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Safety evaluation of amylomaltase from *Thermus aquaticus*

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ABSTRACT

A recombinant amylomaltase, MQ-01, obtained by cultivation of *Bacillus subtilis* expressing the amylomaltase gene from *Thermus aquaticus* is to be used in the production of enzymatically-synthesized glycogen; which is intended for use as a food ingredient. In order to establish the safety of MQ-01, the enzyme was subjected to standard toxicological testing. In a battery of standard *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537) and in *Escherichia coli* WP2 *uvr*A, both with and without metabolic activation, MQ-01 failed to exhibit mutagenic activity. Similarly, MQ-01 did not display clastogenic properties in Chinese hamster lung fibroblast cells (CHL/IU), in an *in vitro* chromosomal aberration assay. In a 13-week subchronic toxicity study in rats, oral administration of MQ-01 at doses of up to 15 mL/kg body weight/day (corresponding to approximately 1230 mg/kg body weight/day) did not produce compound-related clinical signs or toxicity, changes in body weight gain, food consumption, hematology, clinical chemistry, urinalysis, organ weights, or in any gross and microscopic findings. The results of this study support the safety of MQ-01 in food production.

1. Introduction

Amylomaltase is a member of the 4-α-glucanotransferase family, which catalyzes the transfer of a glucan moiety from one α-1,4-glucan molecule to another or to glucose, forming two linear products of different sizes. This reaction, which is an inter-molecular transglycosylation, is often called the disproportionation reaction (Takaha and Smith, 1999). Amylomaltase also can catalyze an intra-molecular glucan transfer reaction or cyclization within a single linear glucan molecule, in which the enzyme cleaves the α-1,4-glucosidic bond, and concomitantly links the reducing end to the non-reducing end to produce cyclic α-1,4-glucan products. This reaction is reversible, and the reverse reaction is often referred to as the coupling reaction (Fujii et al., 2007).

Amylomaltase was first identified in *Escherichia coli* (*E. coli*) as a maltose inducible enzyme (Monod and Torriani, 1950), and is found to be widely distributed in various bacterial species and having different physiological functions (Goda et al., 1997; Hsia et al., 1997; Lacks et al., 1982; Pugsley and Dubreuil, 1988; Terada et al., 1999). A similar enzyme, termed disproportionating enzyme (D-enzyme, EC 2.4.1.25), is present in plants, and is presumed to be involved in starch metabolism (Colleoni et al., 1999). Potato D-enzyme shares 40% sequence identity with amylomaltase from the thermophilic bacterium *Thermus aquaticus* (*T. aquaticus*) (Takaha and Smith, 1999).

A number of thermostable amylomaltase enzymes have been reported to have promising applications in the synthesis of products with potential food uses. Combined use of *Thermotoga maritima* amylomaltase and a maltogenic amylase in the production of isomalto-oligosaccharides from starch resulted in a reduction of reaction time, and a higher yield of isomalto-oligosaccharides. Isomalto-oligosaccharides have been suggested as a potential substitute sugar for diabetes due to low viscosity, resistance to crystallization, and their reduced sweetness (Lee et al., 2002). Moreover, the amylomaltase from the thermophilic bacterium *T. aquaticus* has received interest in the production of cycloamylose (Terada et al., 1999), since it exhibits high thermal stability, and it preferentially produces cycloamyloses with higher degrees of polymerization (DP), ranging from 22 to a few hundred, as compared to the conventional cyclodextrins produced by other 4-α-glucanotransferases (Terada et al., 1999). Cycloamyloses resemble cyclodextrins which have applications in modifying the solubility and stability of some flavoring agents (Kaper et al., 2004). Another potential application of amylomaltase in the food industry is in the production of thermoreversible starch gels from gelatinized potato starch upon treatment with *Thermus thermophilus* amylomaltase (Van der Maarel et al., 2000). The product has gelatin-like properties, and may be used as potential plant-derived alternatives to gelatin (Van der Maarel et al., 2000).

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Recently, *T. aquaticus* amylomaltase obtained from recombinant *E. coli* was used in the enzymatic production of glycogen, for which starch was used as a starting material (Kajiura et al., 2008). In this process the branched linkages of starch are hydrolyzed using isoamylase (EC 3.2.1.68) to produce a mixture of short chain amyloses, which are assembled into glycogen by the action of branching enzyme (BE, EC 2.4.1.18) in the presence of amylomaltase. A large number of small oligosaccharides are produced as a by-product of the synthesis reaction catalyzed by BE. As these oligosaccharides are poor substrates for BE, elongation of the malto-oligosaccharide by amylomaltase increases the substrates available for BE, and consequently increases the yield of glycogen production (Kajiura et al., 2008).

Amylomaltase enzyme evaluated in the present study is to be used in the commercial-scale production of enzymatically-synthesized glycogen. The enzyme is derived from a recombinant strain of *Bacillus subtilis* (*B. subtilis*) (NCIMB12378, a variant of strain 168) that was genetically modified to express the amylomaltase gene *malQ* from *T. aquaticus*, and herein referred to as MQ-01. The safety evaluation of *T. aquaticus* MQ-01 is based on the results of a 13-week subchronic toxicity study in rats, a bacterial reverse mutation assay, and an *in vitro* chromosomal aberration test. The findings of these studies support the safety of MQ-01 for use in the production of enzymatically-synthesized glycogen.

