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Research article

Efficient immobilization of agarase using carboxyl-functionalized magnetic nanoparticles as support

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ABSTRACT

Background: A simple and efficient strategy for agarase immobilization was developed with carboxyl-functionalized magnetic nanoparticles (CMNPs) as support. The CMNPs and immobilized agarase (agarase-CMNPs) were characterized by transmission electron microscopy, dynamic light scattering, vibrating sample magnetometry, scanning electron microscopy, X-ray diffraction, thermogravimetric analysis, and zeta-potential analysis. The hydrolyzed products were separated and detected by ESI-TOF-MS.
Results: The agarase-CMNPs exhibited a regular spherical shape with a mean diameter of 12 nm, whereas their average size in the aqueous solution was 43.7 nm as measured by dynamic light scattering. These results indicated that agarase-CMNPs had water swelling properties. Saturation magnetizations were 44 and 29 emu/g for the carriers and agarase-CMNPs, respectively. Thus, the particles had superparamagnetic characteristics, and agarase was successfully immobilized onto the supports. Agaro-oligosaccharides were prepared with agar as substrate using agarase-CMNPs as biocatalyst. The catalytic activity of agarase-CMNPs was unchanged after six reuses. The ESI-TOF mass spectrogram showed that the major products hydrolyzed by agarase-CMNPs after six recycle uses were neoagarotetraose, neoagarohexaose, and neoagarooctaose. Meanwhile, the end-products after 90 min of enzymatic treatment by agarase-CMNPs were neoagarobiose and neoagarotetraose.
Conclusions: The enhanced agarase properties upon immobilization suggested that CMNPs can be effective carriers for agarase immobilization. Agarase-CMNPs can be remarkably used in developing systems for repeated batch production of agar-derived oligosaccharides.

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1. Introduction

Agar is a polysaccharide in the cell walls of some red algae and consists of agarose and agaropectins [1]. Agar or agarose can be enzymatically degraded using two types of agarases on the basis of their hydrolysis patterns. The two agarases, namely, α-agarase and β-agarase, hydrolyze α-1,3 linkages and β-1,4 linkages in agarose, respectively. α-Agarase cleaves the α-L-(1,3) linkages of agarose to produce oligosaccharides of the agarobiose series with 3,6-anhydro-L-galactopyranose at the reducing end. By contrast, β-agarase cleaves the β-D-(1,4) linkages of agarose to produce neoagaro-oligosaccharides with D-galactopyranoside residues at the reducing end [2]. Neoagaro-oligosaccharides from β-agarase have recently received much attention because of their numerous biological functions, such as inhibition of bacterial growth, retardation of starch degradation, improvement of food qualities as low-calorie additives, and even for moisturizing and whitening the skin [3]. Therefore, agar-derived oligosaccharides have broad applications in the food, cosmetic, and medical industries.

Agarase can be directly used to produce agar-derived oligosaccharides. However, these enzymes are often easily devitalized and difficult to segregate from the reaction system during recovery and recycling. Therefore, the enzymatic activity, stability, and recovery of agarase should be improved before its application as an industrial biocatalyst [4]. Magnetic nanoparticles for immobilizing enzymes and cells are recently considered for the commercial application of biocatalyst-processed products [5,6]. The application of biomolecule immobilization mainly depends on the solid-phase magnetic feature which can be rapidly separated and recovered from the reaction medium using an external

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magnetic field [7,8]. The use of magnetic supports can also reduce the capital and operation costs [9,10].

To the best of our knowledge, the production of agar-derived oligosaccharides with immobilized agarase on magnetic nanoparticles has not been reported yet. In this study, we described a simple and efficient method to immobilize agarase using carboxyl-functionalized magnetic nanoparticles (CMNPs) as support. The successful immobilization of agarase onto the support was confirmed by characterizing the CMNPs and immobilized agarase (agarase-CMNPs) by transmission electron microscopy (TEM), scanning electron microscopy (SEM), dynamic light scattering (DLS), vibrating sample magnetometry (VSM), X-ray diffraction (XRD), thermogravimetric analysis (TGA), and zeta-potential analysis. In addition, the hydrolyzed agar products were detected by thin-layer chromatography (TLC) and ESI-TOF-mass spectroscopy (MS) to determine the catalytic activity of the agarase-CMNPs. The results provide a foundation for further studies on the production of agar-derived oligosaccharide.

2. Materials and methods

2.1. Materials

Analytical grade $FeCl_3 \times 6H_2O$, $FeCl_2 \times 4H_2O$, $NH_3 \times H_2O$, $KMnO_4$, and oleic acid were purchased from Sinopharm Chemical Reagent Co., Ltd. and used directly without further treatment. Agar was purchased from Guangdong Huankai Microbial Sci. & Tech. Co., Ltd. All other chemicals were of analytical grade.

