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Genetic and rat toxicity studies of cyclodextrin glucanotransferase

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ABSTRACT

Introduction: Microbiologically derived cyclodextrin glucanotransferase (CGTase) is used commercially as a processing agent in manufacture of food, pharmaceuticals, and cosmetics. Its toxic potential was evaluated in anticipation of use in the production of *alpha*-glycosyl isoquercitrin, a water-soluble form of quercetin.
Methods: Following OECD guidelines, CGTase, produced by *Bacillus pseudalcaliphilus* DK-1139, was evaluated in a genotoxicity battery consisting of a bacterial reverse mutation assay, an *in vitro* micronucleus (MN) assay and MN and comet assays using B6C3F1 male and female mice. These same genotoxicity assays were also conducted for sodium sulfate, a contaminant of CGTase preparation. In a 90-day Sprague Dawley rat toxicity study, CGTase was administered by gavage in water at daily doses of 0, 250, 500, and 1000 mg/kg/day.
Results: CGTase did not induce mutations with or without metabolic activation in the bacterial reverse mutation assay. Formation of micronuclei was not induced in either *in vitro* or *in vivo* MN assays with or without metabolic activation. No induction of DNA damage was detected in male or female mouse liver, stomach, or duodenum in the comet assay. Sodium sulfate also tested negative in these same genotoxicity assays. In the 90-day repeated dose rat study there were no treatment-related adverse clinical or pathological findings.
Conclusion: The genotoxicity assays and repeated dose toxicity study support the safe use of CGTase in production of *alpha*-glycosyl isoquercitrin.

1. Introduction

Cyclodextrin glucanotransferase (CGTase) is one of several starch-hydrolyzing extracellular enzymes produced by many bacteria to convert large molecules to utilizable small molecules for growth. CGTase, a member of the *alpha*-amylase superfamily, catalyzes cleavage of glycosidic bonds between carbohydrates and/or non-carbohydrate moieties. As an amylolytic enzyme, CGTase is used commercially in manufacture of food, pharmaceuticals, and cosmetics [10,25]. Our interest is in use of CGTase in synthesis of glycosylated molecules such as *alpha*-glycosyl isoquercitrin (EMIQ). CGTase catalyzes addition of one or more glucose units to isoquercitrin to produce EMIQ (Fig. 1); during processing, this step is followed by heat inactivation of the CGTase. Even though not present in the final product, food enzymes are subject to safety evaluation according to EFSA and current Japanese regulations [6,7]. In the present study we provide a safety evaluation of CGTase, produced by *Bacillus pseudalcaliphilus* DK-1139, using a battery of genotoxicity assays and a 90-day repeated dose toxicity study in Sprague Dawley rats.

2. Methods

2.1. Chemical analysis

All genotoxicity assays were conducted according to OECD guidelines and were Good Laboratory Practice (GLP)-compliant.

Abbreviations: CGTase, cyclodextrin glucanotransferase; EMIQ, enzymatically modified isoquercitrin; MN, micronuclei; MN-RET, micronucleated reticulocytes; MN-NCE, micronucleated normochromatic erythrocytes; GLP, good laboratory practice; NOAEL, no observed adverse effect level; OECD, Organization for Economic Co-operation and Development; EFSA, European Food Safety Authority.

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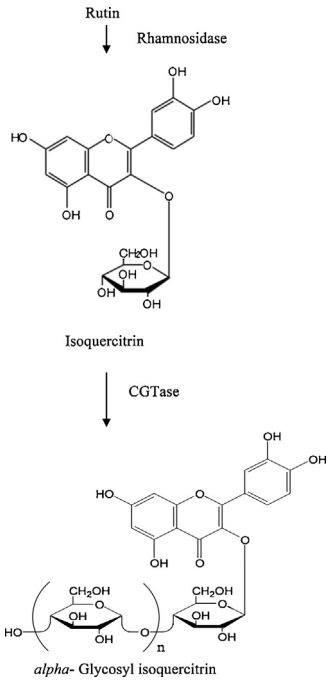

Fig. 1. CGTase modifies isoquercitrin with dextrin to form alpha-glycosyl isoquercitrin.

Samples removed from the top, middle, and bottom fractions of each chemical formulation were submitted for analytical testing (Alera Laboratories, LLC, Durham, North Carolina, USA). Most analyzed formulation concentrations were greater than 15% of the nominal dose or were within the ±15% acceptance criteria. The few formulations that were lower than 15% of the nominal dose were low or mid-range doses that were not used as benchmark doses to determine overall assay responses. CGTase (Composition = 85% carbohydrate, 0.3% protein, 0.7% lipid, 12.1% water, 0.26% sodium sulfate, ≤2 ppm lead, ≤0.5 ppm arsenic, and 1.0% total organic solvent; CAS No. 9030-09-5; San-Ei Gen F.F.I., Inc., Osaka, Japan) was prepared in sterile deionized water and stored between 0 and −30 °C and away from light. The neat test article was determined to be stable, based on enzyme titer, for a period of three months with storage at approximately −15 °C. Continued stability is inferred from repeated formulation analyses in which the formulation concentrations, calculated from enzyme activity measurements, routinely exceeded nominal concentrations [1]. Sodium sulfate (99.7% pure; Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) prepared in sterile deionized water and stored at 1–10 °C and away from light is stable for at least 8 days [1]. Sodium sulfate, present at low levels in preparations of CGTase, was also tested in the genotoxicity battery.