2. Materials and methods

2.1. Enzyme preparation

For the production of MQ-01, a DNA fragment containing the *malQ* gene was amplified by PCR from the recombinant plasmid pFQG8 (Terada et al., 1999). The sequences of the primers were 5′-AATCCAACCTTCGCATGCTGATTAAAAGGAGGTAATAACATATGGAGCTTCCCCGCGCTTTCG-3′ and 5′-GACCCGGGAATTCGGGCTTGGTCTCATTAGAGCCGTTCCGTGG-3′. Underlined sequences in the former and latter primers are start codon and anti-codon of stop codon, respectively. In the DNA fragment, the 5′ and 3′ untranslated region was modified by adding a synthetic Shine-Dalgarno (SD) sequence (ribosomal binding site), and changing the original stop codon (TAG) to TAA, without modifying the coding region. An SphI site was inserted upstream of the transcriptional start site and an EcoRI site was inserted downstream of the stop codon. The amplified DNA fragment was digested with SphI and EcoRI, and then inserted between the SphI and EcoRI restriction sites of vector pUB110 to construct the pUMQ1 plasmid. MQ-01 was prepared by the cultivation of *B. subtilis* strain NCIMB12378, a derivative of Marburg strain 168, carrying the pUMQ1 plasmid, using standard techniques. Following cultivation of *B. subtilis*, the cells were collected and lysed using lysozyme. The enzyme was then heated at 70 °C for 1 h to terminate lysozyme enzymatic activity, followed by filtration to remove insoluble materials. The enzyme was concentrated by ultrafiltration, followed by microfiltration using a 0.45 μm filter unit. The analyses of the test material yielded the following results: amylomaltase activity, 67.4 U/mL; total organic solid value, 82 mg/mL; protein concentration, 3.35 mg/mL; lead, <5 ppm; arsenic (as As_2O_3), <4 ppm; aerobic plate count, 10,000/g; coliforms, negative; *E. coli*, negative. The same test material was used in all of the studies. All processing materials used were of food-grade quality.

For the subchronic toxicity study, MQ-01 was provided as a liquid without added preservatives or diluting agents, and was used as such without further dilution or modification. For the *in vitro* reverse mutation assay and chromosomal aberration test, MQ-01 was provided as a liquid without added preservatives or diluting agents, and was diluted in distilled water prior to testing. The enzyme preparation was stored between −36 and −23 °C prior to use, and its stability was confirmed throughout the course of the study.

2.2. Subchronic toxicity study in rats

This study was conducted in compliance with the Organization for Economic Cooperation and Development (OECD) Principles of Good Laboratory Practices (GLP) (OECD, 1997), and in accordance with the OECD Guidelines for the Testing of Chemicals No. 408 (OECD, 1998).

2.2.1. Animals and treatment

A total of 57 male and 57 female 5-week-old Sprague–Dawley [Crl:CD(SD), SPF] rats was obtained from Charles River Laboratories Japan, Inc. (Atsugi Breeding Center, Japan). The animals were provided a pelleted diet (CRF-1, Oriental Yeast Co., Ltd.) and tap water *ad libitum*, and were housed under controlled conditions: 21–24 °C, relative humidity of 45–66%, ventilation of 10–15 fresh air exchanges/hour, and 12-h light/dark cycles. The animals were quarantined and allowed to acclimate to laboratory conditions for a period of 9 days, during which signs of general health, including body weight and ophthalmology, were evaluated. Based on the results of these observations and examinations during the acclimation period, 40 rats of each sex were selected and randomized to control and treatment groups by an adequate stratification method based on animal body weight gain and body weight on the second last day of acclimatization (i.e., 2 days prior to start of study initiation). Animals were housed individually during the experimental period.

On day 1 of the experimental period, the animals were 6 weeks of age with body weights in the range of 197–222 g for males and 154–190 g for females. MQ-01 was administered by oral gavage, using flexible stomach tubes, once daily for a period of 13 weeks. Control animals were administered water for injection (Japanese Pharmacopeia, Otsuka Pharmaceutical Factory, Inc.) by the same method. The administration volume was calculated based on the animal's most recently measured body weight. The selection of the dose levels for this study was based on the maximum dose volume for administration of 15 mL/kg, and a total of 3 dose levels selected using a common ratio of approximately 3. Considering this, the rats were administered MQ-01 at dose levels of 1.7, 5, or 15 mL/kg body weight (bw)/day, with the control group receiving 15 mL of water. Based on a total organic solid value of 82 mg/mL for MQ-01, doses of 1.7, 5, or 15 mL/kg bw/day corresponded to 139.4, 410, and 1230 mg/kg bw/day, respectively (Table 1).

2.2.2. Observations

The animals were observed for clinical signs three times a day during the administration period. Body weight and food consumption were measured three times during the first week of test article administration, and twice weekly thereafter. Detailed clinical observations (home-cage observations, in-the-hand observations, open-field observation) were recorded once a week and manipulative test, measurement of grip strength, and motor activity assessment were conducted once in the 13th week of the administration period. Ophthalmological examination on each animal from the control and the high-dose (15 mL/kg bw/day) groups was conducted prior to the experimental period and in the 13th week of the study (day 90 of administration).

2.2.3. Urinalysis and water intake

During the final week of the study (week 13), all animals were placed in individual metabolism cages for a 20-h period with free access to water, and urine samples were collected every 4 h. The daily water intake also was recorded for all animals subjected to

urinalysis. Urinalysis was performed to examine the following parameters on 4-h urine samples: pH, protein, ketones, glucose, occult blood, bilirubin, urobilinogen, color, sediment, and 4-h urinary volume. For 20-h urine samples, the following parameters were evaluated: urinary volume, osmotic pressure, sodium (Na^+), potassium (K^+), and chloride (Cl^-) concentrations.

2.2.4. Hematology and clinical chemistry

Hematological analysis was performed on blood samples collected on the day of necropsy. All animals were fasted for 16–20 h and blood samples were collected from the abdominal aorta under ether anesthesia. The plasma was separated from blood and treated with sodium citrate for prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen analysis using a coagulometer (Coagulatometer ACL 100, Instrumentation Laboratory). Determination of the remaining hematological parameters [red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte percentage, platelet count, white blood cell count (WBC), and differential WBC] was conducted on ethylenediaminetetraacetic acid (EDTA)-2K-treated blood.

For clinical chemistry analysis, serum was separated from blood collected similarly and simultaneously as for hematological examination. Serum clinical chemistry parameters examined included alkaline phosphatase (ALP), total cholesterol, triglyceride, phospholipids, total bilirubin, glucose, blood urea nitrogen (BUN), creatinine, Na^+, K^+, Cl^-, and calcium (Ca^{2+}) concentrations, inorganic phosphorus (P), total protein (TP), albumin, albumin/globulin ratio, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and γ-glutamyltranspeptidase (γ-GTP).

