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Toxicological evaluation of neoagarooligosaccharides prepared by enzymatic hydrolysis of agar

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A B S T R A C T

Agar, a heterogeneous polymer of galactose, is the main component of the cell wall of marine red algae. It is well established as a safe, non-digestible carbohydrate in Oriental countries. Although neoagarooligosaccharides (NAOs) prepared by the hydrolysis of agar by β-agarase have been reported to exert various biological activities, the safety of these compounds has not been reported to date. For safety evaluation, NAOs containing mainly neoagarotetraose and neoagarohexaose were prepared from agar by enzymatic hydrolysis using β-agarase DagA from *Streptomyces coelicolor*. Genotoxicity tests such as the bacterial reverse mutation assay, eukaryotic chromosome aberration assay, and *in vivo* micronucleus assay all indicated that NAOs did not exert any mutational effects. The toxicity of NAOs in rat and beagle dog models was investigated by acute, 14-day, and 91-day repeated oral dose toxicity tests. The results showed that NAO intake of up to 5,000 mg/kg body weight resulted in no significant changes in body weight, food intake, water consumption, hematologic and blood biochemistry parameters, organ weight, or clinical symptoms. Collectively, a no-observed-adverse-effect level of 5,000 mg/kg body weight/day for both male and female rats was established for NAO. These findings support the safety of NAO for possible use in food supplements and pharmaceutical and cosmetic products.

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1. Introduction

Agar is the predominant polysaccharide component of the cell wall of marine red algae; it represents up to 60% of the dry weight of algal biomass (Fu and Kim, 2010) and comprises a family of galactans with a hybrid structure that consists of alternating 3-O-linked β-D-galactose (G) and 4-O-linked α-L-galactose (L). According to Knutsen et al. (1994), agar can be classified into agarose and porphyran. Agarose is composed of a repeating moiety of agarobiose (G-LA), with a low level of modifications, in which the L monomer is replaced with 3,6-anhydro-α-L-galactose (LA). Porphyran is composed of a repeating moiety of porphyrobiose (G-L), with many modifications, such as a high degree of 6-O-sulfation in the L-galactose units and of 6-O-methylation in the D-galactose units (Giordano et al., 2006).

Agar is well established as a "generally recognized as safe" (GRAS) food additive in the United States (https://www.fda.gov/food/ingredientspackaginglabeling/gras/scogs/ucm260847.htm), commonly found in gelling agents, diet foods, icings, glazes, processed cheese, jelly sweets, and marshmallows. It has also been used in microbiological media for agarose gel electrophoresis and in various chromatographic techniques. Recently, several biological functions of oligosaccharides prepared from agar have been reported (Chi et al., 2012). For example, agarooligosaccharides (AOs) with LA at the reducing ends, produced by hydrolysis of the α-1,3 glycosidic bonds of agar, were reported to have antitumor-promoting activities against mouse skin carcinogenesis (Enoki et al., 2012), antioxidant activity, and hepatoprotective potential (Chen et al., 2006). Neoagarooligosaccharides (NAOs, Fig. 1) with G at the reducing ends (Sugano et al., 1993), produced by hydrolysis of the β-1,4 glycosidic bonds of agar, were reported to inhibit the growth of bacteria, inhibit the degradation of starch, and function as low-calorie additives for improving food quality (Giordano et al., 2006). These NAOs also exhibited moisturizing and whitening effects in melanoma cells (Kobayashi et al., 1997; Ohta et al., 2004; Hong et al., 2017a). Therefore, (N)AOs have potential applications

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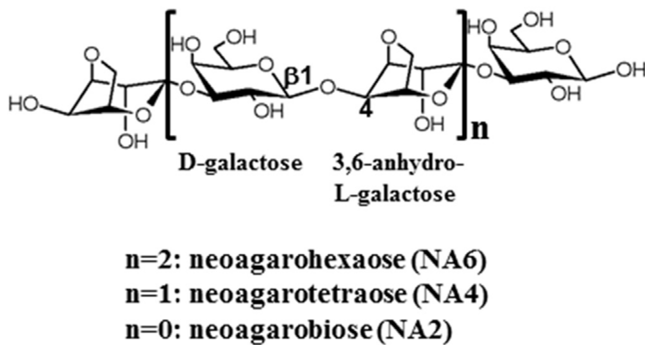

Fig. 1. The chemical structures of neoagarooligosaccharides. Neoagarobiose, α-L-3,6-anhydro-L-galactosyl-(1 → 3)-β-D-galactopyranose, is the basic unit of neoagarooligosaccharides.

in the food, pharmaceutical, and cosmetic industries.

As humans do not express genes coding for α/β-agarases, neither agar nor NAOs can be digested in the gastrointestinal tract. Instead, prolonged exposure to agar may lead to its fermentation by the microbiome in the large intestine. The hydrolyzed end product of agar, D-galactose, is readily metabolized by microbial and human enzymes, but 3,6-anhydro-L-galactose requires further metabolism by two additional enzymes before being incorporated into the central metabolic pathway (Lee et al., 2016). Therefore, NAOs cannot be metabolized without appropriate microbial activity in the human gastrointestinal tract, and in the intact form, NAOs appear to exhibit various biological activities *in vivo*.