2.2. Bacterial reverse mutation assay

GLP mutagenicity assays of CGTase and sodium sulfate, with and without metabolic activation, were conducted as described previously [2,13,14] using the following five *Salmonella* and *E. coli* strains as prescribed in the guideline for the bacterial reverse mutation assay [15]: TA98, TA100, TA97a, TA1535, and *E. coli* WP2 *uvr*A pKM101. All strains (Moltox, Inc., Boone, NC) were checked for maintenance of genetic markers prior to the study. Nominal concentrations of CGTase ranged from 25 to 5000 μg/plate. Concentrations of sodium sulfate ranged from 250 to 5000 μg/plate. Metabolic activation was provided using phenobarbital/benzoflavone-induced rat liver S9 (Moltox, Inc., Boone, NC) with cofactors (Regensys™ NADPH Regeneration System Reagents, Moltox, Inc., Boone, North Carolina, USA). The composition of the S9 mix was: 10% S9, 8 mM $MgCl_2$, 32.6 mM KCl, 4.7 mM glucose-6-phosphate, 4 mM NADP, and 0.1 M phosphate buffer. Cytotoxicity was assessed by changes in the background bacterial lawn (i.e., enhanced or absent lawn), reduced revertant counts, presence of microcolonies, and/or absence of colonies. Strain specific positive controls tested without metabolic activation were 2-nitrofluorene, 3 μg (TA98), sodium azide, 1 μg (TA100 and TA1535), ICR191, 0.25 μg (TA97a), and 4-nitroquinoline-N-oxide, 0.25 μg (*E. coli* WP2). Benzo[a]pyrene, 2 μg (TA100) and 2-aminoanthracene, 2 μg (TA98); 2.5 μg (TA97a, TA1535); 20 μg (*E. coli* WP2) were used as the positive controls with metabolic activation. Following a preliminary incubation at 37 °C for 20 min prior to plating, three test plates per concentration were incubated at 37 °C for 48 h and then counted using the Sorcerer/Ames Study Manager System (Perceptive Instruments, Ltd., Suffolk, UK). To ensure accuracy of the results, reagent sterility and automated scoring checks were conducted. Criteria for a positive response were a ≥ 2-fold increase in the average plate count compared to the solvent control for at least one concentration level and a dose response over the range of tested concentrations in at least one strain with or without metabolic activation. In addition, the average response should fall outside the laboratory historical vehicle control range for the strain/metabolic activation condition.

2.3. In vitro *micronucleus (MN) assay*

The GLP *in vitro* MN assay was conducted in compliance with OECD TG 487 [18]. Human TK6 cells (ATCC, Manassas, VA) were cultured and maintained in RPMI 1640 medium containing 10% heat inactivated horse serum plus 1.0% Pluronic F-68, 0.5% sodium pyruvate, and antibiotics (penicillin at 20 Units/mL and streptomycin at 20 μg/mL) at 37 °C, with 6% CO_2. The normal cell cycle time of these cells is approximately 12 h. Metabolic activation was provided using phenobarbital/benzoflavone-induced rat liver S9 (Moltox, Boone, NC) with added Regensys™ cofactors (Moltox, Boone, NC) at a final concentration of 1% S9. The composition of the S9 mix was: 10% S9, 8 mM $MgCl_2$, 32.6 mM KCl, 4.7 mM glucose-6-phosphate, 4 mM NADP, and 0.1 M phosphate buffer. Triplicate cultures of exponentially growing cells seeded at 0.40×10^6 cells/mL in 12-well plates were exposed to CGTase, sodium sulfate, or controls for approximately four hours in the presence of metabolic activation (+S9) and approximately 24–29 h in the absence of metabolic activation (-S9). Cyclophosphamide (Sigma-Aldrich, St. Louis, MO) and vinblastine (Sigma-Aldrich, St. Louis, MO) were used as the positive controls with and without metabolic activation, respectively. On the basis of preliminary tests, the concentrations of CGTase selected for testing were 6000, 5000, 4000, 3000, 2000, and 1000 μg/mL of CGTase for approximately 4 h with S9 and 24 h without S9. Concentrations of sodium sulfate selected were 5000, 1667, 556, 185, 61.7, and 20.6 μg/mL for 4 h with S9 and 24 h without S9. At the end of the culture period, cells were analyzed for cytotoxicity and micronucleus induction by flow cytometry using the *In Vitro* MicroFlow™ kit (Litron Laboratories, Rochester, NY) according to manufacturer's instructions. Unless limited by cytotoxicity, 20,000 cells from each sample were analyzed for the frequency of micronuclei (MN) using a FACSCalibur™ dual-laser bench top system (Becton Dickinson Biosciences, San Jose, CA).

Cytotoxicity was measured as relative increase in cell count or as relative survival of cells in vehicle control cultures compared to cells in treated cultures using ratios of counted nuclei to counted beads (inert latex microspheres added to each sample). Higher nuclei to bead ratios correspond to greater cell survival. Greater emphasis was placed on relative survival since this flow cytometry-based measurement may provide a more sensitive assessment of cytotoxicity, enhancing assay specificity Avlasevich et al., 2011.