2.2.5. Clinical pathology and histopathology

All surviving animals were terminated by exsanguination *via* the abdominal aorta after blood collection under ether anesthesia. Animals were examined for any external abnormalities at termination. Organs and tissues of all animals were examined macroscopically and organ weights were recorded for the brain (cerebrum and cerebellum), thyroid, adrenal glands, thymus, spleen, heart, lung (with bronchus), salivary gland, liver, kidney, testes, ovaries, uterus, prostate, and seminal vesicles. The weight of each organ relative to 100 g of the final body weight was calculated. All organs and tissues were fixed in 10% phosphate-buffered formalin, with the exception of the testes and epididymis, which were fixed in Bouin's solution, and then preserved in 10% phosphate-buffered formalin. The organs and tissues of all animals in the control and high-dose (15 mL/kg bw/day) groups were examined microscopically.

2.2.6. Statistical analysis

The following parameters were initially analyzed for homogeneity of variance by the Bartlett's test (significance value of 0.01, two-tailed): quantitative items of open-field observation and manipulative test, measurement of grip strength and motor activity, body weight, food consumption and water intake, quantitative items of urinalysis, hematological and clinical chemistry data, and organ weights. If the variances were homogenous, the Dunnett test was performed to compare the difference of the mean values between the control group and each treatment group (significance value of 0.05 and 0.01, two-tailed). If the variances were heterogenous, the mean values of the treatment groups were compared with that of the control group using a Dunnett-type mean rank test (significance levels of 0.05 and 0.01, two-tailed).

2.3. In vitro bacterial reverse mutation assay (Ames test)

The *in vitro* bacterial reverse mutation assay was conducted in compliance with the OECD Principles of GLP (OECD, 1998), and in accordance with the OECD guidelines for the Testing of Chemicals Test No. 471 (OECD, 1997). The reverse mutation assay was conducted using four standard auxotrophic histidine-dependent *Salmonella typhimurium* (*S. typhimurium*) tested strains (TA98, TA100, TA1535, and TA1537), as well as the auxotrophic tryptophan-dependent *E. coli* Wp2 *uvrA* strain. The tester strains of *S. typhimurium* and *E. coli* were obtained from the Division of Genetics and Mutagenesis (National Institute of Health Sciences). The bacterial strains were stored in dimethyl sulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd.) in a deep freezer set at −70 °C.

Prior to testing, a 50 mg/mL stock solution of MQ-01 was prepared by dissolving the test substance in distilled water for injection. Metabolic activation was achieved *via* incubation with an S9 microsomal fraction (S9-mix) obtained from Oriental Yeast Co., Ltd. and Kikkoman Corporation. A sterility test was conducted in which 0.1 mL of the stock solution with and without S9-mix was mixed and added to the minimal glucose agar plates. The plates were then incubated at 37 °C for 49 h. No bacterial growth was observed indicating that both MQ-01 and the S9-mix were sterile. Serial 4-fold dilutions of the stock concentration were subsequently prepared with distilled water for injection (Japanese Pharmacopeia, Otsuka Pharmaceutical Factory, Inc.). Distilled water for injection served as the negative control in all assays, while the following compounds were used as positive controls for assays conducted in the absence of metabolic activation: 0.01 μg/plate 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) (Wako Pure Chemical Industries, Ltd.) for tester strains TA100 and Wp2 *uvrA* and 0.1 μg/plate AF-2 for TA98, 0.5 μg/plate sodium azide (SAZ) (Wako) for TA1535, and 1 μg/plate 2-methoxy-6-chloro-9-[3-(2-chloroethyl)-aminopropylamino]acridine·2HCl (ICR-191) (Polysciences, Inc.) for TA1537. For assays conducted in the presence of metabolic activation, 5 μg/plate benzo[α]pyrene (B[α]P) (Wako) was used as a positive control for tester strains TA98, TA100, and TA1537, while 2 μg/plate 2-aminoanthracene (2AA) (Wako) served as a positive control for tester strain TA1535 and 10 μg/plate 2AA for Wp2 *uvrA*.

The bacterial reverse mutation assay was conducted using the pre-incubation procedure as previously described by Maron and Ames (1983). To prepare the bacterial cultures, frozen pellets were thawed and 20 μL of each *S. typhimurium* TA strain or 10 μL of *E. coli* strain were seeded in L-shaped tubes containing 10 mL of 2.5% nutrient broth. The tubes were shaken and bacteria were cultured at 37 °C for a period of 9 h. The bacterial suspensions were stored at room temperature until use. MQ-01 test solutions and positive or negative controls were added to 0.1 mL of a fully grown culture of each bacterial strain at a volume of 0.1 mL. For the test systems with metabolic activation, 0.5 mL of metabolic activation mix (S9-mix) was added, while for the systems without metabolic activation, 0.5 mL of 0.1 mol/L phosphate buffer (pH 7.4) was added instead to the bacterial culture. The test mixtures were then mixed with 2 mL of soft agar (0.6% agar and 0.5% sodium chloride) supplemented with 0.5 mM D-biotin/0.5 mM L-histidine (1:10) for *S. typhimurium* strains or 0.5 mM D-biotin/0.5 mM L-tryptophan (1:10) for *E. coli* strain, and added to the minimal glucose agar plates. All samples were plated in triplicate and two independent experiments were performed. The plates were then incubated at 37 °C for a period of a period of 48–72 h. After incubation, the number of revertant colonies was counted using an automatic colony counter (Colony Analyzer CA-11D systems, System Sciences Co., Ltd.). A response was considered as positive in the bacterial mutation assay if there was a 2-fold or a dose-dependent or reproducible increase in the number of revertant colonies compared to the negative control value. No statistical analyses were performed.

To determine the dose levels for the main test, a preliminary dose-finding experiment was conducted prior to the main bacterial reverse mutation assay in which MQ-01 was added to each bacterial strain at final concentrations of 19.5, 78.1, 313, 1250, and 5000 μg/plate. MQ-01 did not induce cytotoxicity or increase the number of revertant colonies compared to the negative control at any concentration or in any bacterial strain in either the presence or the absence of S9 metabolic activation (data not shown). Based on the results of the preliminary study, five concentrations of MQ-01 were selected for the main test: 313, 625, 1250, 2500, and 5000 μg/plate.

