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SHORT COMMUNICATION

A magnetically separable biocatalyst for resolution of racemic naproxen methyl ester

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Abstract *Candida rugosa* lipase (CRL) was encapsulated via the sol–gel method, using 5, 11, 17, 23-tetra-tert-butyl-25,27-bis(2-aminopyridine)carbonylmethoxy-26, 28-dihydroxy-calix[4]arene-grafted magnetic Fe_3O_4 nanoparticles (Calix-M-E). The catalytic activity of encapsulated lipase (Calix-M-E) was tested both in the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) and the enantioselective hydrolysis of racemic naproxen methyl ester. The present study demonstrated that the calixarene-based compound has the potential to enhance both reaction rate and enantioselectivity of the lipase-catalyzed hydrolysis of racemic naproxen methyl ester. The encapsulated lipase (Calix-M-E) had great catalytic activity and enantioselectivity ($E > 400$), as well as remarkable reusability as compared to the encapsulated lipase without supports ($E = 137$) for *S*-Naproxen.

Keywords Nanoparticles · Fe_3O_4 · Lipase · Calixarene · Enantioselectivity

Introduction

In recent years, the vast number of potential applications of superparamagnetic nanoparticles of iron oxides have been displayed for many biological fields, such as bioseparation [1, 2], tumor hyperthermia [3], magnetic resonance imaging (MRI) diagnostic contrast agents [4], magnetically guided site-specific drug delivery agents [5], and biomolecule immobilization [6–8].

The sol–gel process is a simplified method that is a more suitable platform for the immobilization of enzymes and other biological molecules [9, 10]. Unlike other immobilization matrices, sol–gel systems provide many advantages such as entrapment of large amounts of enzymes, thermal and chemical stability, simplicity of preparation without any covalent modification, and the flexibility to control pore size and geometry [9, 11, 12].

For more than three decades, calix[4]arenes have drawn attention in the fields of supramolecular chemistry and enantioselective separation of chiral-compounds, especially chiral-drugs, due not only to the molecule's typical bowl-shape which has been exploited in the formation of various nanometer-scale supramolecular architectures, but also to their rigid conformations, which could be utilized in self-assembly [13–15]. In addition, calix[4]arenes have a unique three-dimensional structure that provides almost unlimited derivatization possibilities [15].

Recently, calixarene derivatives were used as additives for lipase immobilization by the sol–gel method [16–18]. The results indicated that calixarene-based encapsulated lipases had particularly higher conversion and enantioselectivity in the hydrolysis reaction of racemic Naproxen methyl ester than the sol–gel free lipase. When introducing magnetic properties to calixarene derivatives, magnetic speciation makes it easier to separate from the reaction mixture, making it easier to reuse the process, as well [16]. The purpose of this study is to see the influences of Calix-M on lipase activity and enantioselectivity as a new additive in the sol–gel lipase encapsulation process.

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Materials and methods

Candida rugosa lipase (CRL), a commercial enzyme obtained from Sigma-chemical Co. (St. Louis, MO), was used in the immobilization. The solvents used in the HPLC analyses were HPLC grade (Merck, Germany). All aqueous solutions were prepared with deionized water that had passed through a Millipore Milli-Q Plus water purification system. All other chemicals used in this work were of analytical or of reagent grade, available from various commercial sources.

Calix-M was prepared according to the procedure in [8]. All sol–gel encapsulated lipases, with and without the magnetic calixarene derivatives, were prepared by adapting a known procedure [12]. The amount of protein in the enzyme solution and the elution solutions was determined by the Bradford method [19], using bovine serum albumin as a standard. The enzymatic activity of the encapsulated lipases was also determined by published method [20].

The reactions were carried out on a horizontal shaker, and samples drawn from the isooctane phase were analyzed by HPLC to calculate the conversion and enantioselectivity [9, 16, 21].

$$E = \frac{\ln[(1-x)\ (1-ee_s)]}{\ln[(1-x)\ (1+ee_s)]}$$

$$x = \frac{ee_s}{ee_s + ee_p} \qquad \mathrm{ee_s} = \frac{C_R - C_S}{C_R + C_S} \qquad \mathrm{ee_p} = \frac{C_S - C_R}{C_S + C_R}$$

.

E, $\mathrm{ee_s}$, $\mathrm{ee_p}$, x, C_R and C_S indicate enantiomeric ratio for irreversible reactions, enantiomeric excess of substrate, enantiomeric excess of product, racemate conversion, concentration of R-enantiomer and concentration of S-enantiomer, respectively.

