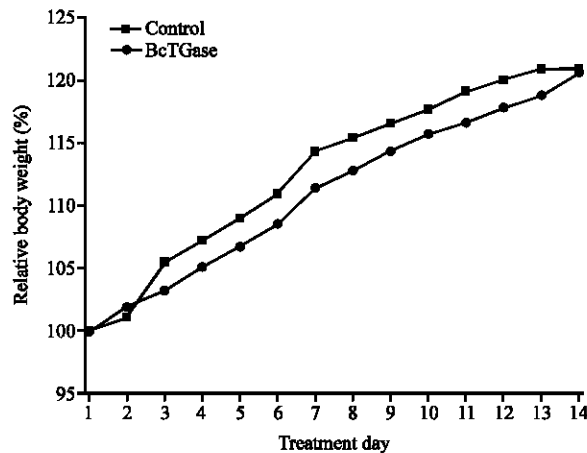


Fig. 1: Relative weight gain of the rats (with respect to day 1, representing 100 %), treated with water (control), or BcTGase

Table 1: Relative organs weight (%) of rats treated with water or BcTGase for 14 days

| Organs | Control group | BcTGase group |
|---------------------|---------------|---------------|
| Liver | 3.01±0.035 | 3.10±0.043 |
| Right kidney | 0.42±0.010 | 0.42±0.011 |
| Left kidney | 0.40±0.008 | 0.41±0.010 |
| Spleen | 0.27±0.008 | 0.29±0.004 |
| Thymus | 0.13±0.009 | 0.15±0.011 |
| Right adrenal gland | 0.01±0.001 | 0.01±0.001 |
| Left adrenal gland | 0.01±0.001 | 0.01±0.001 |
| Heart | 0.34±0.010 | 0.35±0.009 |
| Lungs | 0.42±0.008 | 0.55±0.020* |
| Brain | 0.68±0.019 | 0.71±0.015 |

Data are reported as mean±SE (n = 10) based on the percentage of the organ weight in relation to total body weight. *Significantly different at $p < 0.05$ by Student's t-test

toxic response. The results obtained in this study show that there were no treatment-related changes for the mean body weight or body weight gain of the test group receiving BcTGase preparation at dose of 150 U kg b.wt. day⁻¹. Coenen *et al.* (1995) evaluated the toxicity of a β -glucanase enzyme obtained from *Trichoderma reesei* in a subacute 14 day oral toxicity study. The body weight gain of the animals during the study was considered similar to that expected for untreated rats of the same age, suggesting no evidence of toxicity for this enzyme at feeding concentrations of 20,000 ppm.

The weight of individual organs was measured and the ratio of organ weight per final body weight was calculated. Table 1 shows the relative weight of the organs of animals treated with water (control group) and BcTGase preparation for 14 days. There was significant difference ($p < 0.05$, Student's t-test) only in relation to the weight of the lungs between the groups, but these were normal and healthy. Coenen and Aughton (1998) obtained similar results for the amino peptidase enzyme preparation derived from *Aspergillus niger*, in a subacute 14 day oral toxicity study in rats. The lung relative weight for males receiving 2,000 mg kg⁻¹ b.wt. day⁻¹ was high. According to the authors, this result was due to the fact that two animals showed an incomplete collapse of the lungs at macroscopic examination.

Table 2 shows the results of the haematological and blood chemistry evaluation of animal groups. There was no significant difference ($p > 0.05$) in the serum levels of total protein, CRE and ALT between the two groups. Haematological findings did not show any effects related to treatment with BcTGase. No significant differences were seen between rats of the control and test groups in haemoglobin, haematocrit, erythrocyte count, mean corpuscular volume and mean corpuscular haemoglobin concentration. Similarly, total and differential leukocyte counts in the rats treated with BcTGase were not significantly different from those in the control group. Moreover, all values remained within the normal range of historical data for rats of this age and strain.

