Food and Chemical Toxicology 65 (2014) 1–11

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

The safety of β-carotene from *Yarrowia lipolytica*

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ARTICLE INFO

Article history:
Received 8 August 2013
Accepted 8 December 2013
Available online 16 December 2013

Keywords:
β-Carotene
Yarrowia lipolytica
Subchronic
Toxicity
Genotoxicity

ABSTRACT

Crystalline β-carotene from genetically modified *Yarrowia lipolytica* is an alternative source of β-carotene for use as a nutritional supplement. To support the use of β-carotene from *Y. lipolytica* as a food ingredient, the genotoxic and subchronic toxicity potential of this compound was determined. Genotoxicity was examined using *Salmonella typhimurium* and *Escherichia coli* (Ames test), a chromosomal aberration assay in Chinese Hamster Ovary WBL cells, and the micronucleus test in CD-1 mice. All three assays showed no significant results due to β-carotene from *Y. lipolytica*. In a subchronic toxicity study in SD rats, β-carotene from *Y. lipolytica* was administered by oral gavage for 13 weeks at 0, 125, 250 or 500 mg/kg per day. Adverse effects were not observed following clinical, clinical pathology and gross- and histopathological evaluations of dosed rats; thus, the no-observed-adverse effect level (NOAEL) for β-carotene from *Y. lipolytica* was 500 mg/kg, the highest dose used in the study. In conclusion, β-carotene derived from *Y. lipolytica* was shown in genotoxicity models and a standard rat subchronic rat study to have a safety profile similar to that of the current commercial products (synthetic and natural) with no unexpected finding attributable to the alternative source.

1. Introduction

Crystalline β-carotene produced using the genetically modified microorganism, *Yarrowia lipolytica*, is an alternative source of β-carotene for use as a nutritional supplement (as an important source of vitamin A) and, consistent with existing Generally Recognized As Safe (GRAS) affirmed food categories, as an exempt color additive (21 CFR §73.95). The main dietary sources of β-carotene are carrots, oranges, tomatoes and dark green leafy vegetables. β-Carotene from synthetic sources, from the fungal organism *Blakeslea trispora*, as well as mixed carotenes from plant and algal sources are widely used to impart yellow to orange color in foods, as a nutritional ingredient in foods and as ingredients for use in dietary supplements in order to help individuals meet the recommended dietary allowances for vitamin A as determined by the Institute of Medicine (IOM, 2001).

A number of reviews, monographs and comments on the safety of β-carotene have been published (e.g. Bauernfeind et al., 1981; Heywood et al., 1985; Rock, 1997; IARC, 1998; Omenn, 1998; Palozza, 1998; SCF, 1998; Woutersen et al., 1999). The Scientific Committee on Food (SCF) assembled the scientific data relevant to the safety of use of β-carotene from all dietary sources but limited its conclusions only to food additive uses (SCF, 2000a). As summarized in the 2000 SCF opinion, no adverse effects of high-dose oral β-carotene supplementation were observed in several standard toxicological studies in various experimental animals (rat, mice, rabbits) (IARC, 1998; Woutersen et al., 1999). These studies included acute toxicity, up to 5000 mg/kg bw/day in Sprague Dawley rats (Woutersen et al., 1999) and up to 2000 mg/kg bw/day in Wistar rats (Buser, 1992; Strobel, 1994), sub-chronic/chronic toxicity/carcinogenicity up to 1000 mg/kg bw/day for life in rats (Hummler and Buser, 1983; Heywood et al., 1985) or mice (Buser and Hummler, 1983a; Heywood et al., 1985), and teratogenicity and reproductive toxicity (up to 1000 mg/kg bw/day for 3 generations, or during days 7 to 16 of gestation, in rats; up to 400 mg/kg bw/day during days 7 to 19 of gestation in rabbits) (Komatsu, 1971, cited in Kistler, 1981; Buser and Hummler, 1982; Heywood et al., 1985; Woutersen et al., 1999). In beagle dogs (Buser and Hummler, 1983b; Heywood et al., 1985) no toxic effects were observed (up to 250 mg/kg bw/day for 2 years).

Abbreviations: ANS, Panel on Food Additives and Nutrient Sources added to Food; DMSO, dimethyl sulfoxide; EFSA, European Food Safety Authority; FDA, Food and Drug Administration; GLP, Good Laboratory Practice; GRAS, Generally Recognized As Safe; HOSO, high oleic sunflower oil; IOM, Institute of Medicine; NCE, normochromatic erythrocytes; NOAEL, no observed adverse effect level; OECD, Organization for Economic Cooperation and Development; PCE, polychromatic erythrocytes; SCF, Scientific Committee on Food; SD, standard deviation.

