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Preparation, characterization and alcoholic liver injury protective effects of algal oligosaccharides from *Gracilaria lemaneiformis*

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ABSTRACT

Oligosaccharides derived from seaweeds possess diverse biological functions. However, little is known about their effects on liver damage. In this study, algal oligosaccharides (AOS) were prepared from *Gracilaria lemaneiformis* by biodegradation approaches. HPLC analysis showed AOS were composed of agarooligosaccharides and neoagarooligosaccharides. *In vivo* animal studies showed AOS significantly attenuated alcohol-induced hepatopathy in mice to some extent, as revealed by the normalization of several serum liver-damage markers. Besides, AOS increased antioxidant levels of hepatic glutathione (GSH) and superoxide dismutase (SOD), and ameliorated the elevated formation of malonaldehyde (MDA), suggesting AOS attenuated hepatopathy mainly through their antioxidant activities. Interestingly, AOS could also enhance the activities of hepatic alcohol dehydrogenase (ADH). Histological examination of liver tissues showed AOS reduced the alcohol-induced liver injury in a dose-dependent manner. Moreover, the comparison of different administration strategies suggested AOS were best taken 2 h before alcohol consumption. Therefore, our study provided a novel nutritional strategy for reducing alcohol-induced hepatotoxicity.

1. Introduction

Gracilaria lemaneiformis, one of the marine red algae, is a valuable resource for foods, feeds and medicines, because of its high yields and commercially valuable extracts (Mei-zhen, Hao, Yang, & Liao, 2010; Qi et al., 2010). It contains abundant polysaccharides, proteins, dietary fibers, vitamins and minerals. Agar, a major cell wall component of red algae, is widely used as gelling agents and food additives for more than a thousand years in China and Japan according to the historic records, due to its favorable characteristics of thermo-reversible aqueous gelation (Fu & Kim, 2010). In recent years, algal oligosaccharides (AOS) especially agaro-oligosaccharides that derive from agar, have been reported to possess diverse physiological and biological functions, such as anti-oxidation, immune modulation, antitumor, whitening and skin-moisturizing effects, which make AOS attractive in food, cosmetic and pharmaceutical industries (Enoki et al., 2010; Kim et al., 2010; Liu, Mei, Yi, Chen, & Ying, 2008). AOS are conventionally prepared by acid hydrolysis of agars, but this method produces substantial pollution and wastes. However, novel strategies, such as biodegradation, could simplify the oligosaccharides production process in an environment-friendly way, and increase the recovery of oligosaccharides with low degrees of polymerization (DP), making them promising approaches for rapid AOS production (X. Chen, Hou, Jin, Zeng, & Lin, 2016; Hou, Chen, Chan, & Zeng, 2015).

Alcohol abuse is one of the major risk factors for chronic disease worldwide, as it cause 3.8% of all deaths (Rehm et al., 2009). Alcohol consumption is also a major cause of liver disease as it could trigger serious liver injuries, and thus lead to liver fibrosis and cirrhosis. According to the reports of US National Institute on Alcohol Abuse and Alcoholism, alcohol-induced liver disease is responsible for up to 48% of cirrhosis-related deaths in the USA, and is also major cause for liver injury-associated mortality in other countries (Gao & Bataller, 2011; Paula et al., 2010). Oxidative stress has been implicated in the pathogenesis of alcohol-induced liver diseases, since acute and chronic ethanol treatment has been proved to increase reactive oxygen species (ROS) production, reduce the contents of cellular antioxidant, and enhance oxidative stress in liver (Dey & Cederbaum, 2006). Therefore, developing of antioxidant drugs may be a promising approach to treat and protect liver injuries and liver diseases.

To this date, several polysaccharides and oligosaccharides from a variety of sources have been shown to exhibit antioxidative activities and diverse biological functions (Kang, Yong, Ma'aruf,

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Osman, & Nazaruddin, 2014; Sansone, Sansone, Shiga, & do Nascimento, J. R. O., 2016; Yao, Luo, & Li, 2015; Yao, Xue, Zhu, Gao, & Ren, 2015), but only a few have been evaluated for their hepatoprotective effects. For example, raffinose family oligosaccharides isolated from *Rehmannia glutinosa* Libosch were found to protect mice from chronic carbon tetrachloride (CCl_4) -induced injuries (Zhang, Zhao, Sun, Lu, & Yang, 2013). Chitosan, a cationic polysaccharide prepared from alkaline *N*-deacetylation of chitin, displayed antioxidative effects on CCl_4-caused hepatic injuries in mice (Jeon et al., 2003). However, to our best knowledge, all of the studies were performed over the CCl4-induced liver injury model, and no hepatoprotective effects of oligosaccharides or polysaccharides, especially oligosaccharides derived from algae, on alcohol-induced liver injuries are reported.

In this study, AOS were prepared by biodegradation of *Gracilaria lemaneiformis* with a deep-sea agarolytic bacterium *Flammeovirga pacifica* WPAGA1(Xu et al., 2012). After purification and identification, the AOS were further characterized for their hepatoprotective effects against acute alcoholic liver injuries in mice.

