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Preparation of bioactive neoagaroligosaccharides through hydrolysis of *Gracilaria lemaneiformis* agar: A comparative study

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ABSTRACT

Hydrolysis of *Gracilaria lemaneiformis* agar by β-agarase was compared with HCl hydrolysis. The results showed that optimum catalysis conditions for the β-agarase were pH 7.0 at 45 °C. Mass spectroscopy, thin-layer chromatography and GPC results showed that the polymerization degrees of the hydrolysis products by the β-agarase were mainly four, six and eight (more specific than the hydrolysate by HCl). The enzymatic degradation products of agar were distinctly different from those of HCl hydrolysis in the ratios among galactose and 3,6-anhydro-galactose and sulfate group contents. The NMR spectrometry proved that the products of β-agarase were neoagaroligosaccharides, which was not found in the agarolytic products by HCl. The neoagarotetraose inhibited tyrosinase activity competitively with the K_I value of 16.0 mg/ml. Hydroxyl radical-scavenging ability of neoagaroligosaccharides was much greater than that of agar HCl hydrolysate. This work suggests that neoagaroligosaccharide products produced by our β-agarase could be more effective in function than products from acid hydrolysis.

1. Introduction

Agar is the main matrix component of cell walls of some red algae, applicable in many aspects of the food industry. It is a polygalactan chain composed of 1, 3-linked β-D-galactopyranose and 1,4-linked 3,6-anhydro-L-galactose with some ester sulfate, pyruvic acid acetal or methyl groups (Kang, Ghani, Hassan, Rahmati, & Ramli, 2014). One of the most important applications of agar is the preparation of functional agaroligosaccharides (AOS) or neoagaroligosacchrides (NAOS) through hydrolysis. AOS present excellent prebiotic effect on intestinal microbial flora (Marinho-Soriano & Bourret, 2005) and also could be developed as an edible preservation coat for fruits (Hou, Gao, Gu, Wang, & Zeng, 2015). NAOS could decrease starch degradation rate in the human body for restricting caloric intake as non-digestible food oligosaccharides (Weinberger, 2001) and also stimulate immune function with low cell toxicity (Chen, Yan, Zhu, & Lin, 2006). Moreover, NAOS present inhibitory effects on tyrosinase activity and melanoma cell proliferation, which might facilitate control of the level of melanin for well-being of the human body (Jang et al., 2009).

To prepare AOS or NAOS, there are two agar hydrolysis approaches, including acid hydrolysis and agarase degradation. Acid hydrolysis of agar is convenient for preparation of agaroligosaccharides due to the low cost of chemical agents. However, the type of oligosaccharide products is uncontrollable and their structures are easily destroyed (Lee, Myong, & Lee, 2012). Compared with acid hydrolysis, agarase degradation is more appropriate. The enzymatic method could avoid damage of the structure of pyranose units of agar and polymerization degrees of enzymatic products are limited in a fixed range (Lee, Krishnanchettiar, Lateef, & Gupta, 2005). According to action mode, there are two types of agarases, including α-agarase catalyzing the α-(1→3) linkage disruption to generate AOS, and β-agarase producing NAOS by β-(1→4) linkage split (Lee et al., 2006). To date, most agarases have been identified to be β-agarases and classified into several glycoside hydrolase families, most of which are isolated from agarolytic bacteria in aquatic environments. The NAOS generated by β-agarase, has an even number of galactose units (Flament et al., 2007; Kim et al., 2010). Types of agarolytic products from various β-agarases might be different in polymerization degree with different physiological functions of NAOS (Kang et al., 2014; Weinberger, 2001). Despite described methods of agar hydrolysis, a comparison of products derived from acid and enzyme hydrolysis is still lacking (Chi, Chang, & Hong, 2012; Tran et al., 2016).

Abbreviations: AOS, agaroligosaccharides; NAOS, neoagaroligosaccharides; Gnr, galactose at non-reducing end; Arα, α-anomeric forms of galactose unit at reducing end; Grβ, β-anomeric forms of galactose unit at reducing end; Anr, 3,6-anhydro-galactose at non-reducing end; NA 2, 4, 6 and 8, neoagaro-biose, -tetraose, -hexaose and –octaose; GPC, gel permeation chromatography.

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In this study, agar is extracted from *Gracilaria lemaneiformis* for preparation of NAOS. A potent β-agarase with high specific activity was prepared through heterogeneous expression in this study. In particular, enzymatic hydrolysis of the agar by the β-agarase was compared with acid hydrolysis in terms of product type and antioxidation. The agaroligosaccharide products were then separated and prepared for NMR, mass spectrometry and bioactivity investigation in detail.

2. Materials and methods

2.1. Materials and reagents

Gracilaria lemaneiformis was planted in Fujian Province, China. Agar was extracted from *G. lemaneiformis,* using a hot-water extraction method (Sousa, Borges, Silva, & Goncalves, 2013). The gene of the enzyme, BN3 β-agarase, was amplified from a *Micrococcaceae*. sp. strain (screened out by our lab) with specific primers (Table S1). The gene fragment was digested by restrict nuclease NotI and XhoI and then ligated to pPIC9k vector with T4 ligase (Takara product). The construct was transferred into *Pichia pastoris* GS115 by electrotransformation after linearization by nuclease bglII. To express the BN3 β-agarase, the recombinant *Pichia pastoris* GS115 strain was cultivated as reference (Yu et al., 2015).

