Toxicology Reports 3 (2016) 123–134

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

13-week dietary study and *in vitro* and *in vivo* genotoxicity studies of a structuring fat produced through a microalgal fermentation process

R.A. Matulka[a,*], T. Chan[b], R. Green[b], J.R. Carney[b], S. Franklin[b], P. Licari[b]

[a] *Burdock Group, 859 Outer Road, Orlando, FL 32814, United States*
[b] *Solazyme, Inc., 225 Gateway Blvd., South San Francisco, CA 94080, United States*

ARTICLE INFO

Article history:
Received 21 December 2015
Accepted 23 December 2015
Available online 29 December 2015

Keywords:
Microalgae
Stearic acid
Subchronic toxicity
Kokum
Prototheca moriformis
Structuring fat

ABSTRACT

Microalgae are increasingly being utilized as food ingredients for a variety of applications, including as sources of protein, egg and dairy substitutes, and cooking oils. The dietary safety of a new structuring fat produced using a heterotrophic fermentation process by a strain of *Prototheca moriformis* was evaluated in a 13-week dietary toxicity study and compared with kokum fat, a structuring fat of similar composition used in the food industry and derived from *Garcinia indica* seeds. The algal structuring fat was evaluated for its genotoxic potential using both *in vitro* and *in vivo* assays. No treatment-related adverse events occurred in rats consuming algal structuring fat or kokum fat in the 13-week study; no treatment-related effects were reported for body weight, food consumption, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology. While statistically significant effects occurred in some parameters, none were dose-related or considered adverse. Overall, the NOAELs for the algal structuring fat and the kokum fat were 100 000 ppm, the highest concentrations tested. The algal structuring fat was not mutagenic in the bacterial reverse mutation assay in the *Salmonella typhimurium* or *Escherichia coli* strains tested and was not clastogenic in the *in vivo* mouse bone marrow chromosome aberration assay.

1. Introduction

There are very few natural fats that have the unique melting properties of cocoa butter. The sharp melting profile associated with cocoa butter, which approximates human body temperature, yet its ability to remain solid at room temperature, derives from its high concentration of structuring fats (*i.e.*, symmetrical monounsaturated triglycerides in which oleate (C18:1) occupies the sn-2 position) in greater than 80% of the triacylglycerol (TAG) species present [18]. Kokum butter has been evaluated for supplementation to cocoa butter, but cost still prohibits widespread use of this fat [13]. While fractionation and hydrogenation processes have been developed to increase levels of structuring fats in vegetable and lauric acid-containing (palm kernel) oils and thereby impart melt profiles similar to cocoa butter, their costs of production are relatively high, their uses limited because of the type of TAG species found in the starting oil [4], and there are negative health consequences associated with the generation of trans fats when hydrogenation is used to make cocoa butter-like fats. Trans fats have been found to increase low-density lipoprotein (LDL) levels, increasing the risk of cardiovascular disease [9,15]. Indeed, the food industry is moving away from the use of trans fats and partially hydrogenated oils due to an increased understanding of the potential adverse effects related to their consumption, and the US FDA's decision to rescind the Generally Recognized As Safe (GRAS) status of partially hydrogenated oils (PHOs) [6]. Hence, the search to find replacements and meet additional demand for structuring fats will only become more challenging.

New sources of oils that can either replace or complement currently available oil sources includes the use of microalgae that have been found to produce high levels of potentially useful oils [19]. Advances in production and processing have made