2. Materials and methods

2.1. Preparation of crude AOS from Gracilaria lemaneiformis

The AOS were prepared by biodegradation of *Gracilaria lemaneiformis* with a deep-sea agarolytic bacterium *Flammeovirga pacifica* WPAGA1 (Xu et al., 2012). *Gracilaria lemaneiformis* was bought from local aquaculture market, and was thoroughly oven-dried and pulverized into powder. Fresh single colony of WPAGA1 strain was inoculated into 5 mL 2216E medium (1% tryptone and 0.2% yeast extract in seawater), and was cultured at 28 °C with shaking (200 rpm), until the OD_{600} of the cultures reached 0.4–0.5. Then the culture was diluted 100-fold with 50 mL fresh carboxymethylcellulose sodium (CMC) liquid medium (1% CMC, 0.2% yeast extract and 0.1% agar in seawater), and subsequently subjected to shaking at 200 rpm at 28 °C for another 14 h. For oligosaccharides production, the domesticated WPAGA1 cells were inoculated into degradation medium (3% *Gracilaria lemaneiformis* powder in seawater) with 50–100 folds dilution rate, and were subjected to shaking at 200 rpm at 37 °C for 42 h. Then the culture supernatant containing released oligosaccharides was collected after centrifugation at 12,000*g* at 4 °C for 20 min, and was filtered through 3 kD filter to remove water-soluble macromolecules including polysaccharides and proteins. The resulting sample was mixed with threefold volume of absolute ethanol, and was kept at 4 °C overnight. After centrifugation at 12,000*g* at 4 °C for 10 min, the supernatant was collected and lyophilized as crude AOS.

2.2. Purification of AOS

The crude AOS were purified with the combined separation of a diethylaminoethyl (DEAE) -cellulose 52 column (Whatman, General Electric, USA) and a Bio-gel P2 column (Bio-Rad, USA). 10 mL crude AOS was first loaded onto the DEAE-cellulose 52 column (5.0 i.d. × 100 cm), followed by the wash and elution with 2 M NaCl at 10 °C with the flow rate of 0.8 mL/min. Fractions were collected at the time intervals of 2 min, and the oligosaccharides contents were measured based on the phenol-sulfuric acid method at 490 nm (Taylor, 1995). Oligosaccharides-containing fractions were then combined and concentrated, followed by the second separation over the Bio-gel P2 column (5.0 i.d. × 100 cm). After the load of samples, the column was washed and eluted with 0.4%NH_4HCO_3. The flow rate was 1.0 mL/min, and the fractions were collected and analyzed as described above. Fractions corresponding to the peaks of oligosaccharides were collected and lyophilized as pure AOS for further composition analysis and *in vivo* bioactivity assay.

2.3. Ion exchange chromatography analysis of AOS

The identification of AOS components was performed using an anion exchange chromatograph system (DIONEX, Sunnyvale, CA, USA) equipped with a 250 × 4-mm IonPac column (ASII-HC). The pure neoagarobiose (NA2), neoagarotetraose (NA4), neoagarohexaose (NA6), neoagarooctaose (NA8), agarotriose (AG3), agaropentose (AG5) obtained from Marineoligo, China were used as standards, and were assayed under the same conditions as those of pure AOS samples. After the sample was loaded, the column was washed for 50 min at a flow rate of 0.25 mL/min with mobile phase (100 mM NaOH, 50 Mm NaAc). The eluents were detected with electrical conductivity, and were identified by comparing the peak retention times with those of standards.

2.4. Animals and experimental design

Kunming mice (male, specific pathogen free, weight 18 ± 2 g, 4 weeks old) were purchased from SLAC Laboratory Animal Company (Shanghai, China). All the animal experiments were performed in Xiamen University Laboratory Animal Center (Xiamen, China), and were approved by the Animal Care and Use Committee of Xiamen University (SYXK 2013-0006). Mice were housed under standard conditions at a constant temperature of 22 °C and a humidity of 50%, and exposed to a 12/12 h light-dark cycle. Mice were fed on standard diet, and were allowed free access to drinking waters.

In order to elucidate the protective effects of AOS on acute alcoholic liver injury in mice, and to compare the efficiency of different AOS administration manners, four AOS administration manners were employed (Fig. 2). After environmental adaptation for 1 week, mice were randomly divided into four groups (30 mice in each group), and were treated with four manners. For all manners, mice were randomly divided into five subgroups (normal, water, AOS-50 mg/kg, AOS-150 mg/kg and AOS-250 mg/kg) of 6 animals each. Mice in normal subgroup were only administrated with distilled water (0.1 mL/10 g, ig). Mice in other subgroups were treated as follows:

Manner 1 (M1): Mice were intragastrically administrated with distilled water as negative controls (0.1 mL/10 g) or pure AOS at various dosages (50 mg/kg, 150 mg/kg and 250 mg/kg), followed by administration of alcohol (0.1 mL/10 g, ig) 2 h later. Administrations were conducted once daily for 3 consecutive weeks.

Manner 2 (M2): Mice received distilled water as negative controls (0.1 mL/10 g, ig) or pure AOS at various dosages (50 mg/kg, 150 mg/kg and 250 mg/kg, ig) once daily for 2 consecutive weeks, followed by administration with alcohol (0.1 mL/10 g, ig) once daily for 3 consecutive weeks.

Manner 3 (M3): Mice were intragastrically administrated with distilled water as negative controls (0.1 mL/10 g) or pure AOS at various dosages (50 mg/kg, 150 mg/kg and 250 mg/kg), and then administrated with alcohol (0.1 mL/10 g, ig) immediately. Administrations were conducted once daily for 3 consecutive weeks.

Manner 4 (M4): Mice were intragastrically administrated with alcohol (0.1 mL/10 g) once daily for 3 consecutive weeks, followed by administration with distilled water (0.1 mL/10 g, ig) or pure AOS at various dosages (50 mg/kg, 150 mg/kg and 250 mg/kg, ig) once daily for 2 consecutive weeks.

On completion of the experiment, all mice were fasted for 12 h, and were sacrificed by cervical dislocation after anaesthetized with pentobarbital sodium. The blood was collected from abdominal aorta, and serum was prepared from blood by centrifugation at 1000 g for 15 min at 4 °C, and was then stored at 4 °C until use. Mice liver was excised immediately, and was frozen at − 80 °C for further analysis after several washes with physiological saline.