2.4. In vitro chromosomal aberration assay

The *in vitro* chromosomal aberration assay was conducted in accordance the OECD Principles of GLP (OECD, 1997), and in compliance with the OECD Guidelines for Testing of Chemicals Test No. 473 (OECD, 1997). The Chinese hamster lung fibroblast cells (CHL/IU) were obtained from the Health Science Research Resources Bank. A stock solution of MQ-01 (50 mg/mL) was prepared by dissolving the enzyme in distilled water for injection (Japanese Pharmacopeia, Otsuka Pharmaceutical Factory, Ltd.). Serial dilutions of the MQ-01 stock solution were prepared prior to use to achieve the desired test concentrations. The vehicle (distilled water for injection) served as the negative control, while cyclophosphamide (CP) (Wako Pure Chemical Industries, Ltd.) and mitomycin C (MMC) (Kyowa Hakko Kogyo Co., Ltd.) were used as positive controls in the presence and absence of metabolic activation (Oriental Yeast Co., Ltd.), respectively.

The CHL/IU cells were seeded in each plate containing 5 mL of culture medium and incubated at 37 °C in humidified air containing 5% CO_2 for 3 days. The culture medium consisted of minimum essential medium (GIBCO™), which was supplemented with heat-inactivated (30 min, 56 °C) bovine serum (10%), both obtained from Invitrogen Corporation.

Two separate chromosomal aberration experiments were conducted: short-term treatment (with and without S9-mix) and continuous treatment (24-h and 48-h treatments), and all treatments were performed in duplicate.

In the short-term treatment, after the incubation period, cells were treated with 0.5 mL of the MQ-01 test solutions at final concentrations of 1250, 2500, and 5000 μg/mL, in the absence or presence of S9-mix. For the treatment without metabolic activation, 0.5 mL of the MQ-01 test solution or vehicle only (distilled water for injection) was added to the culture medium, while additional cultures were treated with 0.15 mL of positive control (MMC, 0.075 μg/mL). For the treatment with metabolic activation, cells were co-treated with 0.5 mL of the MQ-01 test solution and 0.5 mL of the S9-mix. The additional cultures were treated with 0.1 mL of the positive control (CP, 14 μg/mL). Cells were observed after the 6-h incubation period, rinsed with isotonic sodium chloride solution, then supplied with 5 mL of freshly prepared culture medium, and incubated for an additional 18 h (i.e., a total of 24 h).

In the continuous treatment, which was conducted in the absence of S9-mix, after the incubation period, cells were treated with 0.5 mL of the MQ-01 test solution at final concentrations of 1250, 2500, and 5000 μg/mL, or vehicle only (distilled water for injection), while the additional cultures were treated with 0.1 mL of positive control (MMC, 0.05 μg/mL). The cells were continuously incubated for 24 or 48 h.

For both chromosomal aberration experiments, cells were arrested in metaphase by the addition of 1 mL colcemid (0.1 μg/mL, Wako Pure Chemical Industries, Ltd.) 2 h prior to the end of the total incubation period. At the end of the incubation period, cells were harvested by 0.25 trypsin solution (Trypsin 0.25%, Invitrogen Co.), treated with 0.075 M hypotonic potassium chloride solution for 15 min, and fixed in methanol:acetic acid solution (3:1). Cells were then transferred to clean microscope slides, with two slides prepared from each culture, air-dried for one day, and stained with a 2% Giemsa solution (source not specified) for 15 min.

Cytotoxicity was assessed by a preliminary cell-growth inhibition test based upon cell-growth inhibition relative to the negative control. In this cytotoxicity assay, 8 MQ-01 concentrations were tested: 39.1, 78.1, 156, 313, 625, 1250, 2500, and 5000 μg/mL, and cell density was measured with a monolayer cell density measuring device (Monocellator, Olympus Optical Co., Ltd.). To assess the induction of structural chromosomal aberrations in each treatment group, 200 well-spread metaphases (100 metaphases/plate) were blindly analyzed by microscopic examination for chromatid-type aberrations (gaps, breaks, fragments, and interchanges), chromosome-type aberrations (gaps, breaks, decentrics, and rings), and other anomalies, including polyploidy and endoreplication. Clastogenicity of the test article was evaluated based on the incidence of cells with structural and numerical chromosomal aberrations according to the method described by Ishidate (1987).

In the chromosomal aberration assay (both short-term and continuous treatments), a response was considered positive if the incidence of cells with structural and numerical aberrations observed was 10% or greater. A response was considered as negative if the incidence of cells with chromosomal aberrations was less than 5%, and equivocal, if the incidence of cells with incidences of chromosomal aberrations was between 5% and 10%. The test article was

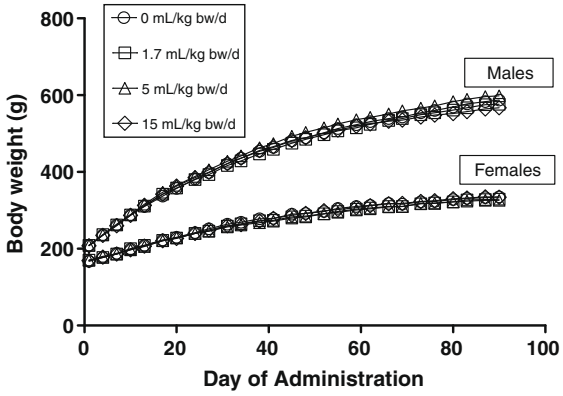

Fig. 1. Body weights of male and female rats during the 13-week toxicity study. There were no significant differences in body weight between treated and control groups ($p > 0.05$).

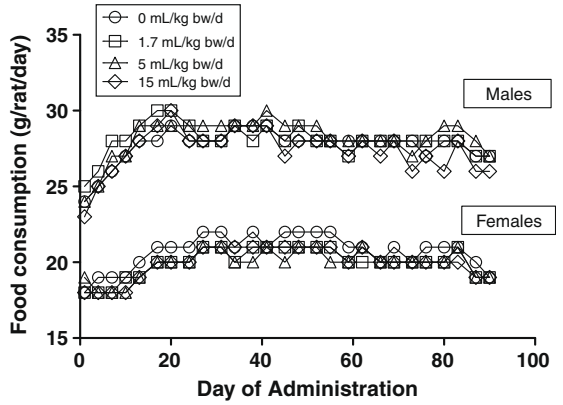

Fig. 2. Food consumption of male and female rats during the 13-week toxicity study. Overall, there were no significant differences between treated and control groups, with the exception of a significant decrease in food intake in mid-dose females on day 38 and day 45 of the study compared to controls ($p < 0.05$).