Histopathological examination of liver, kidneys, spleen, thymus, adrenal glands, heart, lungs and brain revealed no differences between rats receiving BcTGase preparation at dose of 150 U kg⁻¹ b.wt. day⁻¹ and those of the control. The bigger lungs of rats treated with BcTGase presented normal histological appearances (Fig. 2b) when compared with the control group (Fig. 2a). The microscopic analysis of the lungs from animals receiving BcTGase preparation showed no changes on the lung cells in comparison with the control. Cell morphology and tissue characteristics were typical of lung cells. In addition, there were no microscopic changes observed

Table 2: Haematological and blood chemistry evaluation of rats treated with water or BcTGase for 14 days

| Parameters | Control group | BcTGase group |
|---|---------------|---------------|
| Haematocrit (%) | 47.89±0.77 | 47.33±0.50 |
| Haemoglobin (g dL ⁻¹) | 15.14±0.36 | 15.34±0.22 |
| RBC (10 ⁶ µL ⁻¹) | 8.33±0.22 | 8.22±0.20 |
| WBC (10 ⁶ µL ⁻¹) | 6.31±0.59 | 5.59±0.39 |
| MCV (fL) | 57.67±1.29 | 57.74±1.07 |
| MCHC (%) | 31.60±0.29 | 32.42±0.37 |
| Neutrophils (10 ⁶ mL ⁻¹) | 1.07±0.13 | 1.03±0.10 |
| Eosinophils (10 ⁶ mL ⁻¹) | 0.00±0.0 | 0.00±0.0 |
| Basophils (10 ⁶ mL ⁻¹) | 0.00±0.0 | 0.00±0.0 |
| Lymphocytes (10 ⁶ mL ⁻¹) | 5.08±0.53 | 4.39±0.33 |
| Monocytes (10 ⁶ mL ⁻¹) | 0.16±0.04 | 0.05±0.02 |
| Alanine aminotransferase (U L ⁻¹) | 65.16±10.52 | 59.27±11.07 |
| Creatinine (mg dL ⁻¹) | 0.52±0.02 | 0.47±0.01 |
| Total protein (mg mL ⁻¹) | 61.33±0.82 | 62.22±0.85 |

Data are reported as Mean±SE (n = 10); RBC: Red blood cells; WBC: White blood cells; MCV: Mean corpuscular volume; MCHC: Mean corpuscular haemoglobin concentration. There was no significant difference between control and test groups (p>0.05, Student's t-test)

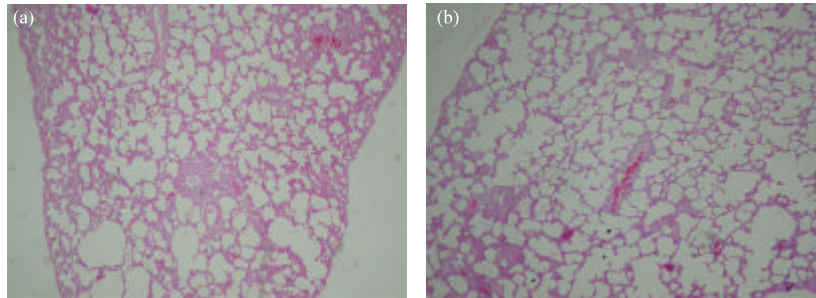


Fig. 2: Histological sections of the lungs of the animals of (a) control group and (b) BcTGase. The sections were stained with haematoxylin-eosin (H and E). Magnification is 4X

in any of the evaluated organs that could indicate any toxicity as a result of the exposure to the enzyme, by daily oral gavage, at dose of 150 U kg b.wt. day⁻¹. Present results strongly suggest that BcTGase did not cause any toxic effects in the examined tissues when the animals were exposed to the enzyme during a short term period. Results obtained by Bernard *et al.* (1998), showed that toxicological studies performed with TGase from *Streptovercillium* sp. did not demonstrate any significant toxicological effects in rats within 14 days after a single oral dose of 1,000 or 2,000 U TGase kg⁻¹ b.wt. In present study, however, animals received daily 150 U kg⁻¹ b.wt. doses over the same period of 14 days.

Genetic toxicity of TGase in mammalian cultured cells: A balance between desirable versus toxicological effects of a substance is an important parameter when evaluating its usefulness as a pharmacological drug or biotechnological product. Cell culture can be used to evaluate basal cytotoxicity and target organ toxicity (Ekwall and Ekwall, 1988). In this study, V79 fibroblasts were used because this cell line is well characterized and commonly used in mutagenicity and cytotoxicity studies (Bradley *et al.*, 1981). The results shown in Table 3 indicate that BcTGase preparation at 150 U treatment⁻¹ had no toxic effects on V79 cells.

