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Improvement of catalytic activity of *Candida rugosa* lipase in the presence of calix[4]arene bearing iminodicarboxylic/phosphonic acid complexes modified iron oxide nanoparticles

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ABSTRACT

In the present study, iron oxide magnetite nanoparticles, prepared through a co-precipitation method, were coated with phosphonic acid or iminodicarboxylic acid derivatives of calix[4]arene to modulate their surfaces with different acidic groups. *Candida rugosa* lipase was then directly immobilized onto the modified nanoparticles through sol–gel encapsulation. The catalytic activities and enantioselectivities of the two encapsulated lipases in the hydrolysis reaction of (R/S)-naproxen methyl ester and (R/S)-2-phenoxypropionic acid methyl ester were assessed. The results showed that the activity and enantioselectivity of the lipase were improved when the lipase was encapsulated in the presence of calixarene-based additives; the encapsulated lipase with the phosphonic acid derivative of calix[4]arene had an excellent rate of enantioselectivity against the (R/S)-naproxen methyl and (R/S)-2-phenoxypropionic acid methyl esters, with $E = 350$ and 246, respectively, compared to the free enzyme. The encapsulated lipases (**Fe-Calix-N(COOH)**) and (**Fe-Calix–P**) showed good loading ability and little loss of enzyme activity, and the stability of the catalyst was very good; they only lost 6–11% of the enzyme's activity after five batches.

1. Introduction

Lipase is a very important enzyme that catalyzes a large number of processes such as hydrolysis, esterification, and transesterification reactions [1,2]. Therefore, this enzyme is among the most sought biocatalysts by the food industry, chemistry, and the pharmaceutical industry [3,4]. Among others, *Candida rugosa* lipase (CRL) has been widely studied for its selectivity with respect to specific functional groups and to stereo- and regioselectivity because of its high activity and broad specificity [5,6]. However, using a native enzyme as a biocatalyst presents several disadvantages, such as poor stability under operational conditions, difficulty of product recovery, high cost of operation, and the impossibility of multiple reuse in industrial processes [7]. The immobilization of free lipase onto solid supports, which have many advantages including easy recycling and reusing; large, specific surface areas; catalytic and operational stability; easy dispersion; reduction of cost; etc., via covalent binding [8–10], entrapment [11–13], or adsorption [14–18] can overcome those problems [19].

The sol–gel encapsulation method is particularly easy for preparation without any covalent modification compared to the other immobilization matrices. It also offers several advantages. It can entrap a large amount of enzymes, is chemically and thermally stable, and acts as an excellent catalyst in the kinetic resolution of chiral aromatic alcohols and carboxylic acids [20–26]. However, researchers would experience difficulty separating the nanoparticles from the solution. This problem can be resolved by using the magnetic property [3,27]. Fe_3O_4 nanoparticles have been considered suitable for the immobilization of enzymes because of their multifunctional characteristics, including small size, low toxicity, easy separation from the reaction medium, reusability, and, indirectly, lower processing costs [3,28,29]. Furthermore, with magnetic separation, there is no need for expensive and time-consuming techniques such as chromatography, centrifugation, and filtration.

Several methods have been recommended to improve the performance of lipase-catalyzed reactions for optical resolution. These methods include the optimization of reaction conditions, modification of substrate, optimization of the nature of the solvent [30] and of the water content [31], chemical and non-covalent modifications of enzymes, and the use of an additive that regulates lipase

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reactivity. Among these options, the additive method is the most advantageous [32]. However, while it is simple to use, only a few compounds have been reported to enhance the enantioselectivity of lipase reactions as an additive [33,34]. Several macrocyclic compounds such as cyclodextrins [35], crown ethers [36] and calix[n]arene derivatives have been used in this capacity [37,38].

Calixarenes are important building blocks in supramolecular chemistry. They can be selectively functionalized at both the phenolic OH groups (lower rim) and the *para* positions of the phenol rings (upper rim) to achieve desirable goals [39]. Regarding the immobilization of lipase via the sol–gel method, we have reported the use of calixarene derivatives as additives [40,41]. The results showed that the calix[n]arene-based encapsulated lipases had higher enantioselective conversion as compared to the sol–gel-free lipase in the hydrolysis reaction of (R/S)-naproxen methyl ester [42–46]. By introducing magnetic properties to organic molecules or to biomolecules, researchers can make the tasks of separation and reusable processes easy due to magnetic speciation.

Within this framework, there have been several works about the synthesis and characterization of the lower rim functionalized calix[4]arene derivatives grafted onto magnetic nanoparticles and evaluated for the enantioselective hydrolysis reactions [19,37,38,45,46]. However, up to this time, there has been no other reported study about upper rim functionalized calix[4]arene derivatives grafted onto magnetic nanoparticles and their catalytic activities toward enantioselective hydrolysis reactions. Therefore, the aim of the present study was to synthesize two upper rim-functionalized calix[4]arene derivatives bearing iminoacetic acid and/or phosphonate groups and immobilize them onto magnetic nanoparticles as the new additives for the sol–gel encapsulation of *C. rugosa* lipase. Furthermore, the activity and enantioselectivity of the encapsulated lipases were also evaluated under different conditions such as temperature and pH influences.

