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Safety evaluation of rebaudioside A produced by fermentation

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ABSTRACT

The safety of rebaudioside A, produced fermentatively by *Yarrowia lipolytica* encoding the *Stevia rebaudiana* metabolic pathway (fermentative Reb A), is based on several elements: first, the safety of steviol glycosides has been extensively evaluated and an acceptable daily intake has been defined; second, the use of *Y. lipolytica*, an avirulent yeast naturally found in foods and used for multiple applications; and third the high purity of fermentative Reb A and its compliance with internationally defined specifications. A bacterial reverse mutation assay and an *in vitro* micronucleus test conducted with fermentative Reb A provide evidence for its absence of mutagenicity, clastogenicity and aneugenicity. The oral administration of fermentative Reb A to Sprague–Dawley rats for at least 91 days did not lead to any adverse effects at consumption levels up to 2057 mg/kg bw/day for males and 2023 mg/kg bw/day for females, which were concluded to be the No Observed Adverse Effect Levels. The results were consistent with outcomes of previous studies conducted with plant-derived rebaudioside A, suggesting similar safety profiles for fermentative and plant-derived rebaudioside A. The results of the toxicity studies reported here support the safety of rebaudioside A produced fermentatively from *Y. lipolytica*, as a general purpose sweetener.

1. Introduction

Rebaudioside A extracted from the leaves of the plant *Stevia rebaudiana* Bertoni is one of the principal steviol glycosides widely used as a non-caloric sweetener in food and beverages. Rebaudioside A can also be produced fermentatively by a strain of *Yarrowia lipolytica (Y. lipolytica)*, genetically engineered to express the steviol glycoside metabolic pathway of the plant *S. rebaudiana*, and can be subsequently purified by crystallization to more than 95%.

The safety of rebaudioside A and other steviol glycosides extracted from plants has been extensively evaluated since the 1970's (Carakostas et al., 2008). The use of *S. rebaudiana* extracts containing mostly steviol glycosides has been authorized as food additives for many years in a number of South American and Asian countries such as Paraguay, Argentina, Brazil, South Korea, and Japan. The U.S. FDA (United States Food and Drug Administration) has reviewed 35 Generally Recognized As Safe (GRAS) Notices for steviol glycosides since 2008. However, as reported in 1999 and 2000, both the European Scientific Committee for Food (SCF) and the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) were not convinced of the safety of steviol glycosides, notably due to mutagenic effects of steviol glycosides reported *in vitro* in the presence of metabolic activation, and due to the paucity of *in vivo* data available at that time (SCF, 1999; JECFA, 2000). Later on, as purification of steviol glycosides improved, their safety was re-

Abbreviations: ADI, Acceptable Daily Intake; EFSA, European Food Safety Authority; FCC, Food Chemical Codex; FDA, Food and Drug Administration; JECFA, Joint FAO/WHO Expert Committee on Food Additives; GRAS, Generally Recognized As Safe; NOAEL, No Observed Adverse Effect Level; NOEL, No Observed Effect Level; Reb A, Rebaudioside A; SCF, European Scientific Committee for Food; *Y. lipolytica*, *Yarrowia lipolytica*.

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tested in several toxicology studies. Subchronic rat studies conducted with purified rebaudioside A or stevioside did not reveal toxic effects on any organs up to doses of 2500 mg/kg bw/day (Curry and Roberts, 2008; Nikiforov and Eapen, 2008; Aze et al., 1991; Geuns et al., 2003). Reproduction studies in rats also showed no effects of purified rebaudioside A on fertility and fetal development (Curry et al., 2008). Overall, the additional mutagenicity studies requested by the SCF did not show evidence of genotoxicity *in vitro* and *in vivo* (Brusick, 2008). In addition, results of a recent study in humans showed the absence of adverse effects of steviol glycosides when consumed at approximately 4 mg/kg bw/day, expressed as steviol equivalent, for up to 16 weeks by individuals with type 2 diabetes mellitus and individuals with normal and low-normal blood pressure for 4 weeks (Maki et al., 2008a, 2008b). In 2008, JECFA established an Acceptable Daily Intake (ADI) for steviol glycosides of 0–4 mg/kg bw/day expressed as steviol using a 100-fold uncertainty factor (JECFA, 2008, 2009) based on a chronic (104 weeks) study in rats. This study, performed with stevioside, led to a No Observed Effect Level (NOEL) of 970 mg steviosides/kg bw/day, equivalent to 388 mg steviol equivalents/kg bw/day (Toyoda et al., 1997). JECFA has also established specifications to ensure that the material on the market is equivalent to the material tested in the safety studies.

