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Immobilization of *Candida rugosa* lipase on MSU-H type mesoporous silica for selective esterification of conjugated linoleic acid isomers with ethanol

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ABSTRACT

Candida rugosa lipases (CRLs) immobilized on MSU-H type mesoporous silicas with different pore sizes (6.0, 7.2 and 13.3 nm) through physical adsorption and a cross-linking method were examined as biocatalysts for esterification of conjugated linoleic acid (CLA) isomers with ethanol in isooctane. N_2 adsorption–desorption isotherms indicated that the MSU-H silicas and the immobilized CRL derivatives had mesoporous structure. Introduction of CRL molecules onto MSU-H was confirmed by Fourier transform infrared (FT-IR) spectra. The optimum conditions of the immobilization process (pH of CRL solution, 7.0; initial concentration of CRL solution, 1.6 mg/ml; contact time, 8–10 h) and the esterification reaction (temperature, 45 °C; molar ratio of CLA to ethanol, 1:1; biocatalyst, 6% by weight of CLA) were determined. The MSU-H material with pore size of 13.3 nm proved to be more suitable to immobilize CRL onto it than others. The CRL immobilized on MSU-H (pore size, 13.3 nm) by cross-linking gave a loading amount 48.2 mg CRL/g-dry support and a maximal hydrolytic activity of 3418.2 U/g-catalyst. It also exhibited high synthetic activity and operational stability and remained 43.2–67.8% of total esterification in 48 h consecutive six runs under the optimized reaction conditions. Moreover, the immobilized CRLs catalyzed 2.0–3.1 times of esterification of *cis*-(*c*)9, *trans*-(*t*)11-CLA faster than that *t*10, *c*12-CLA. The formation of ethyl esters of CLA isomers was identified by GC–MS analysis.

1. Introduction

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of octadecadienoic acid with a pair of conjugated double bonds [1]. Due to its various healthy functions such as anticarcinogenic, antiatherogenic and antiobesity effects and improvement of immunity, CLA has received much attention [2,3]. However, the extensive applications of CLA are limited in food and feed additives, drugs and cosmetics because it is easily oxidized and has unpleasant taste [4,5]. The ethyl ester of CLA (CLAEE) is stable, nontoxic and readily absorbed by living bodies [6,7]. Therefore, CLAEE can be a substitute for CLA. The industrial production of CLAEE is mainly carried out by chemical esterification of CLA in presence of catalysts such as sulfuric acid, sodium or potassium hydroxide [8]. These catalysts have environmental and processing disadvantages and lead to poor quality of the CLAEE product due to peroxidation of the unsaturated bonds in CLA. Hence, it is desirable to develop an eco-friendly technology of biocatalytic process for this conversion. Due to its mild reaction conditions and no pollution, an enzymatic process for producing CLAEE is more attractive than the chemical one.

In addition, the most commonly used CLA is a mixture of more than seven positional and geometric isomers [9]. The *cis*-(*c*)9, *trans*-(*t*)11- and *t*10, *c*12-CLA isomers are believed to have beneficial physiological effects in anticancer activity and decreasing fat in body, respectively [10], whereas a very small amount of the *trans*-isomers of *t*9, *t*11- and *t*10, *t*12-CLA is harmful to health. Methylation of CLA under acid catalysis was commonly used in separation and identification of CLA isomers [11,12]. However, the acid-catalyzed methylation altered the CLA isomer composition

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(formation of *t*, *t* isomers) [8] and formed methoxy and hydroxy artifacts [12]. Ethyl esterification of CLA with ethanol under lipase catalysis provided an alternative to separate and identify of CLA isomers because this method not only prevented CLA isomerization [13] but also was safe in food supplement [14,15].

Candida rugosa lipase (CRL), a globular protein with a molecular weight of 45–60 kDa and a molecular volume of 5 nm × 4.2 nm × 3.3 nm is relatively inexpensive and available biocatalyst for food production and can catalyze esterification, transesterification and interesterification reactions in organic media or a solvent-free system [16]. However, its use as a catalyst is deterred because of the lack of long-term stability under process conditions and difficulties in recovery and recycling. Immobilization of free lipase onto solid supports can overcome such problems [17].

Various solids such as mica [18], bentonite [19] and mesoporous SBA-15 silicas [20,21] have been used as supports for immobilization of CRL. Mesoporous silica materials are excellent candidates as the support for immobilizing enzyme because of their high surface area, large pore volume and nontoxicity [22,23]. It is worth noting that MSU-H is two-dimensional hexagonal ordered mesoporous silica which can be prepared by using a low-cost and convenient reagent of sodium silicate as a silica source and a nonionic surfactant of triblock copolymer Pluronic P123 as template at near neutral pH condition [24]. It has an open porous system with interconnected channels which favors diffusion of enzyme and bulky substrate [25]. Therefore, in the present work, we attempted to immobilize CRL onto MSU-H silicas with different pore sizes and evaluated the performances of the resultant catalysts in esterification reaction of CLA with ethanol using isooctane as a solvent. The factors affecting immobilization of CRL and esterification reaction as well as operational stability of the CRL derivatives were also explored.