Other chemicals, tyrosinase and L-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma-Aldrich. LH-20 gel was a product of Amersham. Other chemical reagents were all of analytical grade.

2.2. Agarase hydrolysis of G. lemaneiformis agar

The β-agarase was characterized by using modified methods from Lee et al. (2006), including specific activity, optimum catalysis temperature and pH, stability under various pH and temperatures. The activity of the β-agarase was determined, using pure agar (from Sigma Aldrich) as substrate. One enzyme unit was defined producing 1 μmol of reducing sugar in 1 min. The extracted agar in this work was hydrolyzed at content of 0.5% (w/v) by the β-agarase in 100 ml of sodium phosphate buffer (pH 6.5, 50 mM) with 10 U/ml of enzyme activity. The reaction was conducted at 55 °C in a rotary shaking water bath for 2 h. The reducing sugar concentration was quantified by the 3,5-dinitrosalicylic (DNS) method, using a standard curve of D-galactose. Compositions of the agar and the enzymatic hydrolysate were determined according to Marinho-Soriano and Bourret (2005). The hydrolysis of the extracted *G. lemaneiformis* agar by the β-agarase was run under the optimum catalysis conditions of the enzyme; agar concentration was 0.5 mg/ml. Hydrolysis degree was equal to the ratio of the released reducing sugar concentration to the agar mass weight in the reaction solution.

2.3. Acid hydrolysis of G. lemaneiformis agar

The dried agar (3%, w/v) was hydrolyzed in 0.3 M HCl solution at 80 °C for 2 h. Effect of acid hydrolysis conditions on the hydrolysis degree were investigated in advance, including kind of acid, acid concentration, hydrolysis time and agar concentration. Reducing sugar of the reaction mixture was quantified by the DNS method after neutralization.

2.4. Structural characterization of the hydrolysis products

The hydrolysis products were analyzed by thin-layer chromatography (TLC), mass spectrometry, gel permeation chromatography (GPC) and nuclear magnetic resonance spectroscopy (NMR). For TLC, hydrolysate samples were applied to a Merck Silica Gel 60 TLC plate (length is 20 cm), about 1.5 centimetres from the bottom edge, and the samples were separated with an *n*-butanol-ethanol-water mixture solution (3:3:1, v/v) as mobile phase in a glass beaker for about 3 h. Sample spots were visualized by spraying the reagent aniline diphenylamine phosphate (Donero & Biller, 1984). Mass spectrometer: Agilent 6220 TOF LC/MS (Agilent, America), ionization source: positive electrospray-ionization (ESI+). GPC-HPLC was performed on a WATERS 2695 system with an OHpak SB-802 HQ column (8.0*300 mm, Shodex) and a refractive index detector. The mobile phase was 0.1 M sodium sulfate acid and the elution velocity was 0.5 ml/min. ^{13}C NMR spectra of agaroligosaccharides were recorded at 400 MHz on a Bruker AVANCE III spectrometer. The chemical shifts were measured in parts per million (ppm).

LH-20 chromatography was used for the separation of enzymatic hydrolysis products, in which distilled water was the elution phase. If the sugar concentration of each fraction eluted from the LH-20 column (100 × 1.6 cm) was too low to be detected by the DNS reagent, the sugar concentration after LH-20 chromatography was determined by the phenol-sulfuric acid method (Masukoa et al., 2005). An aliquot of 0.1 ml of sample was mixed well with 0.5 ml of 5% phenol and 2.5 ml of sulfuric acid and then left at ambient temperature for complete reaction of sugar in 30 min. The optical density of the solution at 490 nm was measured for determination of sugar content of the LH-20 eluted fraction.

2.5. Determination of tyrosinase inhibition effect of NAOS

The effect of neoagarotetraose on mushroom tyrosinase was determined, using the reported method (Qiu et al., 2009). The diphenolase activity of the enzyme was assayed with L-DOPA as substrate. The reaction was performed in 2 ml of 50 mM Na_2HPO_4-NaH_2PO_4 solution (pH 6.8) containing 0.5 mM L-DOPA and 20 μg of mushroom tyrosinase. The reaction was carried out at 30 °C, started with addition of the enzyme. The activity was determined by measuring the absorbance absorption increase rate at 475 nm on a Thermo Multiskan GO spectrophotometer. Different concentrations of neoagarotetraose were first mixed with 1.75 ml of Na_2HPO_4–NaH_2PO_4 solution containing 0.5 mM L-DOPA and then preheated in a water bath; 50 μl of tyrosinase was added to this mixture to start the reaction. The residual tyrosinase activity was expressed as a percentage of the activity assayed without inhibitor.

The Lineweaver-Burk plots of the tyrosinase in the presence of different concentrations of neoagaroteraose were obtained for determination of the inhibition type and constant according to Qiu et al. (2009).

2.6. Hydroxyl free radical-scavenging activity

The hydroxyl radical-scavenging ability of agaroligosaccharides was determined according to a modified method (Falkeborg et al., 2014). An aliquot of sample was added to 4 ml of 0.6 mM $FeSO_4$-EDTA containing 2 mM H_2O_2, 2 mM sodium salicylate and then blended and incubated at 37 °C for 60 min. Absorbance absorption of the reaction mixture was measured at 510 nm. The influence of sample liquid was removed by subtracting the absorbance of reaction mixture without sodium salicylate to obtain the absorbance difference, noted to be As. Distilled water was used instead of sample as blank control (the absorbance absorption was noted as A_{ctrl}). Scavenging effect (%) = [(As − Actrl)/Actrl] * 100.