Results and discussion

In our previous work, *Candida rugosa* lipase was encapsulated in the presence of *N*-methylglucamine derivative of calix[4]arene-grafted magnetic nanoparticles as additive and used for enantioselective hydrolysis of naproxen methyl ester. The results showed that the encapsulated lipase particularly had higher conversion and enantioselectivity. In the literature [8, 15] it has been reported that di-substituted calix[4]arene amide derivative bearing pyridinium units, acted as a receptor for molecules, ions and effective binding sites. Thus, in this study, we aimed to synthesized a calix[4]arene receptor bearing pyridinium units and grafted onto grafted magnetic Fe_3O_4 nanoparticles (Calix-M-E).

When *Candida rugosa* lipase was immobilized on Calix-M (Fig. 1), separating the lipase from the reaction mixture could be easier and reused with consistent results, as well.

Scanning electron micrographs comparing the sol–gel encapsulated lipase (Calix-M-E) with that containing the Calix-M showed that after encapsulation, the irregular surface cavities of the immobilized Calix-M had become a porous surface (Fig. 2).

The hydrolytic activity of the encapsulated lipases was evaluated in the hydrolysis of *p*-Nitrophenyl palmitate (*p*-NPP) (Table 1). The Calix-M-E was performed to give 295 U/g of support while the encapsulated lipase without the Calix-M was found to give 95. The activity results given in Table 1 imply that the encapsulated lipase with pyridyl derivative of calix[4]arene-grafted magnetic nanoparticles (Calix-M-E) was more efficient than the encapsulated lipase without support. This is not a surprising result, because the

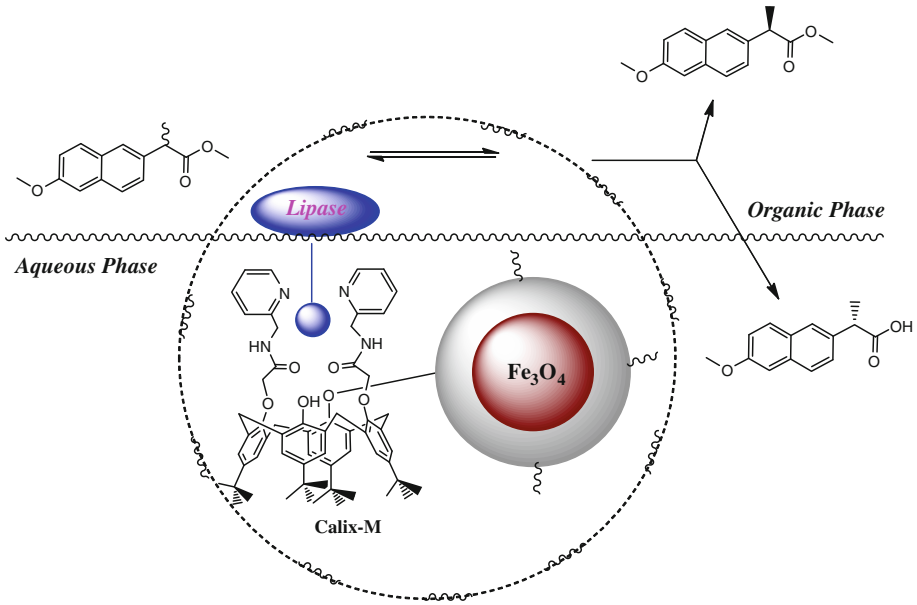

Fig. 1 The suggested resolution mechanism of racemic naproxen methyl ester

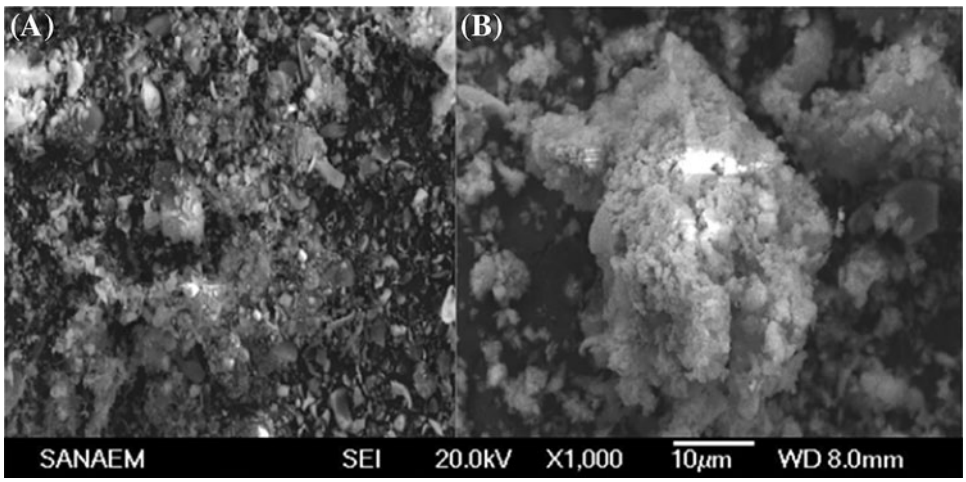

Fig. 2 SEM images of (**a**) Calix[4]arene-grafted magnetic nanoparticles (Calix-M) and (**b**) lipase encapsulated Calix[4]arene-grafted magnetic nanoparticles (Calix-M-E)