Table 1
Composition of treatment groups in the subchronic toxicity study.

Test group	Number of animals and sex	Dose of MQ-01 (mg/kg bw/d)
Control	10 M, 10 F	0
Low-dose	10 M, 10 F	139.4
Middle-dose	10 M, 10 F	410
High-dose	10 M, 10 F	1230

Abbreviations: bw = body weight; F = female; M = male.

considered to be clastogenic if a dose-dependent increase in the percentage of cells with structural chromosomal aberrations was observed, or if a single positive test point was observed in both sets of experiments. No statistical analyses of the study results were performed.

3. Results

3.1. Subchronic toxicity study in rats

One male rat in the high-dose (15 mL/kg bw/day) group died on day 60 of the study period. Interstitial cell infiltration in the harderian gland and prostate were observed, but these changes were minimal. No other clinical symptoms or macroscopic abnormalities were observed at necropsy. All other control and treated animals survived the course of the study and displayed good general condition throughout the study period. Ophthalmological examinations revealed no abnormalities in any treatment group compared to control animals.

No significant differences were observed in body weights between the control group and any of the treatment groups during the course of the study (Fig. 1). Compared to control animals, food consumption was significantly decreased in mid-dose (5 mL/kg bw/day) females on days 38 and 45 only, but these changes were not observed in the high-dose group (Fig. 2).

There was a significant decrease in 24-h water intake in the high-dose female group compared to controls, but no significant differences were observed in the high-dose male group. No significant differences were noted in any of the urinalysis parameters examined (data not shown).

Hematological analysis revealed no significant difference between the treated and control groups (data not shown). Several significant differences were observed in clinical chemistry parameters between the treated and control groups (Table 2). In males, an increase in serum AST and ALT was observed in the high-dose group only. In females, increases in serum Ca^{2+}, TP, and albumin were observed in the mid-dose, but not in the high-dose group.

Macroscopic examination revealed significant decreases in absolute and relative heart weights in the low-dose (1.7 mL/kg bw/day) male group, as well as significant decreases in relative heart weight in mid-dose males, as compared to the control group. No significant changes in absolute or relative heart weights were noted in the high-dose group. No other significant differences were observed in absolute or relative organ weights between the treated and control groups (Table 3).

As noted in Table 4, a comparison of the results of the histopathological evaluation of the control and high-dose groups revealed no significant effects of treatment.

3.2. In vitro bacterial reverse mutation assay

Using the pre-incubation method, the results of the bacterial reverse mutation assay showed that exposure to MQ-01 did not increase the mean number of revertant colonies in any strains and at all concentrations tested (19.5–5000 μg/plate), both in the presence and absence of metabolic activation, as compared to the negative control. Conversely, the number of revertant colonies induced by the concurrent positive controls was greater than 2-fold relative to the negative control, while the negative controls were within the acceptable range (data not shown). This indicates the sensitivity of the test and confirms the activity of the S9-mix. MQ-01 was

Table 2
Clinical chemistry parameters following oral exposure to MQ-01 for 13 weeks.

Measured clinical chemistry parameters	Treatment group							
	Control		1.7 mL/kg bw/day		5 mL/kg bw/day		15 mL/kg bw/day	
	Males (n = 10)	Females (n = 10)	Males (n = 10)	Females (n = 10)	Males (n = 10)	Females (n = 10)	Males (n = 9)	Females (n = 10)
AST (IU/L)	57 ± 5	61 ± 10	59 ± 6	70 ± 24	61 ± 9	60 ± 16	68 ± 11*	67 ± 10
ALT (IU/L)	27 ± 3	25 ± 7	26 ± 3	31 ± 24	29 ± 5	27 ± 9	33 ± 4**	32 ± 12
LDH (IU/L)	47 ± 11	44 ± 12	50 ± 13	52 ± 24	46 ± 7	50 ± 21	54 ± 18	62 ± 39
γ-GTP (IU/L)	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
ALP (IU/L)	298 ± 78	144 ± 22	322 ± 31	186 ± 52	340 ± 84	163 ± 63	362 ± 66	167 ± 26
CHO (mg/dL)	55 ± 7	72 ± 13	53 ± 12	66 ± 16	61 ± 12	78 ± 11	55 ± 11	72 ± 14
TG (mg/dL)	81 ± 30	32 ± 15	59 ± 20	34 ± 14	87 ± 56	38 ± 12	64 ± 27	33 ± 12
PL (mg/dL)	96 ± 9	132 ± 18	87 ± 13	125 ± 23	102 ± 15	147 ± 18	96 ± 11	130 ± 21
TBILL (mg/dL)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
GLU (mg/dL)	143 ± 14	126 ± 10	153 ± 26	124 ± 12	153 ± 10	125 ± 11	151 ± 24	124 ± 6
BUN (mg/dL)	15 ± 2	16 ± 2	14 ± 2	15 ± 1	16 ± 2	16 ± 1	15 ± 2	16 ± 3
CRE (mg/dL)	0.28 ± 0.05	0.34 ± 0.05	0.29 ± 0.04	0.35 ± 0.04	0.28 ± 0.04	0.33 ± 0.04	0.26 ± 0.04	0.34±.04
BNA (mmol/L)	145 ± 1	144 ± 1	144 ± 1	144 ± 1	144 ± 1	143 ± 1	145 ± 1	144 ± 1
BK (mmol/L)	4.8 ± 0.3	4.3 ± 0.3	4.9 ± 0.4	4.2 ± 0.3	4.9 ± 0.4	4.3 ± 0.3	4.7 ± 0.3	4.2 ± 0.2
BCL (mmol/L)	107 ± 1	111 ± 1	108 ± 1	112 ± 1	107 ± 1	110 ± 1	107 ± 1	111 ± 1
BCA (mg/dL)	10.0 ± 0.2	10.0 ± 0.2	9.9 ± 0.2	10.1 ± 0.2	10.1 ± 0.4	10.3 ± 0.3*	10.0 ± 0.3	10.1 ± 0.2
BP (mg/dL)	7.1 ± 0.9	5.4 ± 1.4	7.2 ± 0.7	5.5 ± 1.1	7.1 ± 0.7	5.3 ± 1.0	7.1 ± 0.8	5.7 ± 1.1
TP (g/dL)	6.5 ± 0.2	6.7 ± 0.2	6.4 ± 0.3	6.8 ± 0.2	6.6 ± 0.3	7.3 ± 0.2**	6.5 ± 0.2	6.9 ± 0.2
ALB (g/dL)	2.8 ± 0.1	3.1 ± 0.2	2.9 ± 0.1	3.2 ± 0.2	2.9 ± 0.1	3.4 ± 0.2**	2.9 ± 0.1	3.2 ± 0.1
A/G	0.78 ± 0.05	0.87 ± 0.06	0.81 ± 0.07	0.90 ± 0.07	0.78 ± 0.07	0.90 ± 0.05	0.81 ± 0.05	0.86 ± 0.07