Abbreviations: 2-AA, 2-aminoanthracene; AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; ANOVA, Analysis of Variance; AOAC, Association of Analytical Communities; AOCS, American Oil Chemists' Society; ASTM, American Society for Testing and Materials; bw, Body weight; GLP, Good laboratory practice; CPA, Cyclophosphamide; cps, Centipoise; DHA, Docosahexaenoic acid; g, Gram; EPA, Eicosapentaenoic acid; GRAS, Generally recognized as safe; GRN, GRAS notification; ISO, International Organization for Standardization; kg, Kilogram; LDL, Low-density lipoprotein; mg, Milligram; MMS, Methylmethansulfonate; MTD, Maximum tolerated dose; ppm, Parts-per-million; 4-NOPD, 4-nitro-*o*-phenylene-diamine; NOAEL, No-observed-adverse-effect level; OECD, Organisation for Economic Cooperation and Development; OSD, Open source diet; PHOs, Partially hydrogenated oils; RSD, Relative standard deviation; SOS, Stearic-oleic-stearic triglyceride; TAG, Triacylglycerol; TFA, Total fatty acid; US FDA, United States Food and Drug Administration.

* Corresponding author. Fax: +1 407 802 1405.
E-mail address: rmatulka@burdockgroup.com (R.A. Matulka).

http://dx.doi.org/10.1016/j.toxrep.2015.12.006

the manufacture of new microalgal-derived oils and ingredients through heterotrophic fermentation more cost-effective. The ability to genetically engineer certain species of microalgae, as well as tightly controlling their growing conditions, has resulted in the production of oils with well-defined fatty-acid constituents possessing desirable properties.

Prototheca moriformis is an achlorophyllous (*i.e.*, non-chlorophyll producing) microalga related to *Chlorella protothecoides* (aka *Auxenochlorella protothecoides*) and is found ubiquitously in the environment [16]. A strain of *P. moriformis* was genetically engineered as a stable microorganism that produces significant amounts of a new structuring fat containing mostly stearic (~55%) and oleic (~35%) fatty acids, with minor amounts of other fatty acids. The resulting fat is composed primarily of triglycerides (>98%), with minor levels of diglycerides and monoglycerides (<2%). This structuring fat produced by this engineered organism has been produced for use in a variety of food products, but has not previously been added to food. The algal structuring fat is similar to kokum butter, a fat utilized in the European Union (EU) as a substitute for cocoa butter [5].

The typical fatty acid profile for kokum butter is: palmitic acid (2–6%), stearic acid (50–62%), linoleic (0–2%) and oleic acid (30–42%) [1]. Before introducing a new food ingredient to the market for human consumption, a demonstration of the safety of that ingredient must be completed.

To evaluate the dietary safety of the genetically engineered microalgal-derived structuring fat produced using a heterotrophic fermentation process, the structuring fat was assessed for toxicity in a comprehensive 13-week dietary study in rats and compared to another structuring fat containing similar levels of stearate (kokum fat derived from the seeds of *Garcinia indica*). No safety information could be located in the scientific literature evaluating the safety of kokum fat according to current scientific standards, and this is also the first scientific evaluation of the structuring fat produced by this genetically engineered strain of *P. moriformis*. The comparison was particularly relevant as the algal structuring fat has a fatty acid and TAG composition quite similar to kokum [12], yet the latter is already consumed in the European Union [5] and in India [7]. An *in vitro* mutagenicity study in bacteria (the bacterial reverse mutation study) and the *in vivo* chromosome aberration assay were also conducted on the algal structuring fat to evaluate its clastogenic potential.

2. Materials and methods

2.1. Test substance and diet preparation

The algal structuring fat (lot # RBD735) is an off-white, refined, bleached, deodorized solid, isolated from a genetically engineered strain of *P. moriformis* utilizing a unique manufacturing process to produce the structuring fat with consistent fatty acid ratios. The neutral oil is composed of >95% triglycerides, followed by diglycerides (<2%) and monoglycerides (<0.5%). The major fatty acids are stearic acid (~55%) and oleic acid (~35%), as reported as the area percent of total fatty acids. Nonsaponifiable material is less than 1% and the moisture content is approximately 113 ppm (0.01%). Product characteristics of the algal structuring fat are provided in Table 1.