Table 1 Enantioselective hydrolysis of racemic naproxen methyl ester and activity of the encapsulated lipases under optimum reaction conditions

	Encap. protein (mg/g)	Lipase activity (U/g support)	Specific activity (U/mg protein)	x (%)	ee_s (%)	ee_p (%)	E
Lipase-encap[a]	28.6	95	3.30	20	25	>98	137
Calix-M-E	39.5	295	7.47	49	98	>98	>400

[a] Encapsulated lipase without Calix-M

calix[4]arene-based compounds containing these groups are highly effective complexing agents [15], which means pyridyl groups interact with lipase by a combination of hydrogen bonding and electrostatic interactions. In this sol-gel process, the lipase is not only physically encapsulated, but also bound by additional multipoint interactions through hydrogen bonding, ionic, and hydrophobic interactions. Moreover, this could be explained by the modification in the three-dimensional structure of the enzyme, which leads to a conformation change of the active center. The presence of the matrix hinders the accessibility of substrate to the enzyme-active site, and limits mass transfer of substrate and product [9].

The reaction with Calix-M-E was terminated after 24 h, obtaining naproxen methylate (unreacted *R*-ester) and corresponding acid (ee_p) 98 % at conversion of 49 % and the enantioselectivity being very high ($E > 400$). Whereas the resolution reactions with encapsulated lipase (without Calix-M-E), gave an unreacted naproxen methylate (*R*)-ester, a corresponding acid (ee_p) 98 % at the conversion rate of 20 %, and an enantioselectivity (*E*) of 137. Sol–gel encapsulation led to high enantioselectivity, high conversion, and fast recovery of the product. The reason for improvement of enantioselectivity by immobilization is not yet well understood. However, some literature [22] reported that this phenomenon might be attributed to some distortion in the protein conformation, reducing the overall flexibility of the enzyme molecules generated from the interactions between enzyme and the supports during the immobilization.

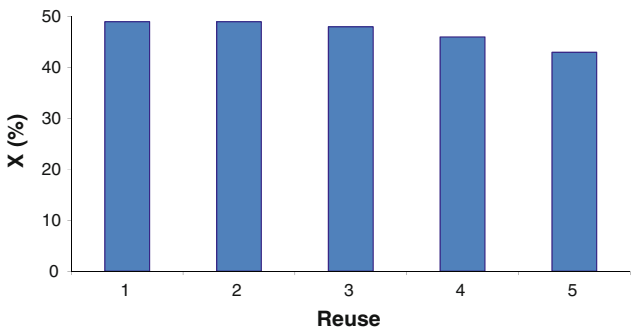

Fig. 3 Reusability on the conversion (x) in the hydrolysis of racemic naproxen methyl ester

The reusability of Calix-M-E is also important for economical use of the enzyme, which is very easy due to its magnetic properties. Figure 3 shows that the immobilized lipases still retained 43 % of their conversion ratios for Calix-M-E after the fifth reuse cycle. These results are due to inactivation of the enzyme denaturation of protein and the leakage of protein from the supports upon use [23].

Conclusions

In conclusion, Calix-M was found to be a useful support for lipase encapsulation. The encapsulated lipase was taken out of the reaction medium via magnetic decantation using a simple magnet, and then reused a couple of times. Reusing

the encapsulated enzyme five times did not result in a significant loss in enantioselectivity.

The resolution studies using sol–gel support have observed more improvement in the enantioselectivity of naproxen in ee_s 98 %, and $E > 400$ (threefold increase in E values) with Calix-M-E than with encapsulated lipase without Calix-M. This work not only shows a significant advancement in improvement of lipase-catalyzed enantioselective hydrolysis reactions but also provides an interesting combined use of calixarene with enzyme. The approach is therefore recommended as a new technique to regulate enzymatic reactions by chemical reagents.

