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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