2. Materials and methods

2.1. Materials

Lipase from *C. rugosa* (type VII), bovine serum albumin used as the standard for protein assay, and *p*-nitrophenylpalmitate (*p*-NPP) used as the substrate to estimate the enzyme activity were supplied by from Sigma-Chemical Co. and OTES and TMOS were obtained by Merck. Without further purification or drying, high-performance liquid chromatography (HPLC) grade organic solvents were used as a mobile phase. All other chemicals used in this work were of reagent grade or analytical grade and were obtained from various commercial sources. IR spectra were performed on a Perkin Elmer spectrum 100 FT-IR spectrometer (ATR). UV/Vis spectra were measured with a Perkin Elmer Lambda 25 spectrometer. High performance liquid chromatography (HPLC) Agilent 1200 Series were performed using a 1200 model quaternary pump, equipped Diode Array detector. Pure *S*-naproxen was provided from TCI. Racemic naproxen was produced in the laboratory by the racemization of optically pure S-naproxen as described by Wu and Liu [47]. Racemic naproxen methyl ester has been prepared according to published method [48].

2.2. Synthesis

The parent compounds (**1**) and (**2**) were prepared according to published procedures. Calix[4]arene (**3**) containing phosphonate groups at the upper rim, was synthesized by the reaction with corresponding chloromethylated derivatives of calix[4]arene (**2**) which was synthesized according to modified literature procedure [51] and trimethyl phosphite in chloroform at reflux [52,53] (Scheme 2) while calix[4]arene (**4**) containing carboxyl groups was synthesized by the reaction with chloromethylated calix[4]arene (**2**) and iminodiacetic acid. Magnetite nanoparticles (Fe_3O_4) and epoxy groups modification on magnetite nanoparticles were prepared according to literature procedures [9,12,29]. The *p*-iminocarboxylic acid and *p*-phosphonate derivative of calix[4]arene immobilized magnetite nanoparticles (**Fe-Calix-N**(**COOH**) and (**Fe-Calix–P**) were herein reported for the first time.

2.2.1. Synthesis of p-iminocarboxylic acid derivative of calix[4]arene (*4*)

To a stirred solution of compound **2** (1 mmol) in dry acetonitrile (20 ml), pyridine (12 mmol) and 2-(carboxymethylamino) acetic acid (4.1 mmol) were added. The color of the mixture changed to pale purple from the colorless after 30 min. The reaction mixture was refluxed under nitrogen atmosphere for 24 h and then the solvent was removed under reduced pressure. The residue was treated with a 1 N HCl solution (50 mL) and extracted with dichloromethane 2 × 50 mL. The combined organic extracts were washed with water, dried over $MgSO_4$ and then the solvent was removed. The residue was treated with ethyl ether and chloroform and each time the solvent removed under reduced pressure. Product **4** was first obtained by precipitation with ethyl ether from the dichloromethane solution. The compound **4** was obtained in 42% yield as a cream colored solid with m.p. 158–159 °C. IR (ATR) (ν): 1733s (C=O) cm^{-1}. ^1H NMR (CD_3OD, 400 MHz): 6.98 (s, 8H, Ar-*H*), 4.23–4.15 (m, 20H, N*CH*$_2$CO and AB system ArCH$_2$Ar), 3.53 (d, 4H, *J* = 13.2 Hz, AB system Ar*CH*$_2$Ar), 3.21 (s, 8H, Ar*CH*$_2$N). Anal. Calc.: $C_{48}H_{52}N_4O_{20}$. C, 57.37; H, 5.22; N, 5.58%. Found: C, 57.41; H, 5.13; N, 5.66%.

2.2.2. Preparation of *Fe-Calix-N*(*COOH*) and *Fe-Calix–P*

The **Fe-Calix–P** (**5**) and **Fe-Calix-N**(**COOH**) (**6**) magnetite nanoparticles were prepared by the ring-opening polycondensation of the epoxy groups of bisglycidyl magnetite nanoparticles and the phenolic hydroxy groups of calix[4]arenes (**3**) and/or (**4**). A solution of corresponding calix[4]arene derivative (**3**) or (**4**) (1.12 mmol) and K_2CO_3 (4.5 mmol) was stirred in anhydrous acetonitrile (20 mL) and then magnetite nanoparticles modified with bisglycidyl groups (1.5 g) in 30 mL anhydrous acetonitrile was added dropwise to this mixture with continuous stirring at room temperature for about 30 min under nitrogen atmosphere. Then the reaction mixture was refluxed under a nitrogen atmosphere for 5 days. After magnetic separation, obtained magnetite nanoparticles were washed in sequence three times with warm toluene, methanol, acetone, dichloromethane and distilled water. The product was dried under vacuum at 70 °C for 3 h to give 1.5 g of magnetite nanoparticles modified with calixarene skeleton (**5**) and/or (**6**) and kept in desiccators before use.

2.2.2.1. ***Fe-Calix–P*** *(**5**).* According to the elemental analysis, the bonded calixarene amount onto Fe_3O_4 was found to be approximately 0.076 mmol of **3**/g of **Fe-Calix–P**. IR (ATR) mmax/cm^{-1}: 1603; 1475; 1227; 1196; 1029; 686; elemental analysis, C, 22.67; H, 2.55; P, 0.95%.

2.2.2.2. ***Fe-Calix-N***(***COOH***) (***6***). According to the elemental analysis, the bonded calixarene amount onto Fe_3O_4 was found to be approximately 0.134 mmol of **4**/g of **Fe-Calix-N**(**COOH**). IR (ATR) mmax/cm^{-1}: 1613; 1479; 1221; 1151, 626; 562, elemental analysis, C, 23.51; H, 3.21; N, 0.75%.

2.3. Sol–gel encapsulation of lipases

A modified method of Reetz [36] was carried out in the sol–gel encapsulation of lipase with or without additives including