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Genotoxicity, acute and subchronic toxicity evaluation of savory food ingredients

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ABSTRACT

The potential toxicity of two savory food ingredients produced by fermentation of enzymatically hydrolyzed corn starch (Savory Base 100 and Savory Base 200) was evaluated individually in a bacterial reverse mutation assay, an *in vitro* mammalian cell gene mutation assay, an acute oral study and as a mixture in a 90-day dietary study. In the bacterial reverse mutation and *in vitro* mammalian cell gene mutation assays, neither ingredient was mutagenic at concentrations up to 5000 μg/plate and 5000 μg/mL, respectively in the presence and absence of metabolic activation. In the acute study, the no-observed-adverse-effect level (NOAEL) for each Savory Base 100 and Savory Base 200 in male and female rats was 2000 mg/kg body weight. In the 90-day study, the hematology and clinical chemistry findings and histopathological changes noted in the liver, heart and kidneys were deemed to be of no toxicological significance, as the mean values were within the historical control range, were not dose-dependent, occurred at a similar frequency in control groups, or only occurred in the control group. Considering these findings, the NOAEL for Savory Base 100 and Savory Base 200 was 2333 and 1167 mg/kg body weight, respectively, the highest dose tested in each case.

1. Introduction

Corynebacterium glutamicum, a Gram-positive bacterium, has been widely used for industrial production of amino acids, such as L-glutamic acid (Kinoshita et al., 2004), L-lysine (Krömer et al., 2004), L-ornithine (Hwang et al., 2008; Schneider et al., 2011) and L-threonine (Dong et al., 2011). Industrial production of purine nucleotides was reported to mainly involve microbial fermentation using microorganisms such as *Bacillus subtilis* (Demain et al., 1966) or *Corynebacterium ammoniagenes* (Furuya et al., 1968, 1970; Mori et al., 1997; Abbouni et al., 2004).

This manuscript details studies evaluating the genotoxicity and oral toxicity of two savory ingredients containing a specific intrinsic mix of various compounds, including amino acids, organic acids, Maillard reaction products and minerals and their salts. These ingredients contain a mixture of L-alanine, formic acid, glutamic acid and succinic acid (Savory Base 100) or formic acid, glycine and inosine 5'-monophosphate (IMP) (Savory Base 200) as marker substances contributing to the good fermentation quality, intended to improve the flavor of culinary products. Savory Base 100 and Savory Base 200 are produced by fermentation of enzymatically

Abbreviations: 2-AA, 2-aminoanthracene; 9-AA, 9-aminoacridine; A/G, albumin/globulin; APTT, activated partial thromboplastin time; bw, body weight; DMBA, 7,12-dimethyl benzanthracene; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethyl sulfoxide; EFSA, European Food Safety Authority; EMS, ethylmethane sulphonate; GLP, good laboratory practice; IMP, inosine-5'-monophosphate; MMS, methylmethanesulfonate; MSG, monosodium glutamate; NaN_3, sodium azide; NOAEL, no-observed-adverse-effect level; NPD, 4-nitro-1,2-phenylene-diamine; OECD, Organisation for the Economic and Cooperative Development; QPS, qualified presumption of safety; RBC, red blood cells; RCDW, red cell distribution width.

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hydrolyzed corn starch substrates using non genetically-modified proprietary strains of *Corynebacterium* sp. (*Corynebacterium glutamicum* ATCC 13032 and *Corynebacterium ammoniagenes* ATCC6872, respectively). The fermentation microorganisms are removed during the production process, and therefore, are not present in the finished ingredients. *Corynebacterium* sp. have a long history of safe use in food production, including preparation of fermented maize, sorghum, millet, African oil bean seed, rice, soybean and cassava (Caplice and Fitzgerald, 1999; Osungbaro, 2009; FAO, 2010), and in the manufacture of amino acids (Kinoshita et al., 2004; Krömer et al., 2004; Hwang et al., 2008; Dong et al., 2011). *Corynebacterium glutamicum* has been listed on the European Food Safety Authority (EFSA)'s qualified presumption of safety (QPS) list, indicating that there are no concerns for safety when the species is used for amino acid production purposes (EFSA, 2014). The ingredients are compositionally comparable to the commercially available yeast extracts that are high in both glutamic acid and IMP (Dermiki et al., 2013), and have a long-history of safe use in foods in the United States and European Union for their savory flavoring and flavor balancing attributes.

The non-essential amino acid, L-glutamic acid and its salt glutamate, are naturally occurring substances present in unprocessed or processed plant and animal-based products that are commonly consumed by humans, such as tomato, seaweed, seafood, meat, poultry, cheese, fish sauce, soy sauce, *etc.* (Kurihara, 2015). Despite their natural presence in food, glutamic acid salts are frequently added to processed food as a flavor enhancer. Monosodium glutamate (MSG), the salt form of L-glutamic acid, is the prototypical stimulus for the purported fifth basic taste "umami", further described as a "savory" or meat/broth-like taste, which has been used for over a century to enhance the savory flavor of various foods (Chaudhari et al., 2009; Jinap and Hajeb, 2010).

