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Biosynthesis of oleyl oleate in solvent-free system by *Candida rugosa* Lipase (CRL) immobilized in macroporous resin with cross-linking of aldehyde-dextran

Yicheng Bi, Min Yu, Haiyan Zhou, Hua Zhou*, Ping Wei

School of Pharmaceutical Science, NanJing Tech University, Nanjing 211-816, China

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ABSTRACT

In our study, oleyl oleate was synthesized through enzymatic esterification of oleic acid with oleyl alcohol using *Candida rugosa* lipase (CRL) immobilized on macroporous resin in a solvent-free system. CRL was immobilized flexibly on the support via aldehyde-dextran, and the effectiveness of aldehyde-dextrans, as cross-linking agents with different molecular weights, was compared and discussed. The impact of various factors, such as different molecular weights of dextran, molar ratio between oleic acid and oleyl alcohol, and the temperature and stability of enzymes, on esterification was investigated as well. Our study indicated that lipase immobilized by 20000 Da dextran yielded the best result. The optimal condition to produce oleyl oleate using immobilized CRL was found in our study and turned out to be molar ratio of 1, reaction temperature of 40 °C, and a 12-h reaction time to reach reaction equilibrium. Under the optimal reaction conditions, a high percentage yield of ester was achieved (92.6%). The immobilized CRL also reached a conversion rate of 86.7% with excellent stability even after 8 cycles.

1. Introduction

Liquid wax esters are valuable organics and widely used in cosmetic, lubricant or food industries due to their non-toxic characteristics and excellent wetting behavior in interfaces [1]. The natural sources of wax ester are animals and plant materials, such as sperm whales and jojoba oil, which are quite expensive for mass exploitation. Therefore, focus has been put on the synthesis of analogue products in large quantities. Oleyl oleate, as a synthetic analogue of jojoba oil [2], shares similar characteristics of wax ester, but with much lower cost. Oleyl oleate can be synthesized by either chemical or enzymatic method. Both methods have pros and cons. The chemical method involves high-temperature processes, which may lead to degradation of esters and excessive cost on energy [1]. The enzymatic method, on the other hand, is preferable with the mild reaction conditions, high conversion, low energy cost, and less waste. Lipase is known to be useful in producing functional wax esters by hydrolyzing long-chain triacylglycerols in the presence of water and catalyzing esterification and transesterification in non-aqueous media or solvent-free systems [3–5].

Several studies have conducted on the synthesis of oleyl oleate using lipase. It was reported that Novozyme 435 was used in high performance enzymatic synthesis of oleyl oleate [6]. Kapucu et al. [7] and Radzi et al. [8] have reported that the response surface methodology has been applied to optimize the process in batch and stirred tank reactors, respectively. Kinetics of reaction in stirred tank reactors were also studied by Salina's group and other researchers [9,10]. The commercial lipase Lipozyme was also used to produce oleyl oleate in other research [11,12]. Besides those commercial lipases, CRL was used [13] to synthesize oleyl oleate as well. Even though the formation of ester reaches to 80% in 2 h, lipase used in such system was not immobilized and could not be utilized repeatedly. Zaidi et al. [14] improved the process of oleyl oleate synthesis by immobilizing CRL on nylon, but the process was completed in hexane, which increased the complexity of products' purification.

Activity of immobilized enzyme is greatly related to the way it connects to the carriers. In general, direct immobilization of enzyme by covalent bonds receives low activity recovery due to steric hindrance effects or conformation limitation, even though the immobilization ratio is higher [15]. The introduction of spacer arms solves this problem to some extent. It has been shown that recovery activity can be improved by various coupling reagent, such as the application of ethylenediamine (EDA) [15], 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [16],

* Corresponding author.
E-mail address: zhouhua@njtech.edu.cn (H. Zhou).

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succinic anhydride [17], and glutaraldehyde [18]. But the improvement in recovery activity is limited by the short chain, which is not flexible enough for enzyme to exhibit the full conformation. Therefore, long and flexible chains are developed as the coupling agent for enzymes. Carbohydrates contain plenty of hydroxyls and have strong water-holding capacity. The presence of carbohydrates or polyols can further modify the microenvironment of the enzyme and maintain the hydration of the biocatalyst [19]. The application of poly ethylene glycol (PEG) [15], dextran [19] and dextran derivatives [20] not only reduces the steric hindrance effect mentioned before, but also maintains traces of water around the protein, which was used to decorate the free enzyme [21]. Dextrans with appropriate length were also proposed as new spacer arms to improve the performance of immobilized enzymes and proteins acting on macromolecular substrates, and steric hindrances were prevented [22]. However, activity of enzyme experienced irreversible loss due to the structure change. Carbohydrates were also used as additives in our previous work to protect the hydration state of catalyst in synthesizing of phytosterol esters [23], but purification got further complicated.