Table 3: Genotoxic evaluation of BcTGase during 3 h in FBS-free medium in V79 cells

| Treatment | Cytotoxicity | Comet assay | | Micronuclei | |
|-----------------|---------------------|--------------|------------------|--|------------------------------------|
| | Clonal survival (%) | Damage index | Damage frequency | Cell proliferation (% binucleated cells) | Micronuclei/2000 binucleated cells |
| NC | 100.0±0.0 | 21.3±8.7 | 20.3±6.9 | 93.0±2.1 | 11.0±1.1 |
| PC ^a | 51.4±2.2* | 143.0±19.9* | 88.3±9.7* | 62.1±0.9* | 52.4±8.0* |
| BcTGase | 94.1±3.5 | 17.2±2.0 | 21.3±1.4 | 95.9±3.0 | 9.2±0.5 |

^aThe symbol *represents p<0.05, as tested by one way ANOVA (Tukey's test): BcTGase treatment was compared to negative control; Mutagen treatment was compared against negative control. NC: negative control (distilled water). PC: Positive control (4×10⁻⁵ mol L⁻¹ MMS)

Considering the need of a complete toxicological evaluation, we investigated whether this enzyme could induce DNA damage under these experimental conditions. The *in vitro* alkaline (pH>13) comet test and micronucleus test are the most frequently used assays for routine screening of potential genotoxic agents (Hartmann *et al.*, 2001) and can be performed with a variety of cell types, including V79 cell lines, which have stable karyotype, short generation time and are easy to maintain. While the alkaline version of the Comet assay (Singh *et al.*, 1988) detects primary (repairable) DNA single and double strand breaks and alkali-labile sites, the micronucleus test detects DNA lesions that lead to chromosome mutations. As can be seen in Table 3, BcTGase did not generate significant DNA damage at concentration of 150 U treatment⁻¹. Micronuclei are the results of acentric fragments or lagging chromosomes that fail to incorporate into either of the daughter nuclei during telophase of the mitotic cells (Albertini *et al.*, 2000). The treatment with BcTGase did not alter the percentage of binucleated cells, reinforcing the absence of cytotoxic effect of this enzyme on cell proliferation. Again, the concentration of 150 U treatment⁻¹ caused no significant increase in the incidence of micronuclei, suggesting that the treatment with BcTGase is safe.

Since, the commercial use of microbial TGases possibly increases the human exposure to these preparations, this study is relevant to determine health hazards or food safety. However, few studies have been carried out to test aspects of genetic toxicity of microbial TGase preparations. Bernard *et al.* (1998) showed that *Streptovorticillium* sp. TGase was not mutagenic for bacteria, for V79 cells using the approach of chromosomal aberrations and for male mouse by micronucleus assay. In our study, the BcTGase did not show cytotoxic, genotoxic, or mutagenic effects in V79 cells, reinforcing the fact that this enzyme may be safe for food preparations.

CONCLUSIONS

Microbial TGases can be employed in many attractive food and non-food applications. Although, many reports on production and purification of microbial TGases have been published, little information about toxicological aspects is available, despite its well known role on gluten intolerance. In this study, the microbial TGase from *B. circulans* BL32 was submitted to toxicological evaluation. No evidence of toxicity was found for the enzymatic preparation in the subacute 14 day oral toxicity test in rats and enzyme was neither cytotoxic, genotoxic or mutagenic to V79 cultured cells, showing its safety. It can be concluded that no safety concerns were identified in this study for the BcTGase preparation produced under controlled cultivation conditions. Present results suggest that the microbial TGase from *B. circulans* BL32 has potential to be used in the food industry.

ACKNOWLEDGMENTS

This study, was partially financed by grants from CNPq and CAPES. All animal experimentation was approved by the Bioethics Committee of UFRGS. Our group supports the ethically and morally justified use of animals in experimentation, but we also support the search for proper models to completely replace animal experimentation in the near future.

