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COMMUNICATION

Improvement of catalytic properties of *Candida Rugosa* lipase by sol–gel encapsulation in the presence of magnetic calix[4]arene nanoparticles

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***Candida rugosa* lipase (CRL) was encapsulated within a chemically inert sol–gel support prepared by polycondensation with tetraethoxysilane (TEOS) and octyltriethoxysilane (OTES) in the presence of *N*-methylglucamine based calix[4]arene magnetic nanoparticles. The results indicate that the magnetic calix[4]arene based encapsulated lipase particularly has shown high conversion and enantioselectivity. It has also been noticed that the magnetic calix[4]arene based encapsulated lipase has excellent enantioselectivity (E = 460) as compared to the free enzyme (E = 166) with an ee value of >98% for *S*-Naproxen.**

Calixarenes are important building blocks in supramolecular chemistry.[1] They can be selectively functionalized both at the phenolic OH groups (lower rim) and at the *para* positions of the phenol rings (upper rim).[2] Calixarenes can be functionalized to achieve desirable goals. In this study, we prepared *N*-methylglucamine based calix[4]arene magnetic nanoparticles to use enantioselective hydrolysis of Naproxen methyl ester in order to study the role of *N*-methylglucamine based calix[4]arene magnetic nanoparticles binding site on the lipase activity and stability.

2-Arylpropionic acids (*e.g.* Naproxen), as an important class of non-steroidal anti-inflammatory drug (NSAID), have their pharmacological activity mainly in the (*S*)-enantiomer, which is widely used for the treatment of rheumatic diseases and related painful conditions.[3,4] The pro-drugs of Naproxen with the highest aqueous solubility were the most effective prodrug to deliver Naproxen through the skin. In this case, it is important to obtain enantioselectively pure Naproxen.

Recently, magnetic nanoparticles of iron oxide have shown great potential applications in many fields like bioseparation,[5,6] tumor hyperthermia,[7] magnetic resonance imaging (MRI), diagnostic contrast agents,[8] magnetically guided site-specific drug-delivery agents,[9] biomolecule immobilization,[10,11] as well as having been used as support material for some selective calixarene derivatives toward arsenate or dichromate ions.[12] By introducing magnetic properties to organic or biomolecules, separation and reusable processes becomes an easy task due to magnetic speciation. Herein, we report the stability and enzymatic activity of *Candida rugosa* lipase immobilized on *N*-methylglucamine based on calix[4]arene magnetic nanoparticles.

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Lipases are enzymes that catalyse a variety of reactions such as esterification, interesterification and hydrolysis. Because of their selectivity, lipases are important biocatalysts in several applications such as the synthesis of chiral drug intermediates and nutraceutical lipids.[13–15] In particular, *Candida rugosa* lipase is an important industrial lipase. It is well known that *Candida rugosa* is used in a wide variety of hydrolysis and esterification reactions.[16] Recently, *Candida rugosa* has been used in the enantioselective hydrolysis reaction of Naproxen methyl ester.[15–17] Here, it has been shown that *Candida rugosa* lipase, when immobilized on *N*-methylglucamine based calix[4]arene magnetic nanoparticles, can easily be separated from the reaction mixture, stored, and also reused with consistent results. To obtain the desired goal, we synthesized *p-tert*-butylcalix[4]arene (**1**), calix[4]arene (**2**), *N*-methylglucamine based calix[4]arene derivative **3** and silica based Fe_3O_4 magnetic nanoparticles according to the our previous work.[12] Typically, the substitution of calix[4]arene (**2**) at its upper rim was conducted with a secondary amine (*N*-methylglucamine) and formaldehyde (Mannich reaction) to afford the cone conformer **3** (see Fig. 1).[12] Fe_3O_4 nanoparticles and 2-hydroxypropoxy propyl silica based Fe_3O_4 nanoparticles were prepared according to the stated method.[12] *N*-Methylglucamine based calix[4]arene was grafted onto silica based Fe_3O_4 magnetic nanoparticles[18] (see Fig. 1). This compound was characterized by a combination of IR, TGA and TEM. From the IR spectrum, it was observed that the stretching vibration modes of the Fe–O band, amine band, aromatic C=C bands, and Si–O bands are at 568, 1657, 1467, 1199, 1081, 958 and 796 cm^{-1}, respectively.

Thermal properties of immobilized calix[4]arene magnetite nanoparticles were analyzed by thermogravimetric methods. The indication of coating formation on the magnetic nanoparticles surface can be obtained from TGA measurements. Upon heating, the weight loss of silica-based magnetic nanoparticles (EPPTMS-MN) was shown to be about 5% within a broad temperature range of 250 to 650 °C by decomposition of 3-(2,3-epoxypropoxy)-propyl groups. Similarly, the weight loss of calix[4]arene immobilized with magnetic nanoparticles indicated one-step thermal degradation in the same temperature range. The step arises from decomposition of both calix[4]arene units and 3-(2,3-epoxypropoxy)-propyl groups (26%).