2.2. Preparation of oleic acid-coated magnetic Fe_3O_4 nanoparticles

The oleic acid-coated Fe_3O_4 nanoparticles were prepared by co-precipitating Fe^{3+} and Fe^{2+} ions with a molar ratio of 2:1 in ammonia and oleic acid solution [11]. $FeCl_3 \times 6H_2O$ (8.10 g) and $FeCl_2 \times 4H_2O$ (2.98 g) were transferred into an Erlenmeyer flask with 142.5 mL of deionized water, and the mixture solution was heated to 70°C. Then, 18 mL of $NH_3 \times H_2O$ (25%, w/v) was quickly added into the flask with vigorous stirring. Oleic acid (4.66 g) was added dropwise into the reaction mixture under constant stirring for 1 h at 70°C. The resulting Fe_3O_4 magnetic fluid was isolated magnetically and repeatedly washed with deionized water and ethanol. Finally, the magnetic fluid was dried by lyophilization for use in the next procedure.

2.3. Preparation of CMNPs

Carboxyl-functionalized Fe_3O_4 magnetic nanoparticle was prepared using a modified method described by Lv et al. [12]. The oleic acid-coated magnetic nanoparticles were dispersed in 160 mL of 10 mg/mL $KMnO_4$ solution under continuous sonication for 8 h. The resulting CMNPs were subsequently separated by additional magnetic field, washed thrice with distilled water, and freeze-dried for the subsequent step.

2.4. Preparation of the crude agarase

Agarase-producing strain *Vibrio natriegens* CICC 23820 was used to produce agarase. The optimal culture medium and conditions of agarase production on the *V. natriegens* CICC 23820 were as follows: agar, 0.3%; yeast extract, 0.6%; NaCl, 2%; $MgSO_4 \times 7H_2O$, 0.5%; KCl, 0.1%; $CaCl_2$, 0.02%; K_2HPO_4, 0.01%; $FeSO_4 \times 7H_2O$, 0.002%; initial, pH 6.5; autoclave temperature, 121°C; and treatment time, 20 min. The samples were cooled, inoculated with 2% seed broth, and incubated at 25°C. The samples were cultivated for 24 h, and the supernatant was collected from the culture broth by centrifugation at 12,000 × g for 10 min. The resulting crude agarase solution was directly used for immobilization. The activity of free agarase was determined by measuring the rate of agar hydrolysis [13]. One unit of agarase activity was defined as the amount of enzyme which hydrolyzes agar to liberate 1 μg of the reducing sugar per minute under the assay conditions.

2.5. Preparation of the agarase-CMNPs

The CMNPs (20 mg) were suspended in 5 mL of 4% (v/v) glutaraldehyde (GA) and then ultrasonically treated for 5 min. The mixture was then left to stand at room temperature for 2 h. The CMNPs were then isolated magnetically and washed thrice with PBS (0.05 M, pH 7.0). Subsequently, the crude agarase solution (1 mL, 850 U/mL) was combined with the obtained CMNPs at a constant temperature of 5°C for 1 h under a stationary state. Finally, agarase-CMNPs were collected and rinsed thrice with PBS (0.05 M, pH 7.0).

2.6. Characterization methods

The size and morphology of the CMNPs and agarase-CMNPs were observed by TEM (FEI Tecnai G20, USA) with an acceleration voltage of 200 kV, and hydrodynamic sizes were surveyed by DLS (Malvern Zetasizer NANO ZS, UK). The magnetization curves of the dried CMNPs and agarase-CMNPs were recorded using VSM (Quantum Design MPMS XL7, USA) at room temperature. The crystalline properties of the magnetic microspheres were examined by XRD (Bruker D8 ADVANCE, Germany), and thermal stability was measured by TGA (TA SDT Q600, USA) under nitrogen atmosphere at 20–1000°C at a heating rate of 10°C/min. Zeta potential was measured by dispersing the CMNPs and agarase-CMNPs in water at 25°C in the Nano-Zetasizer (Malvern Zetasizer NANO ZS, UK).

2.7. Reusability of agarase-CMNPs

Hydrolysis reactions were conducted at 40°C in an Erlenmeyer flask containing 20 g of agarase-CMNPs and 10 mL of 0.2% agar. Agarase-CMNPs were isolated by a magnet after each enzymatic reaction, and the supernatant was collected. Then, the agarase-CMNPs were washed thrice with deionized water for the next reaction.

The enzymatic hydrolysate was identified by TLC and ESI-TOF-MS. Then, the samples were first applied to a silica Gel 60 TLC plate and developed with an n-butanol-acetic acid-water solution (2:2:1 by volume). The developed oligosaccharides were detected by submerging the plae with 10% (by volume) H_2SO_4 in ethanol and heating at 90°C. The spots corresponding to the hydrolyzed products were compared with the standard compounds. The total oligosaccharides produced after six reuses were dried in vacuum and extracted with ethanol. The molecular mass distribution was then determined using an ESI-TOF mass spectrometer.

2.8. Preparation and analysis of agaro-oligosaccharides

The different amounts of agarase-CMNPs and agar solution (20 mL, 0.2%) were incubated at 40°C in an Erlenmeyer flask. Samples were collected at different time points to determine the reducing sugar in the reaction liquid. Then, the reaction solutions were separated using an external magnetic field to terminate the reaction. The oligosaccharides were obtained by alcohol precipitation after centrifugation and then analyzed by ESI-TOF-MS after the samples were freeze-dried.

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