Electronic Journal of Biotechnology 19 (2016) 1–7

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Electronic Journal of Biotechnology

Research Article

Preparation and characterization of κ-carrageenase immobilized onto magnetic iron oxide nanoparticles

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ARTICLE INFO

Article history:
Received 9 July 2015
Accepted 16 October 2015
Available online 31 October 2015

Keywords:
Carrageenase
Characterization
Immobilization
Nanoparticles

ABSTRACT

Background: Carboxyl-functionalized magnetic nanoparticles were synthesized via chemical co-precipitation method and modified with oleic acid which was oxidized by potassium permanganate, and κ-carrageenase from *Pseudoalteromonas* sp. ASY5 was subsequently immobilized onto them. The immobilization conditions were further optimized, and the characterizations of the immobilized κ-carrageenase were investigated.

Results: The κ-carrageenase was immobilized onto magnetic iron oxide nanoparticles, and the bonding was verified by Fourier transform infrared spectroscopy. The optimal conditions for κ-carrageenase immobilization were 2.5% (w/v) glutaraldehyde, 13.9 U κ-carrageenase for 20 mg of magnetic nanoparticles, a 2-h cross-linking time, and a 2-h immobilization time at 25°C. Under these conditions, the activity of the immobilized enzyme and the enzyme recovery rate were 326.0 U · g^{-1} carriers and 46.9%, respectively. The properties of the immobilized κ-carrageenase were compared with those of the free enzyme. The optimum temperatures of the free and immobilized κ-carrageenase were 60 and 55°C, respectively, and the optimum pH of κ-carrageenase did not change before and after immobilization (pH 7.5). After immobilization, κ-carrageenase exhibited lower thermal stability and improved pH stability, as well as better storage stability. The immobilized κ-carrageenase maintained 43.5% of the original activity after being used 4 times. The kinetic constant value (Km) of κ-carrageenase indicates that the immobilized enzyme had a lower binding affinity for the substrate.

Conclusions: Under optimal conditions, the activity of the immobilized enzyme and enzyme recovery rate were 326.0 U · g^{-1}·κ-carrageenase-CMNPs and 46.9%, respectively. The thermal, pH, and storage stabilities of κ-carrageenase-CMNPs were relatively higher than those of free κ-carrageenase.

1. Introduction

Carrageenans are gel-forming, linear, and sulfated galactans extracted from certain marine red algae and consist of D-galactose residues with alternating α-1,3 and β-1,4 linkages [1]. Recent studies have shown that carrageenan oligosaccharides with small molecular weight possess certain biological activities, such as anti-oxidation [2], anti-viral [3], anti-angiogenic [4], anti-inflammatory [5], and immunomodulation [6], compared with other carrageenans. Therefore, carrageenans are often degraded into oligomers by using acid, active oxygen, microwave, sonication, or carrageenase to improve their bioactivity and application performance [7].

Compared with chemical and physical degradation, enzyme hydrolysis is more likely to produce carrageenan-derivatives with uniform molecular weights, which can be more advantageous because the observed physiological activities of oligo-carrageenans are associated with their molecular weights [8]. However, the industrial application of the enzyme as a biocatalyst is economically unattractive because of its high cost and inconvenience in separation, recycling, and reusing [9]. Enzyme immobilization presents interesting advantages over these problems, including highly concentrated enzymatic activity and high stability and reusability. Tang and Lee [10] immobilized β-glucosidase on κ-carrageenan hybrid matrix, and the immobilized enzyme tolerated broader range of pH values and higher reaction temperature up to 60°C compared with the free β-glucosidase. Bezerra et al. [11] immobilized laccase in green coconut fiber, and the immobilized enzyme retains up to 100% of the initial activity after the assay was reused 10 times. Among the various supports used in immobilization, the magnetic nanoparticles are

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

http://dx.doi.org/10.1016/j.ejbt.2015.10.001

receiving considerable attention because of their large specific surface area, low mass transfer resistance, less fouling, easy separation from reaction solution with magnetic field, less capital and operating costs, and surface modification with various active groups [12,13,14].

To our knowledge, κ-carrageenase immobilization with magnetic nanoparticles has not been reported yet. In the present study, carboxyl-functionalized magnetic nanoparticles were synthesized via chemical co-precipitation method, and κ-carrageenase was subsequently immobilized onto carboxyl-functioned magnetic iron oxide nanoparticles (CMNPs). The immobilization conditions were further optimized, and the characterizations of the immobilized κ-carrageenase were investigated.

2. Materials and methods

2.1. Materials

$FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$, NH_4OH, $KMnO_4$, oleic acid, 3,5-dinitrosalicylic acid, and potassium sodium tartrate were of analytical grade and purchased from Sinopharm Chemical Reagent Ltd., Corp. (China). κ-Carrageenan was provided by Greenfresh (Fujian) Foodstuff Co. Ltd.

2.2. Microorganism and κ-carrageenase preparation

Pseudoalteromonas carrageenovora CICC 23819, deposited at the China Center of Industrial Culture Collection, was used to produce κ-carrageenase. κ-Carrageenase production was performed by shaking flask fermentation using carrageenan as the sole carbon source. The flasks were incubated at 20°C for 60 h in a shaker at 180 rpm. Culture supernatant as crude enzyme was collected by centrifugation at 9800 × g and 4°C for 10 min.