REFERENCES

- Albertini, R.J., D. Aderson, G.R. Dauglas, L. Hagmar and K. Hemminki *et al.*, 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in human. *Mutat. Res.*, 463: 111-172.
- Allen, D.G., J.K. Pringle, D.A. Smith and K. Pasloske, 1998. Handbook of Veterinary Drugs. 2nd Edn., Lippincott Williams and Wilkins, Philadelphia. ISBN-13: 978-0397584352, pp:886.
- Arrizubieta, M.J., 2007. Transglutaminases. In: Industrial Enzymes: Structure, Function and Applications, Polaina, J. and A.P. MacCabe (Eds.). Springer, New York, pp: 567-581.
- Askin, O.O. and B. Kilic, 2009. Effect of microbial transglutaminase, sodium caseinate and non-fat dry milk on quality of salt-free, low fat turkey döner kebab. *Food Sci. Technol.*, 42: 1590-1596.
- Bernard, B.K., S. Tsubuku and S. Shioya, 1998. Acute toxicity and genotoxicity studies of a microbial transglutaminase. *Int. J. Toxicol.*, 17: 703-721.
- Bonacker, D., T. Stoiber, M. Wang, K.J. Bohm and I. Prots *et al.*, 2004. Genotoxicity of inorganic mercury salts based on disturbed microtubule function. *Archiv. Toxicol.*, 78: 575-583.
- Bradley, M., B. Bhuyan, M.C. Francis, R. Langenbach, A. Peterson and E. Huberman, 1981. Mutagenesis by chemical agents in V79 chinese hamster cells: A review and analysis of literature. *Mutat. Res.*, 87: 81-142.
- Burlinson, B., R.R. Tice, G. Speit, E. Agurell and S.Y. Brendler-Schwaab *et al.*, 2007. Fourth international workgroup on genotoxicity testing: Results of the *in vivo* comet assay workgroup. *Mutat. Res.*, 627: 31-35.
- Ciofalo, V., N. Barton, K. Kretz, J. Baird, M. Cook and D. Shanahan, 2003. Safety evaluation of a phytase, expressed in *Schizosaccharomyces pombe*, intended for use in animal feed. *Regul. Toxicol. Pharmacol.*, 37: 286-292.
- Coenen, T.M.M., A.C.M. Schoenmakers and H. Verhagen, 1995. Safety evaluation of b-glucanase derived from *Trichoderma reesei*: Summary of toxicological data. *Food Chem. Toxicol.*, 33: 859-866.
- Coenen, T.M.M. and P. Aughton, 1998. Safety evaluation of amino peptidase enzyme preparation derived from *Aspergillus niger*. *Food Chem. Toxicol.*, 36: 781-789.
- Collighan, R., J. Cortez and M. Griffin, 2002. The biotechnological applications of transglutaminases. *Minerva Biotecnol.*, 14: 143-148.
- Collins, A.R., 2004. The comet assay for DNA damage and repair: Principles, applications and limitations. *Mol. Biotechnol.*, 26: 249-261.
- Cortez, J., P.L.R. Bonner and M. Griffin, 2004. Application of transglutaminases in the modification of wool textiles. *Enzyme Microb. Technol.*, 34: 64-72.
- Ekwall, B. and H. Ekwall, 1988. Comments on the use of diverse cell systems in toxicity testing. *Alternat. Laborat. Anim.*, 15: 193-200.
- Fenech, M., 2000. The *in vitro* micronucleus technique. *Mutat. Res.*, 455: 81-95.