All values are reported as means ± standard deviation.
Compared to control group (0 mg/kg bw/d), *$p < 0.05$, **$p < 0.01$ (Dunnett's test).
Abbreviations: A/G = albumin/globulin ratio; ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; BCA = calcium; BCL = chloride; BK = potassium; BNA = sodium; BP = phosphorus; BUN = blood urea nitrogen; bw = body weight; CHO = total cholesterol; CRE = creatinine; GLU = glucose; γ-GTP = γ-glutamyltransferase; LDH = lactate dehydrogenase;;PL = phospholipids; TBILL = total bilirubin; TG = triglycerides; TP = total protein.

Table 3
Absolute and relative organ weights following oral exposure to MQ-01 for 13 weeks.

Organ weights	Treatment group							
	Control		1.7 mL/kg bw/day		5 mL/kg bw/day		15 mL/kg bw/day	
	Males (n = 10)	Females (n = 10)	Males (n = 10)	Females (n = 10)	Males (n = 10)	Females (n = 10)	Males (n = 9)	Females (n = 10)
Absolute organ weight (g, unless otherwise indicated)								
Brain	2.20 ± 0.07	1.98 ± 0.05	2.15 ± 0.10	1.99 ± 0.09	2.22 ± 0.06	2.04 ± 0.07	2.18 ± 0.05	1.96 ± 0.08
Pituitary (mg)	13.0 ± 1.9	17.3 ± 2.8	12.7 ± 0.9	16.1 ± 1.3	12.1 ± 1.4	18.1 ± 2.5	12.4 ± 1.4	15.9 ± 2.5
Thyroid (mg)	24.5 ± 2.9	17.5 ± 3.1	21.4 ± 4.8	14.7 ± 2.2	22.8 ± 4.7	16.7 ± 3.2	22.7 ± 3.8	15.6 ± 3.3
Salivary gland (mg)	727 ± 59	450 ± 48	663 ± 47	455 ± 48	746 ± 106	449 ± 53	681 ± 36	449 ± 41
Thymus (mg)	308 ± 49	248 ± 78	285 ± 91	267 ± 46	260 ± 43	234 ± 37	240 ± 60	285 ± 70
Heart	1.54 ± 0.11	0.92 ± 0.09	1.39 ± 0.15*	0.93 ± 0.06	1.46 ± 0.11	0.94 ± 0.06	1.41 ± 0.12	0.92 ± 0.06
Lung	1.54 ± 0.09	1.18 ± 0.10	1.51 ± 0.10	1.16 ± 0.07	1.50 ± 0.12	1.16 ± 0.08	1.47 ± 0.10	1.13 ± 0.08
Liver	14.01 ± 0.83	7.43 ± 0.74	13.28 ± 1.90	7.17 ± 0.35	14.54 ± 2.96	7.71 ± 0.60	13.49 ± 1.41	7.70 ± 1.04
Spleen	0.78 ± 0.13	0.53 ± 0.10	0.70 ± 0.12	0.52 ± 0.04	0.79 ± 0.13	0.55 ± 0.09	0.70 ± 0.12	0.55 ± 0.10
Kidney	3.29 ± 0.19	1.93 ± 0.18	3.24 ± 0.36	1.87 ± 0.15	3.36 ± 0.36	2.00 ± 0.18	3.42 ± 0.22	2.00 ± 0.20
Adrenals (mg)	63 ± 12	71 ± 9	61 ± 12	69 ± 8	62 ± 11	68 ± 9	59 ± 11	69 ± 8
Testes	3.39 ± 0.24	N/A	3.40 ± 0.17	N/A	3.43 ± 0.23	N/A	3.32 ± 0.31	N/A
Seminal vesicle	1.49 ± 0.19	N/A	1.53 ± 0.17	N/A	1.47 ± 0.21	N/A	1.39 ± 0.23	N/A
Prostate	1.34 ± 0.20	N/A	1.47 ± 0.20	N/A	1.38 ± 0.21	N/A	1.44 ± 0.18	N/A
Ovary (mg)	N/A	81.7 ± 13.8	N/A	78.±12.0	N/A	80.8 ± 13.0	N/A	84.3 ± 19.1
Uterus (mg)	N/A	654 ± 120	N/A	605 ± 133	N/A	575 ± 144	N/A	582 ± 143
Relative organ weight (g/100 g bw, unless otherwise indicated)								
Brain	0.39 ± 0.02	0.63 ± 0.03	0.39 ± 0.04	0.64 ± 0.04	0.39 ± 0.04	0.66 ± 0.02	0.41 ± 0.05	0.62 ± 0.03
Pituitary (mg/100 g bw)	2.3 ± 0.3	5.5 ± 0.9	2.3 ± 0.2	5.2 ± 0.4	2.1 ± 0.2	5.8 ± 0.8	2.3 ± 0.2	5.0 ± 0.5
Thyroid (mg/100 g bw)	4.4 ± 0.5	5.5 ± 0.9	3.9 ± 0.9	4.7 ± 0.7	4.0 ± 0.9	5.4 ± 1.2	4.2 ± 0.6	5.0 ± 1.2
Salivary gland (mg/100 g bw)	130 ± 10	142 ± 10	121 ± 15	145 ± 13	132 ± 19	144 ± 13	128 ± 16	142 ± 13
Thymus (mg/100 g bw)	55 ± 10	78 ± 24	52 ± 16	86 ± 15	46 ± 8	75 ± 10	45 ± 12	89 ± 17
Heart	0.28 ± 0.02	0.29 ± 0.02	0.25 ± 0.02*	0.30 ± 0.02	0.26 ± 0.01*	0.30 ± 0.01	0.26 ± 0.02	0.29 ± 0.02
Lung	0.27 ± 0.02	0.37 ± 0.02	0.28 ± 0.02	0.37 ± 0.01	0.26 ± 0.02	0.37 ± 0.02	0.27 ± 0.03	0.36 ± 0.02
Liver	2.50 ± 0.13	2.34 ± 0.16	2.41 ± 0.19	2.29 ± 0.11	2.53 ± 0.33	2.47 ± 0.11	2.51 ± 0.15	2.43 ± 0.15
Spleen	0.14 ± 0.02	0.17 ± 0.03	0.12 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.18 ± 0.03	0.13 ± 0.02	0.18 ± 0.02
Kidney	0.59 ± 0.04	0.61 ± 0.04	0.59 ± 0.06	0.60 ± 0.04	0.59 ± 0.04	0.64 ± 0.05	0.64 ± 0.06	0.63 ± 0.05
Adrenals (mg/100 g bw)	11 ± 2	22 ± 2	11 ± 2	22 ± 2	11 ± 3	22 ± 3	11 ± 3	22 ± 2
Testes	0.61 ± 0.04	N/A	0.62 ± 0.07	N/A	0.61 ± 0.06	N/A	0.62 ± 0.08	N/A
Seminal vesicle	0.27 ± 0.03	N/A	0.28 ± 0.04	N/A	0.26 ± 0.05	N/A	0.26 ± 0.06	N/A
Prostate	0.24 ± 0.03	N/A	0.27 ± 0.04	N/A	0.24 ± 0.05	N/A	0.27 ± 0.5	N/A
Ovary (mg/100 g bw)	N/A	25.8 ± 3.7	N/A	25.2 ± 3.4	N/A	26.0 ± 4.2	N/A	26.5 ± 5.0
Uterus (mg/100 g bw)	N/A	207 ± 36	N/A	193 ± 40	N/A	186 ± 52	N/A	184 ± 47