2. Materials and methods

2.1. Materials

Pluronic P123 triblock copolymer ($EO_{20}PO_{70}EO_{20}$, $M_w = 5800$) and crude CRL (type VII, activity 7030 U/g-solid) were purchased from Sigma–Aldrich. CLA (78–84 wt%) was obtained from Puritan's Pride (USA) and it was composed of 37.8% *c*9, *t*11-CLA, 38.3% *t*10, *c*12-CLA and 5.6% other CLA isomers, 15.4% oleic acid, 2.9% linoleic acid. Coomassie brilliant blue G-250 was produced by Sinopharm Chemical Reagent Co., Inc. (Shanghai, China). Bovine serum albumin (BSA) was purchased from Shanghai Bio Life Science & Technology Co., Ltd. (Shanghai, China). All other chemicals used were AR grade and obtained locally.

2.2. Synthesis of support

MSU-H silica was prepared by the following procedure: sodium silicate solution (27 wt% SiO_2, 14 wt% NaOH) was added dropwise to a vigorously stirred solution of P123 in deionized water at an ambient temperature. The pH of reaction mixture was adjusted to about 6.5 by addition of acetic acid in it. The overall molar ratio of the reactant was $1.00SiO_2$:*x*P123:$0.5CH_3COOH$:$115H_2O$. The resultant mixture was stirred at 60 °C for 20 h. The solid product was filtered, washed with deionized water, air-dried, and then calcined at 550 °C in air for 4 h. For preparation of different samples, *x* equal to 0.0081, 0.012 and 0.0162 was used individually. The pore diameters of the resulting materials were measured and calculated according to the BJH method based on the desorption branch and was 7.2, 6.0 and 13.3 nm. Accordingly, the mesoporous materials were designated as MSU-H(7), MSU-H(6) and MSU-H(13), respectively.

2.3. Immobilization of CRL

Immobilization of CRL was performed by physical adsorption and a cross-linking method. CRL solution was prepared by adding an appropriate amount of crude CRL powder to phosphate buffer (0.025 M, pH 6.0–8.0). The experiment of physical adsorption was carried out by mixing 100 mg of support and 25 ml of CRL solution (0.4–2.0 mg/ml). The mixture was magnetically stirred with 200 rpm at 37 °C for 0–24 h. The subsequent treatment with 5 ml of 0.5 wt% glutaraldehyde was conducted in cross-linking CRL under another 2 h stirring after the physical adsorption. The immobilized CRL was collected by centrifugation at 12 000 rpm, and washed with a pH 7.0 phosphate buffer solution, then dried at 30 °C. The immobilized CRLs prepared by the cross-linking method at 1.6 mg/ml of CRL solution, pH 7.0 and 37 °C using MSU-H(6), MSU-H(7) and MSU-H(13) as supports were designated as CRL/MSU-H(6), CRL/MSU-H(7) and CRL/MSU-H(13). These samples contained 10.8 wt%, 11.2 wt% and 11.5 wt% of water, respectively.

2.4. Esterification reaction and reuse test

The prepared biocatalyst was assayed in the direct esterification of CLA with ethanol using isooctane as a solvent. The esterification reaction was carried out in a 25 ml glass reactor in a shaking water bath at 120 rpm and 37–65 °C for 4–20 h. The reaction mixture contained 4.70 mmol of CLA, 2.35–9.40 mmol of ethanol (1:0.5, 1:1 and 1:2 of molar ratios of CLA to ethanol) and 20–130 mg of biocatalysts (between 2 and 10%, w/w with respect to CLA) in 3 ml isooctane. The reaction products were collected after centrifugation and analyzed with a gas chromatography coupled to mass spectrometry (GC–MS). The products were quantified by a GC equipped with a DB-5ms capillary column and a flame ionization detector. For recycling, the used biocatalyst was separated from the reaction mixture by centrifugation and washed with a phosphate buffer solution. After drying at 30 °C, the biocatalyst was introduced into a fresh medium for a next run under the same reaction conditions. This process was repeated for six cycles.

2.5. Characterization of MSU-H and immobilized CRL

The nitrogen adsorption–desorption isotherms were measured at −196 °C on a Micromeritics ASAP 2020 sorptometer. Prior to analysis the samples were outgassed at 150 °C and 10^{-6} Torr for a minimum of 12 h. BET surface areas were calculated from the linear part of the adsorption curve. Pore size distributions were calculated by using the desorption branches of the N_2 isotherms and the Barret–Joyner–Halenda (BJH) method. FT-IR spectra of the samples were measured by using a Nicolet 6700 FT-IR spectrometer (Thermo-Nicolet, USA) and the KBr-sample pellet was used. The spectra were collected in the spectral range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}.

2.6. Measurement of hydrolytic activity

Hydrolytic activity of crude CRL or immobilized CRL was assayed titrimetrically by using an olive oil emulsion method [26]. The substrate was prepared by mixing 50 ml of olive oil with 150 ml of polyvinyl alcohol solution (2%, w/v) to obtain emulsion. The reaction mixture consisting of 5.0 ml of phosphate buffer (0.025 M, pH 7.0) and 5 ml of the substrate emulsion above and a certain amount of crude CRL/immobilized CRL was incubated for 15 min at 37 °C in a vessel. The reaction was terminated by adding 15 ml of 95 wt% ethanol. The liberated fatty acids were titrated with 0.05 M of sodium hydroxide solution in the presence of phenolphthalein indicator. In addition, a blank experiment for comparison without adding crude CRL/immobilized CRL was carried by the above assay