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http://dx.doi.org/10.1016/j.fct.2013.12.010

Y. lipolytica is an avirulent yeast species historically used for the production of citric acid, γ-decalactone and long-chain poly-unsaturated fatty acids (Groenewald et al., 2013). It is approved by the United States' Food and Drug Administration (FDA) as a secondary direct food additive in citric acid production. *Y. lipolytica* is also routinely found associated with cheeses and meats. Although in rare cases the organism may lead to opportunistic infections in severely immunocompromised or otherwise seriously ill people, these infections either resolve spontaneously or can be effectively treated with standard antifungals. Therefore, *Y. lipolytica* is deemed "safe-to-use".

Y. lipolytica has been developed as an alternative source of bio-based β-carotene that is composed predominantly of all-*trans* β-carotene with minor amounts of 9-*cis* β-carotene, 13-*cis* β-carotene, 15-*cis* β-carotene and other naturally-occurring carotenoids. To support any intended use of β-carotene from *Y. lipolytica* as a food ingredient, the safety of this product was evaluated. Toxicological studies have been conducted to assess the genotoxic potential of β-carotene from *Y. lipolytica*, and to assess its subchronic toxicity. The following standard genotoxicity assays were performed: the Ames test for assaying bacterial reverse mutation, the chromosome aberration assay in Chinese Hamster Ovary WB_L cells, and the *in vivo* mouse micronucleus assay. To investigate the subchronic toxicity of β-carotene from *Y. lipolytica*, a 13-week-oral toxicity study in rats was performed.

2. Materials and methods

2.1. Test article preparation

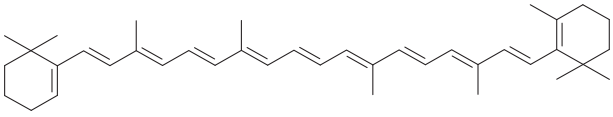
Crystalline β-carotene (CAS No. 7235-40-7;molecular weight 536.88; molecular formula: $C_{40}H_{56}$) produced by fermentation using genetically modified yeast *Y. lipolytica*, was obtained from Microbia, Inc. (Lexington, MA; currently DSM Nutritional Products). Briefly, the process involved biomass isolation from the fermentation media, washing, and physical rupturing of the cells. The β-carotene was isolated by solvent extraction, crystallized from the mother liquor, washed, dried, and packaged. The production process was controlled by Good Manufacturing Practice procedures, with appropriate hygiene controls and appropriate control of raw materials. The final crystalline β-carotene from *Y. lipolytica* met the specifications as outlined in the Food Chemicals Codex (8th ed.) (i.e., purity not less than 96%; Table 1). The specifications and test methods for β-carotene from *Y. lipolytica* were the same as those incorporated in 21 CFR §184.1245, direct food substances affirmed as Generally Recognized As Safe. β-Carotene from *Y. lipolytica* is predominantly all-*trans* β-carotene with minor amounts of 9-*cis* β-carotene, 13-*cis* β-carotene, 15-*cis* β-carotene and other carotenes. The compositional attributes are similar to those of commercial counterparts, especially synthetic β-carotene and β-carotene from the fungal organism *B. trispora* (data not shown).

Table 1
Technical and chemical description of β-carotene from *Yarrowia lipolytica*.

Chemical name: β,β-carotene; 1,18-(3,7,12,16-tetramethyl-1,3,5,7,9,11,13,15,17-octadecanonaen-1,18-diyl)-bis-(2,6,6-trimethylcyclohexene)

Common and usual name: β-carotene obtained by a fermentation process using the genetically modified yeast *Yarrowia lipolytica*

Chemical structure:



CAS No.: 7235-40-7
Molecular formula: $C_{40}H_{56}$
Molecular weight: 536.88

Physical state: Solid
Melting range: 176–182 °C, with decomposition
Color: Red to purple-violet in color
Solubility: Insoluble in water; practically insoluble in ethanol, slightly soluble in vegetable oil
Purity: Not less than 96%
$A_{455/484}$: 1.14–1.18
$A_{455/340}$: Not less than 1.5
1% solution in chloroform: clear
Loss of weight on drying: Not more than 0.2%
Lead (as Pb): Not more than 2 ppm

For the *in vivo* studies, the β-carotene was provided as a 31% wt/wt suspension in high oleic sunflower oil (HOSO) and was a red, viscous liquid. The control compound used for the *in vivo* studies was HOSO, CAS No.: 8001-21-6, also provided by Microbia, Inc.

The *in vivo* and *in vitro* genotoxicity studies and the 90-day subchronic toxicity study were performed by Nucro-Technics (Scarborough, Ontario, Canada). Experiments were performed in compliance with Good Laboratory Practice (GLP) requirements as described in the "Good Laboratory Practice for Nonclinical Laboratory Studies" of the US FDA (FDA, 2006) and "OECD Principles of Good Laboratory Practice and Compliance Monitoring" (OECD, 1998b). The use and the number of animals utilized in the *in vivo* studies were approved by the institutional Animal Care Committee of Nucro-Technics (AUP211905).