2.3. Synthesis of oleic acid-coated magnetic nanoparticles and CMNPs

Oleic acid-coated magnetic nanoparticles were prepared via chemical co-precipitation method as described by Chen et al. [13]. $FeCl_3 \cdot 6H_2O$ (8.1 g) was dissolved in a flask with 142.5 mL of distilled water and then heated to 70°C. $FeCl_2 \cdot 4H_2O$ (3.3 g) was dissolved in 7.5 mL of water, and the mixture was subsequently added into the flask. Under rapid stirring condition, 18 mL of ammonia (25%, w/v) was quickly added. After 1 min, 5.3 mL of oleic acid was added dropwise into the flask and maintained at 70°C for 1 h under continuous stirring. The black precipitate was separated using an external magnet and washed several times with alcohol and water to remove the excess oleic acid.

Thereafter, 160 mL of 10 g · L^{-1} $KMnO_4$ solution was added into the oleic acid-coated magnetic nanoparticles obtained earlier. The precipitate was separated using a magnet after 8 h of sonication treatment and washed several times with water to remove excess KMnO4. Powdered CMNPs were obtained after drying at ¯80°C under vacuum.

2.4. Immobilization of κ-carrageenase onto CMNPs

CMNPs (20 mg) were added into 5 mL glutaraldehyde solution (5%, w/v) and incubated at 4°C for 3 h for cross-linking. The magnetic nanoparticles were separated magnetically and washed five times with sodium phosphate buffer (50 mM, pH 7.5) to remove the excess glutaraldehyde. Subsequently, the mixture of the supports obtained above and 1 mL of κ-carrageenase solution were immobilized at 4°C for 3 h. CMNPs bound with κ-carrageenase were collected using an external magnetic field, washed with sodium phosphate buffer (50 mM, pH 7.5), dried at ¯80°C under vacuum using a freeze dryer, and stored at 4°C for activity assay.

2.5. Enzyme activity assay

The activities of free κ-carrageenase and κ-carrageenase-CMNPs were determined using the 3,5-dinitrosalicylic acid (DNS) method. The substrate used was 0.5% (w/v) κ-carrageenan dissolved in sodium phosphate buffer (50 mM, pH 7.5); the resulting solution was uniformly mixed with carrageenase and incubated at 55°C for 20 min. Subsequently, the DNS was added into the reaction solutions and incubated in boiling water for 10 min; the absorbance was measured at 520 nm thereafter. The amount of sugar was determined based on the D-galactose in standard curve. One unit of κ-carrageenase activity is defined as the amount of enzyme that produced 1 μmoL of D-galactose in 1 min at 55°C and pH 7.5.

Enzyme recovery rate was calculated according to the residual enzyme activity after immobilization. Free κ-carrageenase and κ-carrageenase-CMNP activities were assayed, and the enzyme activity recovery rate was calculated based on the following equation:

$$\text{Activity recovery rate } (\%) = \text{ai}/\text{af} \times 100\% \quad \text{[Equation 1]}$$

where ai is the activity of the immobilized κ-carrageenase, and af is the activity of the free κ-carrageenase added into immobilization system.

2.6. Determination of optimal conditions for κ-carrageenase immobilization

Glutaraldehyde concentration (0%–3.5%, w/v), enzyme dosage (6.95–83.4 U κ-carrageenase mL^{-1}), cross-linking time (0.5–6 h), immobilization time (0.5–6 h), and immobilization temperature (4 and 25°C) were investigated to determine the optimal immobilization conditions of κ-carrageenase. The immobilization efficiency during the immobilization process was evaluated using the activity recovery rate of κ-carrageenase.

2.7. Fourier transform infrared spectra (FTIR) of CMNPs and κ-carrageenase-CMNPs

FTIR spectra were obtained on a NEXUS 670 FTIR instrument using KBr discs in the 4000–400 cm^{-1} region at room temperature (25°C).

2.8. Properties of the free and immobilized κ-carrageenase

The effect of temperature on the κ-carrageenase activity was determined at various temperature levels from 45°C to 65°C, pH 7.5. The effect of pH on κ-carrageenase activity was assayed at 55°C with pH values that ranged from 4.0 to 10.0 in the following buffers: acetic acid-sodium acetate (50 mM, pH 4.0–5.0), Na_2HPO_4–NaH_2PO_4 (50 mM, pH 5.0–7.0), Tris–HCl (50 mM, pH 7.0–9.0), and glycine–sodium hydroxide (50 mM, pH 9.0–10.0). The maximum activity was considered to be 100% and used as the reference to determine the relative activity at different catalytic reaction conditions.

The thermal stability of κ-carrageenase was evaluated by measuring the residual activity of the free and immobilized enzyme after incubation from 45°C to 60°C without substrate. The residual activities of the free and immobilized enzymes were assayed after incubating κ-carrageenase at 4°C for 24 h in the aforementioned pH buffers to determine pH stability. The residual enzyme activity was defined as the proportion of the final value of enzyme activity to the initial activity (100%).

2.9. Reusability and storage stability assays

The reusability of the immobilized κ-carrageenase was evaluated by repeated utilization to catalyze the κ-carrageenan hydrolysis at 55°C and pH 7.5. The activity obtained in each round was compared with the initial activity (defined as 100%) to calculate the relative activity.