To the best of our knowledge, there is no report on the use of *N*-methylglucamine based calix[4]arene magnetic nanoparticles as support for immobilization of *Candida rugosa* lipase. It has been thought that the sol–gel procedure could be interesting for

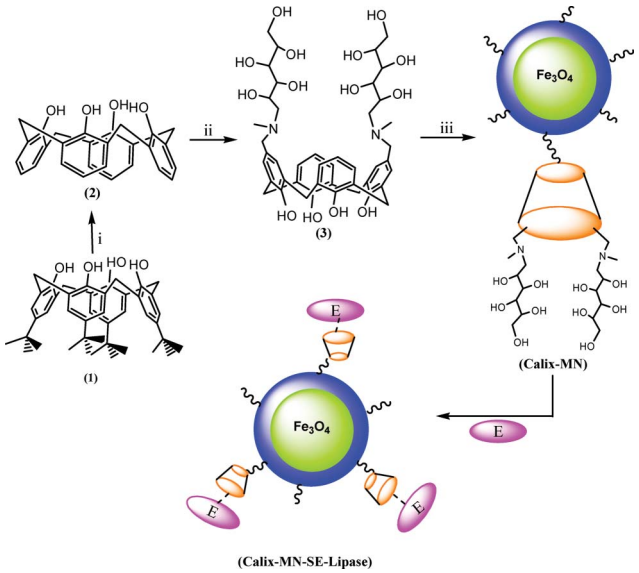

Fig. 1 The sol–gel encapsulation procedure of Calix-MN-SE-Lipase. Reaction conditions: (i) $AlCl_3$, phenol, toluene; (ii) *N*-methylglucamine, formaldehyde, CH_3COOH, THF; (iii) EPPTMS-MN, K_2CO_3, CH_3CN.

lipase immobilization (see Fig. 1). Using sol–gel materials for mechanical entrapment of enzymes allowed stabilization of the proteins tertiary structure because of the tight gel network.[15,19] In the sol–gel encapsulation method, crown ether derivatives and cyclodextrins as macrocyclic compounds have been used whereas the calixarene magnetic nanoparticles have not been studied up until now.

All sol–gel encapsulated lipases with and without the magnetic calixarene derivatives were prepared by adapting a known procedure.[20] For the lipase immobilization onto *N*-methylglucamine based calix[4]arene magnetic nanoparticles, *Candida rugosa* lipase was reacted with *N*-methylglucamine based calix[4]arene magnetic nanoparticles under vigorous stirring on a horizontal shaker at room temperature. Then aqueous PVA, aqueous sodium fluoride and isopropyl alcohol were added, and the mixture homogenized using a shaker. To that solution, the OTES and TEOS were added and the mixture agitated once more. The resulting encapsulated lipases were held at 4 °C prior to use.[21] The amount of protein in the enzyme solution and the elution solutions was determined by the Bradford method[22] using bovine serum albumin as a standard.

In order to obtain more direct information on particle size and morphology, TEM micrographs of Fe_3O_4 magnetic nanoparticles and encapsulated lipase were recorded (Fig. 2a and 1b). From the photograph (Fig. 2a) the nanoparticles are observed (Fig. 2a) as dense aggregates due to the lack of any repulsive force between the magnetite nanoparticles. It is mainly due to the nano-size of the silica-based Fe_3O_4, which is about 10 ± 2 nm. This may be considered as indirect evidence that the magnetic core of the silica-based magnetic particles consisted of a single magnetic crystallite with a typical diameter of 8 ± 3 nm, and that the difference corresponds to the EPPTMS coating. After calix[4]arene immobilization, the dispersion of particles was improved (Fig. 2b). Following encapsulated lipase, the dispersion was increased greatly to about 15 ± 2 nm. This situation can easily be explained by the electrostatic repulsion force and steric hindrance between the calix[4]arenes or amplitude lipase enzyme on the surface of Fe_3O_4 nanoparticles.

The amount of protein in the enzyme solution and the elution solution was determined by the Bradford method[22] using bovine serum albumin as a standard. The enzymatic activity of the encapsulated lipases was also determined by published methods.[23,24]

From the catalytic activity results of the sol–gel encapsulated lipases (see Table 1), it was concluded that the encapsulated lipase with *N*-methylglucamine based calix[4]arene magnetic nanoparticles was more efficient than the activity of free lipase. This is not a surprising result owing to the higher number of –OH groups and also amine groups of *N*-methylglucamine based calix[4]arene magnetic nanoparticles. It is well known that compounds containing these groups are highly effective complexing agents, which means –OH and amine groups interact with lipase by a combination of hydrogen bonding and electrostatic interactions.