Previously, we reported that β-agarase DagA from *Streptomyces coelicolor* A3(2) was an endo-type β-agarase that effectively degraded agar into neoagarotetraose (NA4) and neoagarohexaose (NA6) (Temuujin et al., 2011). Recently, we also reported that the intake of NAOs exerted statistically significant anti-obesity and antidiabetic effects in high-fat diet-induced obese mice (Hong et al., 2017b). Because of the importance of their biological functions, we tested the toxicity of NAOs prepared from agar by DagA hydrolysis using the bacterial reverse mutation assay, the chromosome aberration assay, the *in vivo* mouse micronucleus assay, and *in vivo* acute- and subchronic-oral toxicity assays in rat and beagle dog models. To the best of our knowledge, this is the first report on the *in vivo* and *in vitro* toxicity evaluation of NAOs.

2. Materials and methods

2.1. Experimental conditions

These studies were performed in accordance with the guidelines established by Good Laboratory Practice (2015-82, Ministry of Food and Drug Safety (MFDS), Republic of Korea, November 11, 2015) and the OECD (Organization for Economic Co-operation and Development) Principles of Good Laboratory Practice (1997), where applicable. All mutagenicity studies and oral dose toxicity tests in animals were conducted at the Nonclinical Research Institute, ChemOn Inc. (Republic of Korea). The use of experimental animals was approved by the Institutional Animal Care and Use Committee (IACUC) of the Nonclinical Research Institute, ChemOn Inc., which has been accredited by the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International, 2010) with the following permission numbers: 13-R279, 13-R321, 13-M351, 15-D361, and 16-R222.

2.2. Test materials and formulation

The recombinant plasmid pUWL201-DagA containing the *dagA* gene from *S. coelicolor* A3(2) on an *Escherichia-Streptomyces* shuttle vector, pUWL201PW, was used for overexpression of β-agarase (DagA) in *Streptomyces lividans* TK24 (Temuujin et al., 2011). The recombinant β-agarase (DagA) prepared from *S. lividans* TK24/pUWL201-DagA was used for the preparation of NAOs by agar hydrolysis, as previously described (Hong et al., 2017b). The reactant containing NAOs was then further purified by sequential filtration through Whatman filter paper Grade 2 (GE Healthcare, Chicago, IL, USA) and Labscale TFF (tangential flow filtration) system (5 kDa cut-off) (Millipore, Billerica, MA, USA). The final filtrate was completely lyophilized and the NAO powder was stored at −20 °C in the dark and resuspended in sterile distilled water (DW) before use. The precise composition of the NAO powder was analyzed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), as previously described (Park et al., 2014; Temuujin et al., 2011).

2.3. Mutagenicity studies

2.3.1. Bacterial reverse mutation test (Ames test)

The bacterial reverse mutation assay was performed using the plate-incorporation and pre-incubation methods in the presence and absence of S9 metabolic activation (Maron and Ames, 1983; Green, 1984). The histidine-auxotrophic mutant strains of *Salmonella typhimurium,* TA100, TA1535, TA98 and TA1537, and the tryptophan-auxotroph of *Escherichia coli* WP2 *uvrA* were purchased from Molecular Toxicology Inc. (Boone, NC, USA). Oxoid Nutrient Broth No.2 liquid medium was used for precultivation of the strain. The positive controls (2-aminoanthracene, sodium azide, benzo[α]pyrene, acridine mutagen ICR 191, 4-nitroquinoline *N*-oxide, and 2-nitrofluorene) were dissolved in DMSO. For each treatment, 0.1 mL of NAOs in sterile DW or control solution was added into a sterilized test tube and 0.1 mL of bacterial suspension was added. For the preparations with S9 (Molecular Toxicology Inc., Boone, NC, USA), 0.5 mL S9 mix was also added; for preparations without S9, 0.5 mL 0.1 M sodium phosphate buffer solution was added. The mixtures were then incubated with gentle shaking (120 rpm) for 20 min at 37 °C. After incubation, 4 mL of top agar (0.6% Bacto-Difco agar + 0.5% NaCl, 45 °C) was added and then all substances were spread evenly on minimal glucose agar plates. For *S. typhimurium* strains, the minimal glucose agar medium contained Vogel-Bonner medium E, 2% glucose, and 1.5% Bacto agar (Difco Laboratories Inc., Detroit, MI, USA), and 0.5 mM histidine/biotin was supplemented to the top agar. For *Escherichia* strain, the minimal glucose agar medium supplemented with 0.25 mL of 0.1% tryptophan solution per liter was used. After the top agar solidified, the plates were inverted and incubated for 48 h at 37 °C. The numbers of revertant colonies were counted by either an automatic colony analyzer or manual counting. The test substance was judged positive for mutagenicity when (1) substances induced a dose-dependent increase in the number of revertant colonies to a level greater than two-fold of the negative control value and (2) the dose-dependent increase was reproducible.

2.3.2. In vitro mammalian cell gene mutation assay in Chinese Hamster Lung cell lines

This experiment was carried out in triplicate to determine the effects of NAOs at concentrations of 0, 1,250, 2,500 and 5,000 μg/mL on the induction of chromosomal aberrations in Chinese Hamster Lung (CHL) cells. CHL fibroblast-derived cell lines (CHL/IU) were purchased from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in reconstituted minimum essential

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