All values are reported as means ± standard deviation.
Compared to control group (0 mg/kg bw/d), *$p < 0.05$ (Dunnett's test).
Abbreviations: bw = body weight; N/A = not applicable.

therefore, considered to be without mutagenic activity in the bacterial reverse mutation assay.

3.3. In vitro chromosomal aberration test

In the initial cytotoxicity experiments, short-term and continuous 24-h treatments with MQ-01 did not result in cell-growth inhibition, in the presence or absence of metabolic activation. Reduced cell growth was only observed following continuous treatment with MQ-01 at a concentration of 5000 μg/mL for 48 h in which cell growth was reduced to 29% of the negative control. The 50% cell-growth inhibitory concentration was determined to be approximately 3581.1 μg/mL, in 48-h continuous treatment without metabolic activation.

In the chromosomal aberration experiments, the incidence of CHL/IU cells displaying structural or numerical chromosomal aberrations was less than 5% following short-term or continuous treatment with MQ-01 at all the concentrations and time points assessed, either in the presence or absence of metabolic activation. The positive control agents induced a marked increase in the percentage of cells with structural chromosome damage, while the number of aberrant cells in the negative control cultures was within the negative range of the judging criteria (i.e., <5%), confirming the sensitivity of the assay. Based on the results of these experiments, MQ-01 was considered to have no clastogenic activity in CHL/IU cells.

4. Discussion

Recently, a novel role for amylomaltase has been proposed in the *in vitro* synthesis of glycogen, which is based on the catalytic property of amylomaltase characterized by its ability to perform disproportionation reactions, and consequently increase the yield of the synthesized glycogen (Kajiura et al., 2008). MQ-01 is to be used as a processing aid in the commercial-scale production of enzymatically-synthesized glycogen. The product then undergoes several processing steps, including heating at high temperatures and pH adjustment to inactivate the enzyme, and filtration to remove residual proteins. As such, no residual MQ-01 activity is anticipated to be present in the final product; this is confirmed by the results of protein analysis on enzymatically-synthesized glycogen in which no protein was detected (data not shown). Thus, human exposure to MQ-01 is expected to be negligible to none. The MQ-01 used in the food production industry, however, must be preceded by an established safety of the enzyme preparation. In the following, we report on the safety of MQ-01, an enzyme derived from a recombinant strain of *B. subtilis* expressing the amylomaltase gene *malQ* from *T. aquaticus*.

Table 4
Histopathological findings following oral exposure to MQ-01 for 13 weeks.

Measured parameters	Number of animals			
	Control		15 mL/kg bw/day	
	Male	Female	Male	Female
Epididymis				
Minimal cell infiltration	1	N/A	1	N/A
Eyeball				
Minimal dysplasia	1	0	0	0
Mild dysplasia	1	0	0	0
Harderian gland				
Minimal increase of porphyrin accretion	4	1	5	3
Minimal interstitial cell infiltration	0	1	1	0
Mild interstitial cell infiltration	0	1	0	0
Heart				
Minimal focal myocarditis	0	1	0	1
Cecum				
Minimal mucosal cell infiltration	0	1	0	1
Kidney				
Minimal tubular regeneration	1	1	1	0
Minimal interstitial mineralization	2	5	3	1
Minimal cell infiltration	0	0	2	0
Liver				
Minimal periportal hepatocyte vacuolation in lobular marginal zone	0	1	0	1
Mild periportal hepatocyte vacuolation	0	0	0	1
Minimal extramedullary hematopoiesis	0	0	1	0
Minimal microgranuloma	5	2	7	4
Minimal cell infiltration in Glisson's sheath	0	0	0	1
Pancreas				
Minimal focal acinar atrophy	0	0	1	0
Minimal interstitial cell atrophy	2	0	3	0
Pituitary				
Minimal aberrant craniopharyngeal tissue	0	0	1	0
Prostate				
Minimal interstitial cell infiltration	6	N/A	1	N/A
Mild interstitial cell infiltration	1	N/A	2	N/A
Salivary gland				
Minimal interstitial cell infiltration	0	0	1	0
Stomach				
Minimal epidermal cyst	0	0	1	0
Thyroid				
Minimal ectopic thymus	0	0	1	0
Minimal ultimobranchial body remnant	1	2	3	0

Abbreviations: bw = body weight; N/A = not applicable.