- Gan, C., W. Ong, L. Wong and A.M. Easa, 2009. Effects of ribose, microbial transglutaminase and soy protein isolate on physical properties and *in-vitro* starch digestibility of yellow noodles. *Food Sci. Technol.*, 42: 174-179.
- Gauche, C., P.L.M. Barreto and M.T. Bordignon-Luiz, 2010. Effect of thermal treatment on whey protein polymerization by transglutaminase: Implications for functionality in processed dairy foods. *Food Sci. Technol.*, 43: 214-219.
- Gauche, C., T. Tomazi, P.L.M. Barreto, P.J. Ogliari and M.T. Bordignon-Luiz, 2009. Physical properties of yoghurt manufactured with milk whey and transglutaminase. *Food Sci. Technol.*, 42: 239-243.
- Grossowicz, N., E. Wainfan, E. Borek and H. Waelsch, 1950. The enzymatic formation of hydroxamic acids from glutamine and asparagine. *J. Biol. Chem.*, 187: 111-125.
- Harbak, L. and H.V. Thygesen, 2002. Safety evaluation of a xylanase expressed in *Bacillus subtilis*. *Food Chem. Toxicol.*, 40: 1-8.
- Hartmann, A. and G. Speit, 1997. The contribution of cytotoxicity to DNA-effects in the single cell gel test (comet assay). *Toxicol. Lett.*, 90: 183-188.
- Hartmann, A., A. Elhajouji, E. Kiskinis, F. Poetter and H. Martins *et al.*, 2001. Use of the alkaline comet assay for industrial genotoxicity screening: Comparative investigation with the micronucleus test. *Food Chem. Toxicol.*, 39: 843-858.
- Hiller, B. and P.C. Lorenzen, 2009. Effect of phosphatase/transglutaminase treatment on molar mass distribution and techno-functional properties of sodium caseinate. *Food Sci. Technol.*, 42: 87-92.
- Jahn, A.I. and P.K.H. Günzel, 1997. The value of spermatology in male reproductive toxicology: Do spermatologic examinations in fertility studies provide new and additional information relevant for safety assessment? *Reprod. Toxicol.*, 11: 171-178.
- Jülicher, P., L. Haalck, M. Meusel, K. Cammann and F. Spener, 1998. In situ antigen immobilization for stable organic-phase immunoelectrodes. *Anal. Chem.*, 70: 3362-3367.
- Kessler, D.A., M.R. Taylor, J.H. Maryanski, E.L. Flamm and L.S. Kahl, 1992. The safety of foods developed by biotechnology. *Science*, 256: 1747-1749.
- Nonaka, M., H. Tanaka, A. Okiyama, M. Motoki, H. Ando, K. Umeda and A. Matsuura, 1989. Polymerization of several proteins by Ca²⁺-independent transglutaminase derived from microorganism. *Agric. Biol. Chem.*, 53: 2619-2623.
- Pariza, M.W. and E.M. Foster, 1983. Determining the safety of enzymes in food processing. *J. Food Prot.*, 46: 453-468.
- Pariza, M.W. and E.A. Johnson, 2001. Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century. *Regul. Toxicol. Pharmacol.*, 33: 173-186.
- Singh, N.P., M.T. McCoy, R.R. Tice and E.L. Schneider, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, 175: 184-191.
- Soares, L.H.B., F. Assmann and M.A.Z. Ayub, 2003. Purification and properties of a transglutaminase produced by a *Bacillus circulans* strain isolated from the Amazon environment. *Biotechnol. Appl. Biochem.*, 37: 295-299.
- Souza, C.F.V., S.H. Flores and M.A.Z. Ayub, 2006. Optimization of medium composition for the production of transglutaminase by *Bacillus circulans* BL32 using statistical experimental methods. *Proc. Biochem.*, 41: 1186-1192.
- Souza, C.F.V., D.J.L. Faccin, O. Mertins, J.X. Heck, N.P. Silveira, A.R. Secchi and M.A.Z. Ayub, 2009. Kinetics of thermal inactivation of transglutaminase from a newly isolated *Bacillus circulans* BL32. *J. Chem. Technol. Biotechnol.*, 84: 1567-1575.

- Souza, C.F.V., R.C. Rodrigues and M.A.Z. Ayub, 2009. Effects of oxygen volumetric mass transfer coefficient on transglutaminase production by *Bacillus circulans* BL32. *Biotechnol. Bioprocess Eng.*, 14: 571-576.
- Tice, R.R., E. Agurell, D. Anderson, B. Burlinson and A. Hartmann *et al.*, 2000. Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutag.*, 35: 206-221.
- Vader, L.W., A. Ru, Y. Wal, Y.M.C. Kooy and W. Benckhuijsen *et al.*, 2002. Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J. Exp. Med.*, 195: 643-649.
- Zhu, Y., A. Rinzema, J. Tramper and J. Bol, 1995. Microbial transglutaminase: A review on its production and application in food processing. *Applied Microbiol. Biotechnol.*, 44: 277-282.