2.2. Bacterial reverse mutation assay (Ames test)

The mutagenic potential of β-carotene from *Y. lipolytica* was evaluated using the *Escherichia coli* strain WP2 *uvrA* and *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, with and without metabolic activation. The tester strains were exposed to β-carotene according to the direct plate incorporation and preincubation methods. The experimental design followed the "OECD Guideline for Testing of Chemicals – 471, Bacterial Reverse Mutation Test" (OECD, 1997a).

Bottom agar plates for *S. typhimurium* were made of minimal glucose agar that was based on a standard formula: 2% glucose, Vogel-Bonner medium E and 1.5% Bacto™ agar (Becton Dickinson Co., Sparke, USA). Bottom agar plates for *E. coli* contained 1.44% agar, 0.38% glucose, 0.24% casamino acids, 0.23 μg per mL tryptophan and 23.9% v/v Davis Mingioli Salt Solution. Top agar for the selection of *S. typhimurium* revertants was 0.6% Bacto™ agar, containing 0.5% NaCl and supplemented with histidine and biotin to 50 μM each. Top agar for the selection of *E. coli* revertants contained 0.7% agar only. Liver microsomal 9000*g* fraction from liver homogenate of male Sprague–Dawley rats treated with Aroclor 1254 was used (Moltox Inc., Boone, USA).

The solvent used to dissolve the test article was DMSO (CAS No. 67-68-5); DMSO alone was therefore used as the negative control for this assay. Positive controls for experiments without S9 were aqueous solutions of sodium azide (CAS No. 26628-22-8); and DMSO solutions of 2-nitrofluorene (CAS No. 607-57-8), methyl methanesulfonate (CAS No. 66-27-3) and 9-aminoacridine (CAS No. 52417-22-8). For experiments with S9, benzo[α]pyrene (CAS No. 50-32-8) and 2-aminoanthracene (CAS No. 613-13-8) were dissolved in DMSO; and cyclophosphamide monohydrate (CAS No. 6055-19-2) in water. All positive controls were purchased from Sigma–Aldrich Canada Ltd. (Oakville, Canada). Experiments were performed as described by Maron and Ames (1983).

β-Carotene was dissolved in DMSO and tested at concentrations of 0, 0.062, 0.19, 0.56, 1.7 and 5.0 mg per plate. The selection of doses was based on the results of a previously conducted range-finding study (data not shown). Assays were performed in two independent experiments, using identical procedures, both with and without metabolic activation. Each concentration, including the controls, was tested in triplicate. The colonies were manually counted. For a test substance to be considered positive it had to generate at least a twofold increase in the number of reversions and present a dose-dependent increase in the number of revertants.

2.3. Chromosome aberration assay in Chinese Hamster Ovary cells WB_L

The potential for β-carotene from *Y. lipolytica* to induce structural chromosome aberrations in Chinese Hamster Ovary cells WB_L was evaluated *in vitro*. The experimental design followed the "OECD Guideline for the Testing of Chemicals – 473, *In Vitro* Mammalian Chromosome Aberration Test" (OECD, 1997b).

β-Carotene was dissolved in DMSO, and WB_L cells were exposed to β-carotene, both with and without metabolic activation. The liver microsomal fractions were obtained from rats treated with phenobarbital and 5,6-benzoflavone (Moltox Inc. Boone, USA). Cultures were treated with β-carotene for 3 h with metabolic activation and for 3 or18 h without metabolic activation. The concentrations of β-carotene investigated were 0, 0.139, 0.417 and 1.25 mg per mL. Several solvents compatible with the chromosome aberration test were tested (data not shown), but none produced solutions at the highest concentration (5 mg/mL) indicated by OECD guidelines. Therefore, the highest used dose (1.25 mg/mL) was selected based on the maximum solubility of the test article in accordance with such guidelines (OECD, 1997b). Duplicate flasks were used for each dose level.

Cells were cultured in McCoy's 5A Modified Medium with 2 mM L-glutamine and 25 mM HEPES supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Co., Burlington, Canada). Incubation was performed in a humidified tissue culture incubator at 37 ± 2 °C and 5 ± 2% CO_2. On the day before the experiment, 1×10^5 cells were seeded into each T-25 cm^2 Falcon flask. The cultures were incubated overnight. Fifty μL of a dosing solution was added to each culture for all exposure conditions. DMSO was used as negative control. Mitomycin C (CAS No. 50-07-7) was used as a positive control (for cultures not treated with S9) at 1.0 μg/mL for