Ribonucleotides, more specifically, the purine nucleotide IMP, belong to another group of naturally-occurring substances that contribute to the umami taste in many food products (Halpern, 2000). While glutamate is naturally present in both plant and animal foodstuffs, IMP is mainly present in animal products, such as dried sardine, bonito, tuna, pork and chicken (Kurihara, 2015). Many amino acids, when present in proteins are tasteless, and while free amino acids do not have a strong taste, their taste is intensified in the presence of other compounds, such as nucleotides (Halpern, 2000; Chaudhari et al., 2009; Yamamoto and Ishimaru, 2013) or inorganic salts (Fuke and Konosu, 1991; Ugawa and Kurihara, 1993). When MSG is combined with IMP, they act synergistically to further increase the taste of umami (Kawai et al., 2002; Chaudhari et al., 2009; Yamamoto and Ishimaru, 2013). The synergism between glutamate and the 5′-ribonucleotides varies greatly with species of animals, with that being largest in dogs and humans, as compared to rodents (Kurihara, 2015).

L-alanine and glycine are non-essential amino acids that are natural constituents of proteins in plants and animals (IOM, 2005; Burdock, 2009). Large amounts of free amino acids such as L-alanine, glutamic acid and glycine account for the characteristic taste of hoshi-nori and edamame, which is favored by many Japanese consumers (Ragan and Bird, 1987; Johnson et al., 1999; FAO, 2003). Mixtures of amino acids such as glycine, alanine and arginine, in addition to umami flavoring ingredients and salts, are used to mimic the taste of crabmeat (Kurihara, 2009). When the level of glycine in a food substance is increased, it can elicit different seafood flavors, such as scallops (Kurihara, 2009).

Formic acid is a natural constituent of many foods consumed by humans, such as apple, papaya, pear, raspberry, strawberry, cheeses, breads, yogurt, milk, cream and fish (Burdock, 2009). It is also a metabolite in intermediary metabolism and a precursor in the biosynthesis of several body constituents (FASEB, 1976). Succinic acid, an intermediate metabolite of the tricarboxylic acid cycle and an end-product of aerobic and anaerobic metabolism (Song and Lee, 2006), can be produced from yeast fermentation in the processing of sake and wine (Arikawa et al., 1999; Song and Lee, 2006). Succinic acid was isolated from short-necked clam and hard clam, and was confirmed to be critical to the characteristic flavor of these shellfish (Fuke and Konosu, 1991).

In the following series of studies, the genotoxicity of Savory Base 100 and Savory Base 200 was assessed using both bacterial reverse mutation and *in vitro* mammalian cell gene mutation assays. A single oral dose study was conducted to assess the acute toxicity of each of the ingredients, as well as a 90-day feeding study evaluating the subchronic toxicity of a Savory Base 100/Savory Base 200 mixture in the diet of rats.

2. Materials and methods

2.1. Materials

The test articles used in the genotoxicity and acute toxicity studies were Savory Base 100 and Savory Base 200 as provided by Nestec Ltd. (Nestlé Research Center, Lausanne, Switzerland). The test articles were supplied as a light brown, hygroscopic powder and stored at room temperature and protected from light and humidity. Product compositions for Savory Base 100 and Savory Base 200 are presented in Table 1. Two lots of Savory Base 100 (Batch No. DA8-00028) and Savory Base 200 (Batch No. DA8-00029), meeting product specifications, were used for the studies. Savory Base 100 and Savory Base 200 preparations were considered stable for a period of 1 year under typical storage conditions. A 2:1 mixture of Savory Base 100 and Savory Base 200 was provided as ready-to-use formulations by Nestlé Research Center (Lausanne, Switzerland) for use in the 90-day dietary study.

As previously indicated, Savory Base 100 and Savory Base 200 contain a mixture of several marker substances, including L-alanine, formic acid, glutamic acid and succinic acid (Savory Base 100) or formic acid, glycine and IMP (Savory Base 200) that along with other components listed in Table 1 contribute to the overall savory taste of these ingredients. More specifically, the Savory Base 100/Savory Base 200 mixture contained 37.8 ± 0.2% glutamic acid and 14.5 ± 0.4% IMP. These markers were used to analyze stability and consumption of the test products through the rat diet.

2.2. Genotoxicity studies

2.2.1. Bacterial reverse mutation assay (Ames test)

The bacterial reverse mutation assay was performed in accordance with Organisation for the Economic and Cooperative Development (OECD) Test Guideline No. 471 (OECD, 1997), US EPA Health Effects Test Guidelines (OPPTS 870−5100 and EPA 712-C-98-247) (US EPA, 1996; 1998), and ICH Guidance S2A and S2B (ICH, 1995, 1997), and OECD Good Laboratory Practice (GLP) (OECD, 1998). The test was conducted using the plate incorporation and pre-incubation methods with *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537, and *Escherichia coli* WP2*uvr*A in the presence and absence of metabolic activation. All test strains were obtained from Molecular Toxicology Inc. (Boone, USA). Metabolic activation was achieved by incubation with the S9 microsomal fraction obtained from phenobarbital- and β-naphthoflavone-induced rat liver (Trinova Biochem GmbH, Giessen, Germany). Distilled water (Phoenix Pharma Co., Hungary) was used as the negative vehicle control, while dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Taufkirchen, Germany) was used as the negative solvent control. The following compounds were used as positive controls in the absence of metabolic activation: 4 μg/plate 4-nitro-1,2-