Guidelines for evaluating the safety of microbial enzyme preparations used in foods have been previously established (Pariza and Foster, 1983; Pariza and Johnson, 2001). Based on these guidelines, the first consideration in the safety assessment of an enzyme preparation intended for use in food production is the safety of the production strain or source organism, and in particular the production strain's toxigenic and pathogenic potential (Pariza and Foster, 1983; Pariza and Johnson, 2001). Once safety of the production strain has been established, it may be genetically modified with a gene of interest from another bacterial strain provided that all new DNA introduced are determined to be safe, and the procedures that have been used to modify the host organism are appropriate for food use (IFBC, 1990; Pariza and Johnson, 2001). Consequently, the second point of consideration is the safety of the protein product encoded by the inserted DNA sequence, if the source is a recombinant microbial strain.

Thermus aquaticus amylomaltase enzyme preparation used in this study, MQ-01, is derived from a recombinant production strain of *B. subtilis* (strain 168). *B. subtilis* is a ubiquitous, non-pathogenic, non-toxigenic, Gram-positive bacterium that has a long history of use in food processing as a safe source of native enzymes, mainly, α-amylases and proteases (de Boer and Diderichsen, 1991; Olempska-Beer et al., 2006). In addition, the wild type *B. subtilis* strain 168 is the progenitor of many recombinant *B. subtilis* strains that have been used as sources of food processing enzymes (Olempska-Beer et al., 2006). As a result, the production strain, *B. subtilis* (strain 168) is derived from a safe lineage, and its use in the production of MQ-01 is unlikely to raise a safety concern.

As per Pariza and Johnson (2001), the safety of the DNA introduced into *B. subtilis* also was evaluated in order to assess the safety of the genetically-modified organism. Plasmid pUB110 carrying the *malQ* gene was used as an expression vector in *B. subtilis* to encode the amylomaltase from *T. aquaticus*. The use of pUB110 is documented to be safe for introducing new genetic material in *B. subtilis* for the purpose of producing enzymes used in the manufacture of food products (IFBC, 1990; Olempska-Beer et al., 2006). Although pUB110 carries antibiotic resistance genes, these genes do not transfer to the enzyme products, as they are considered to be stably associated with the vector (IFBC, 1990). Considering this, the pUB110 vector used to carry the gene encoding amylomaltase is established as safe for constructing recombinant enzymes that are to be used to produce food-grade products. The DNA sequence inserted into the pUB110 vector comprised genes (*malQ)* encoding amylomaltase from *T. aquaticus* strain ATCC33923. The expression product of these DNA inserts is the desired product, MQ-01, when produced with the use of *B. subtilis* as the host organism.

MQ-01 does not have a history of use in food; however, the safety of the enzyme preparation was evaluated directly using standard toxicological experiments designed to assess its potential genotoxicity and subchronic oral toxicity. In the standard bacterial reverse mutation assay, MQ-01 failed to induce increases in the incidence of reverse mutations above negative control values in all the *S. typhimurium* and *E. coli* strains tested, and thus was shown to be non-mutagenic in either the presence or absence of metabolic activation. In addition, MQ-01 did not display clastogenic properties in a chromosomal aberration assay conducted in CHL/IU cells even at concentrations that induced cytotoxicity.

In the 13-week oral toxicity study, all treatment and control animals survived the experimental period in good general health, with the exception of one male in the high-dose group for which the cause of death was determined not to be compound-related. In addition, no significant differences were observed in body weights, ophthalmological examinations, urinalysis, and hematological examinations between the control and treated animals.

The small, but statistically significant increases in the liver AST and ALT enzymes in the high-dose males, and an increase in serum Ca^{2+}, albumin and TP in the mid-dose females, were not attributed to treatment with MQ-01, as these changes were not dose-dependent, occurred in only one sex, and were not accompanied by any macroscopic abnormalities. Microscopic examination revealed several histopathological changes in the hepatic tissues; however, there were no significant differences in the incidence of these changes between the treatment and control groups.

Significant changes in absolute and relative heart weights were not specifically MQ-01-related, as the response was not dose-dependent, was only observed in one sex, and was not accompanied by histopathological changes. Similarly, all other histopathological changes were observed to occur at a similar frequency in treatment and control groups or only occurred in the control group. Based on the results of the 13-week oral toxicity study, the no-observed-adverse-effect level for MQ-01 in rats was established to be greater than 15 mL/kg bw/day (equivalent to 1230 mg/kg bw/day), the highest dose tested.

The lack of any toxicological findings directly related to MQ-01 is consistent with the history of safe consumption of enzymes de-

rived from *B. subtilis*, as well as with the nature of the microorganism itself. Furthermore, D-enzyme, a closely-related plant-derived enzyme, has a long history of human consumption, due to its natural occurrence in potatoes and barley (Takaha and Smith, 1999). Although bacterial-derived MQ-01 does not have a history of use in the production of foods for human consumption, other closely-related bacterial-derived enzymes such as cyclomaltodextrin glucanotransferase (EC 2.4.1.19), the enzyme responsible for cyclodextrin synthesis, or the recombinant branching enzymes used in the production of highly-branched cyclic dextrin are considered safe for use in food processing (Bär et al., 2004; JECFA, 1999; Pariza and Johnson, 2001; Choi et al., 2009). The safety of the latter for its intended use was supported by results of a 13-week oral toxicity study, as well as *in vitro* mutagenicity and genotoxicity studies.

In summary, MQ-01 was shown to not possess mutagenic or clastogenic activity *in vitro*. Repeated exposure to MQ-01 also was not associated with any adverse effects of toxicological significance in Sprague–Dawley rats. Furthermore, the production strain for MQ-01 (*B. subtilis* strain 168) is derived from a safe non-toxigenic lineage, and the DNA sequence encoding for MQ-01 also is considered to be safe. Collectively, these data support the safety of MQ-01 for use as a processing aid in the production of enzymatically-synthesized glycogen, especially when considering that potential human exposure is minimized following inactivation and removal of the enzyme preparation from the final food product by down-stream purification processes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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