For the production of rebaudioside A via fermentation, a strain of *Y. lipolytica* was used. *Y. lipolytica* is an avirulent oleaginous yeast species that is used for multiple industrial applications such as the production of citric acid, γ-decalactone, long-chain poly-unsaturated fatty acids and biodiesel fuel (Gonçalves et al., 2014; Zhu and Jackson, 2015). *Y. lipolytica* is naturally found in foods, primarily in foods with high proportions of fat and/or protein, such as in (fermented) dairy products and meat. This yeast is also used to produce food additives such as aroma compounds, organic acids, polyalcohols, emulsifiers, surfactants and carotenoids (Zinjarde, 2014; Zhu and Jackson, 2015). *Candida (=Yarrowia) lipolytica* was approved by the U.S. FDA as a secondary direct food additive for human consumption used for citric acid production and recognized as a nonpathogenic organism (FDA 21 CFR 173.165). More recently, the U.S. FDA also listed erythritol and eicosapentaenoic acid-rich triglyceride oil produced with *Y. lipolytica* on its inventory of Generally Recognized As Safe (GRAS) notifications for which it has no questions (FDA No Questions Letter. DuPont. GRN 000355; FDA No Questions Letter. Baolingbao Biology Co. GRN 000382). The safety of products obtained by fermentation of *Y. lipolytica* was extensively tested: acute (3–6 weeks), subchronic (90 days), chronic (1.5–2 years) and reproduction toxicity was assessed using rats and mice, guppies, chickens and quail. The safety of products obtained by fermentation of genetically engineered strains of *Y. lipolytica* was also assessed. The absence of genotoxic potential of eicosapentaenoic acid-rich triglyceride oil and beta-carotene produced by genetically engineered *Y. lipolytica* was shown in several genotoxicity tests, and no test substance-related adverse effects were observed in a 28-day oral toxicity study performed with eicosapentaenoic acid-rich triglyceride oil and in a 90-day oral toxicity study performed with beta-carotene (Belcher et al., 2011; Grenfell-Lee et al., 2014). A recent review of the safety of *Y. lipolytica* concluded that the yeast did not exert a harmful effect in rats at dietary levels up to 30% of dried biomass for 2 years and over 3 generations, and that, in rare cases, the organism may lead to opportunistic infections in severely immunocompromised or otherwise seriously ill people. However, these infections can be effectively treated with standard antifungals or, in some cases, they resolve spontaneously (Groenewald et al., 2014). In a recent publication, microbiological data from clinical specimens collected over ten years were reviewed for growth of *Y. lipolytica*. It appeared that none of the patients who harbored *Y. lipolytica* had developed an infection despite the low immunity of most patients (Irby et al., 2014), which supports previous reports describing *Y. lipolytica* as a nonpathogenic yeast species. The high prevalence of *Y. lipolytica* reported in distal lung tissues even suggests that the yeast should be considered as normal human flora, especially of the adult respiratory tract (Irby et al., 2014). No reports exist on the production of substances by *Y. lipolytica* that are toxic in humans or animals, apart from its potential contribution to the formation of biogenic amines in cheese and meat, at concentrations not toxicologically relevant. *Y. lipolytica* was concluded to be "safe-to-use" (Groenewald et al., 2014).

Rebaudioside A is produced by a strain of *Y. lipolytica* genetically engineered to contain and express the steviol glycoside metabolic pathway, by using the technology described in the patent application WO2013/110673 filed in the name of DSM IP Assets B.V. To further support the use of rebaudioside A from *Y. lipolytica* (subsequently referred to as fermentative Reb A) as a food ingredient, the safety of this product was assessed by conducting *in vitro* genotoxicity studies and a 90-day subchronic oral toxicity study in rats. These studies are described in the present paper.

2. Materials and methods

2.1. Test article preparation

Rebaudioside A (CAS No. 58543-16-1; molar mass: 967.01 g/mol; chemical formula: $C_{44}H_{70}O_{23}$) produced by fermentation using a genetically engineered yeast, *Y. lipolytica*, was obtained from DSM Food Specialties. After separation of the biomass from the supernatant by centrifugation and heat treatment, the process involved clarification, capture of rebaudioside A by chromatography, followed by elution with alcohol, purification and drying. Purified rebaudioside A was then isolated by crystallization, dried and packaged. The production process was performed following Good Manufacturing Practices, with appropriate controls of raw materials. The final rebaudioside A from *Y. lipolytica* met the specifications as outlined in the JECFA and Food Chemical Codex. The characteristics of the fermentative Reb A batches tested in the toxicity studies are shown in Table 1.

For the genotoxicity and 90-day subchronic toxicity studies, fermentative Reb A was provided as a white powder. The rebaudioside A content of the test article was >95%. The test article was stored at room temperature, protected from light, and was considered stable under these conditions. The genotoxicity studies and 90-day subchronic toxicity study were performed by WIL Research Laboratories, LLC ('s-Hertogenbosch, The Netherlands, Ashland, Ohio, U.S.A. and Hillsborough, North Carolina, U.S.A, respectively). These studies were performed in accordance with

Table 1
Characteristics of fermentative Reb A batches 1 and 2 that were used respectively in the *in vitro* and *in vivo* toxicity studies.

Characteristic	Specification	Batch 1	Batch 2
Moisture by loss on drying (%)	≤6	Conform	Conform
Ash (%)	≤1	Conform	Conform
Solubility in water at RT (%)	>0.3	Conform	Conform
Rebaudioside A (on dry basis) (%)	≥95	Conform	Conform
Total steviol glycosides (on dry basis) (%)	>95	Conform	Conform
Residual solvents (Ethanol) (ppm)	<5000	Conform	Conform
pH	4.5–7.0	6.2	6.4
Lead (ppm)	<1	Conform	Conform
Mercury (ppm)	<1	Conform	Conform
Cadmium (ppm)	<1	Conform	Conform
Arsenic (ppm)	<1	Conform	Conform
Total plate count (CFU/g)	≤1000	Conform	Conform