A comprehensive screen for toxins was carried out on the oil. Pheophorbide A is a naturally-occurring degradation product of chlorophyll that is associated with photosensitive dermatitis [10]. Although this organism is achlorophyllous, the test fat was analyzed for pheophorbide A by high-performance liquid chromatography with fluorescence detection at UBE Analytical Laboratories (Fullerton, CA).[1] The fat was also analyzed for the following algal and cyanotoxins: amnesic shellfish poisoning toxins (domoic acid), diarrhetic shellfish poisoning toxins (okadaic acid, dinophysistoxin-1, pectenotoxin-2, azaspiracid-1, yessotoxin, and homo-yessotoxin), paralytic shellfish poisoning toxins (gonyautoxins 1–6; decarbamoylgonyautoxins 2 and 3; saxitoxin, decarbamoylsaxitoxin, neosaxitoxin and ciguatoxins 1–4), cyanobacterial toxins (microcystin-RR, -YR, -LR, -LW, -LF, -LA, -WR, -LY and -HtyR and dm-microcystin-RR and -LR), nodularin, anatoxin and cylindrospermopsin. The algal and cyanotoxin assays were conducted by liquid chromatography with tandem mass spectrometric detection[2] at the Food GmbH Jena Analytik - Consulting (Jena, Germany). No toxins were reported above detection limits (data not shown).

Diets were formulated using the supplied basal Open Standard Diet obtained from Research Diets, Inc. (New Brunswick, NJ) to which kokum and the algal structuring fat were added to achieve the target doses (with soybean oil as the balance) and to provide comparable fat, protein and carbohydrate content across dose groups. All test and control diets were prepared approximately weekly and stored under refrigeration until used. The concentration of test substance and the reference kokum, stability and homogeneity were evaluated via analysis of the fats in collected feed samples (samples frozen until assayed).

2.2. Chemicals and materials

Corn oil and cyclophosphamide (CPA) were purchased from Sigma–Aldrich Corp. (St. Louis, MO) for the chromosomal aberration assay. Kokum and algal structuring fats were provided by Solazyme, Inc. (South San Francisco, CA) for the 13-week subchronic dietary study. The kokum fat was obtained from Essential Wholesale & Labs (Portland, OR). The S9 metabolic activation mix for the bacterial reverse mutation assay was purchased from Molecular Toxicology, Inc. (Boone, NC) where it was prepared from livers of male Sprague-Dawley rats induced with phenobarbital and benzoflavone. The bacterial reverse mutation assay positive control substances, sodium azide (NaN_3), ICR 191 acridine, daunomycin, methylmethanesulfonate (MMS) and 2-aminoanthracene (2-AA), were also purchased from Molecular Toxicology, Inc. (Boone, NC), as well as the overlay agar (supplemented with biotin and limited amounts of histidine and tryptophan) and minimal glucose agar plates.

2.3. Animals and organisms

CRL Sprague-Dawley (SD) CD® IGS rats (male and female) were obtained from Charles River Laboratories (Raleigh, NC) for the 13-week subchronic dietary study. Veterinarian staff visually inspected all rats at delivery and during a five-day acclimation phase prior to study initiation. The rats were 7–8 weeks of age at study initiation. Body weight variations remained within ±20% of the measured mean for both sexes at study start (220.7 g mean for the males with a range of 220.3–221.5 g and a 182.0 g mean for the females with a range of 181.7–182.3 g). The rats were individually housed in suspended stainless steel caging in a temperature (19–23 °C) and humidity (32–55%) controlled room with a 12-h light/dark cycle. Filtered water and the test diet formulations were provided *ad libitum*.

The bacterial strains (*Salmonella typhimurium* TA1535, TA1537, TA98, TA 100 and *Escherichia coli WP2 uvrA) utilized for the bacterial reverse mutation assays performed at Product Safety Laboratories*

[1] Limit of detection was 0.5 ppm for pheophorbide A.
[2] Limits of detection ranged from 0.0008 to 0.1 μg/g in the fat.