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References

1. Gupta AK, Gupta M (2005) Synthesis and surface engineering of iron oxide nanoparticles for biomedical applications. Biomaterials 26:3995–4021
2. Krizzova J, Spanova A, Rittich B, Horak D (2005) Magnetic hydrophilic methacrylate-based polymer microspheres for genomic DNA isolation. J Chromatogr A 1064:247–253
3. Ito A, Shinkai M, Honda H, Kobayashi T (2005) Medical application of functionalized magnetic nanoparticles. J Biosci Bioeng 100:1–11
4. Mornet S, Vasseur S, Grasset F, Goglio G, Demourgues A, Portier J et al (2006) Magnetic nanoparticle design for medical applications. Prog Solid State Chem 34:237–247
5. Neuberger T, Schopf B, Hofmann H, Hofmann M, von Rechenberg B (2005) Superparamagnetic nanoparticles for biomedical applications: possibilities and limitations of a new drug delivery system. J Magn Magn Mater 293:483–496
6. Campo AD, Sen T, Lellouche JP, Bruce IJ (2005) Multifunctional magnetite and silica-magnetite nanoparticles: synthesis, surface activation and applications in life sciences. J Magn Magn Mater 293:33–40
7. Saiyed ZM, Sharma S, Godawat R, Telang SD, Ramchand CN (2007) Activity and stability of alkaline phosphatase (ALP) immobilized onto magnetic nanoparticles (Fe_3O_4). J Biotechnol 131:240–244
8. Sayin S, Yilmaz M (2011) Preparation and uranyl ion extraction studies of calix[4]arene-based magnetite nanoparticles. Desalination 276:328–335
9. Yilmaz E, Sezgin M, Yilmaz M (2011) Immobilization of Candida rugosa lipase on magnetic sol-gel composite supports for enzymatic resolution (R, S)-Naproxen methyl ester. J Mol Catal B Enzym 69:35–41
10. Brinker CJ, Scherer GW (1990) Sol–gel Science: the physics and chemistry of sol-gel processing. Academic Press, Boston
11. Avnir D, Braun S, Lev O, Ottolenghi M (1994) Enzymes and other proteins entrapped in sol-gel materials. Chem Mater 6:1605–1614
12. Reetz MT, Tielmann P, Wisenhofer W, Konen W, Zonta A (2003) Second generation sol–gel encapsulated lipases: robust heterogeneous biocatalysts. Adv Synth Catal 345:717–728
13. Sirit A, Yilmaz M (2009) Chiral Calixarenes. Turk J Chem 33:159–200
14. Bozkurt S, Durmaz M, Naziroglu HN, Yilmaz M, Sirit A (2012) Chiral calix[4]arenes bearing amino alcohol functionality as membrane carriers for transport of chiral amino acid methylesters and mandelic acid. Chirality 24:129–136
15. Yilmaz M, Sirit A, Deligoz H (2011) Calixarene based materials for cations and anions in sorption processes and pollution: conventional and non-conventional sorbents for pollutant removal from wastewaters. In: Badot PM, Crini G (eds) Universitaires de Franche-Comté, Besancon-Cedex
16. Sayin S, Yilmaz E, Yilmaz M (2011) Improvement of catalytic properties of Candida rugosa lipase by sol–gel encapsulation in the presence of magnetic calix[4]arene nanoparticles. Org Biomol Chem 9:4021–4024
17. Sahin O, Erdemir S, Uyanik A, Yilmaz M (2009) Enantioselective hydrolysis of (R/S)-Naproxen methyl ester with sol-gel encapsulated lipase in presence of calix[n]arene derivatives. Appl Catal A 369:36–41
18. Uyanik A, Sen N, Yilmaz M (2011) Improvement of catalytic activity of lipase from *Candida rugosa* via sol-gel encapsulation in the presence of calyx (aza) crown. Bioresource Technol 102:4313–4318
19. Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254
20. Chiou SH, Wu WT (2004) Immobilization of *Candida rugosa* lipase on chitosan with activation of the hydroxyl groups. Biomaterials 25:197–204
21. Chen CS, Fujimoto Y, Girdaukas G, Sih CJ (1982) Quantitative analyses of biochemical kinetic resolutions of enantiomers. J Am Chem Soc 104:7294–7299
22. Fernandez-Lorente G, Terreni M, Mateo C, Bastida A, Fernandez-Lafuente R, Dalmases P (2001) Modulation of lipase properties in macroaqueous system by controlled enzyme immobilization: enantioselective hydrolysis of a chiral ester by immobilized Pseudomonas lipase. Enzyme Microb Technol 28:389–396
23. Yilmaz E, Sezgin M (2012) Enhancement of the activity and enantioselectivity of lipase by sol-gel encapsulation immobilization onto β-Cyclodextrin based polymer. App Biochem Biotechnol 166:1927–1940