In our current study, instead of decorating the free enzyme directly or using additives, aldehyde-dextran was used as cross-linking reagent to modify the common macroporous resin to overcome the afore-mentioned problems, and CRL covalently immobilized on the modified resin was used to catalyze the esterification of oleic acid and oleyl alcohol. The study indicated flexible immobilization gave high activity recovery. The impact of aldehyde-dextran with different molecular weights on synthesis of oleyl oleate was also investigated. To simplify the downstream process, organic solvent was not involved in the study. Reaction parameters, including time, lipase loading, molar ratio of the substrates and temperature, were evaluated. The stability of immobilized CRL was also tested.

2. Materials and methods

2.1. Materials

The lipase from *Candida rugosa* (≥7 U/mg) is supplied by Sigma-Aldrich. Oleic acid (85%) and oleyl alcohol (85%) was purchased from Aladdin Industrial Inc. Amino macroporous resin, LX-1000HA (with average diameter 0.8 mm and pore size around 48 nm, HA for short), was generously donated by Sunresin Co., Ltd (Xi'an, China). Dextrans with different molecular weights of 6000, 20,000, 40,000, and 70,000 Da were purchased from Pharmacia (New Jersey, USA). All other reagents used were of analytical grade and used as received. All the solutions were prepared with double-distilled water.

2.2. Preparation of the aldehyde-containing dextrans and the modification of support

The oxidation of dextran was prepared according to the method previously reported by our group [24]. Dextrans with molecular weights of 6000, 20,000, 40,000 and 70,000 Da (hereafter, T6, T20, T40 and T70) were chosen. The oxidation was carried out by first adding 600 mg of sodium periodate to 10 mL of sodium acetate buffer (pH 4.0, 0.05 M) containing 400 mg of dextrans at 4 °C for 9 h in dark conditions. Then, the solution was dialyzed for 6 h at 4 °C using a 3000 Da membrane to eliminate the unreacted sodium periodate.

Aldehyde-containing dextrans are used to modify the support as follows:

1. The concentration of aldehyde-containing dextrans was diluted from 40 to 8 mg/mL using sodium acetate buffer with pH of 7.0.
2. 0.1 g amino macroporous resin was mixed with 5 mL of aldehyde-dextran solution and left in 30 °C shaker for covalent bonding.
3. The supports were washed with sodium acetate buffer (pH 7.0, 0.02 M) after 24-h reaction for further use.

As control, 1% glutaraldehyde (GA) was used to react with the supports. 5 mL of GA was mixed with 0.1 g amino macroporous resin and was shaked for covalent bonding. Due to the higher activity of aldehyde groups in GA, the reaction time between GA and support was set to 1 h, and other conditions and steps remained unchanged.

2.3. Immobilization of CRL on macroporous support

The lipase solution (4 mg/mL) was prepared in 0.02 M sodium acetate buffer (pH 5.0). The lipase solution was mixed with 0.1 g support modified by aldehyde-dextran and stirred at 30 °C for 36 h. As for GA group, the reaction time between lipase and modified support was 6 h due to the higher activity of aldehyde groups in GA. Then the support was separated from the mixture by filtration and washed with sodium acetate buffer (pH 5.0, 0.02 M) till no protein could be found in effluent. Bradford assay was used here to confirm the protein in effluent [25].

2.4. Lipase activity assay

The activities of free and immobilized CRL were determined according the method reported by our group [23]. The substrate solution was composed of 0.5 mL of acetonitrile containing *p*-nitrophenyl acetate (*p*-NPA) (0.05 M) and 3.4 mL (3.5 mL for immobilized CRL) of sodium phosphate buffer (pH 7.0, 0.02 M). The reaction commenced upon the addition of 0.1 mL of free CRL (1 mg/mL) or an appropriate amount of immobilized CRL. When the reaction mixture was incubated at 37 °C for 10 min, 10 mL of the phosphate buffer was added, and the absorbance value was measured at 410 nm. One unit (U) was defined as the amount of lipase that liberated 1 μmol *p*-nitrophenol per min under the assay conditions.

2.5. Synthesis of oleyl oleate

The esterification experiment was carried out in a 10 mL flask. First, 2 mmol oleyl alcohol and a corresponding amount of oleic acid were pre-mixed at 30 °C for 30 min. Then, a specific amount of immobilized lipase was added to start the reaction at 150 rpm at specific temperature. After 2-h reaction, 0.5 g 4 Å molecular sieve was added to remove the water produced in the process. The reactant mixture was stirred till the reaction completed. During the process, the samples were withdrawn every 2 h and passed through 0.22-μm filters for test. All experiments were analyzed in triplicate and the mean value was calculated.

To investigate the reusability, immobilized CRL was filtered and washed after each reaction cycle with *n*-hexane for 3 times to remove any substrate or product. The immobilized CRL was then dried and repetitively used in the next reaction batch.

2.6. Identification of reaction product

Qualitative and quantitative analysis of samples were facilitated by thin layer chromatography (TLC) and gas chromatography (GC). For TLC, the developing solvent system used in this study was petroleum ether: diethyl ether:formic acid (210:90:0.4, v/v). Reactants were quantitated by injecting 1 μL diluted samples