To elucidate the hydrolysis capability of encapsulated lipase against Naproxen methyl ester, HPLC was used to calculate the conversion and enantioselectivity.[25] For this purpose, hydrolysis reactions were maintained in an aqueous phase–organic solvent batch reaction system that consisted of isooctane as solvent

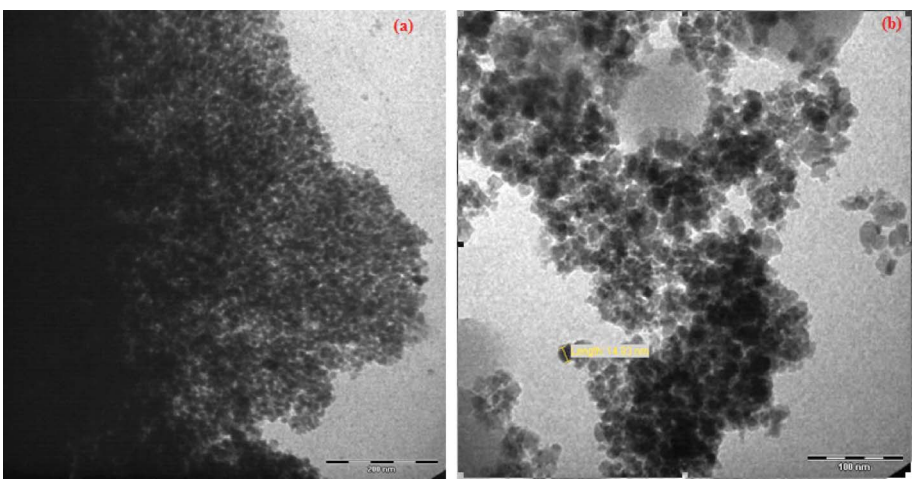

Fig. 2 TEM micrograph images of (a) Fe_3O_4 magnetic nanoparticles, (b) Calix-MN-SE-Lipase.

Table 1 Activities and protein amounts of encapsulated lipases

Additives used in the sol–gel process	Protein loading (mg per g of sol–gel)	Catalytic activity (U min−1 per g of sol–gel)	Specific activity (U per mg of protein)	Activity yield (%)
Calix-MN-SE-Lipase	37.8	227.7	6.0	181
Free Lipase[a]	28.6	95.1	3.3	100[b]

[a] Encapsulated free lipase without magnetic calixarene nanoparticles. [b] Activity yield for free lipase was defined as 100%.

Table 2 The enantioselective hydrolysis of racemic Naproxen methyl ester of using sol–gel encapsulated lipases as catalysts

Additives used in the sol–gel process	X (%)	ee_s (%)	ee_p (%)	E
Calix-MN-SE-Lipase	50.0	98.0	>98	460
Free Lipase[a]	37.9	60.2	>98	166

[a] Encapsulated free lipase without magnetic calixarene nanoparticles. Where E, ee_s, ee_p, X, are the enantiomeric ratio for irreversible reactions, enantiomeric excess of substrate, enantiomeric excess of product and racemate conversion, respectively.

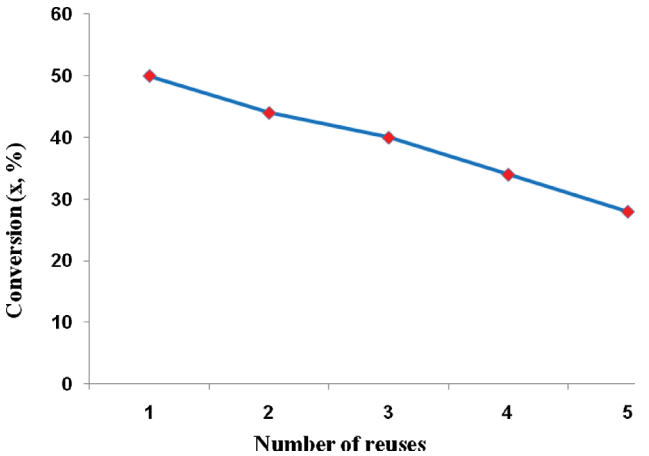

Fig. 3 Reusability *vs.* the conversion (X) in the hydrolysis of racemic Naproxen methyl ester.

dissolving racemic Naproxen methyl ester and buffer solution including encapsulated lipase (Calix-MN-SE-Lipase). The reactions were carried out on a horizontal shaker and samples drawn from isooctane phase were analyzed by HPLC to calculate the conversion and enantioselectivity.[26] The enantioselectivity was expressed as the enantiomeric ratio (E) calculated from the conversion (X) and the enantiomeric excess of the substrate (ee_s) and the product (ee_p) using the equation given in literature.[27]

Table 2 depicts the enantioselective hydrolysis of racemic Naproxen methyl ester by sol–gel encapsulated lipases in the system of aqueous buffer solution and isooctane. The resolution reaction with encapsulated lipase (Calix-MN-SE-Lipase) was terminated after 24 h, giving Naproxen methylate (*R*-ester) and the corresponding acid $ee_p > 98\%$ at a conversion of 50% and the enantioselectivity being very high at 460. Whereas, in the resolution reactions with encapsulated free lipase, unreacted Naproxen methylate (*R*-ester) and the corresponding acid $ee_p > 98\%$ were obtained at a conversion of 37.9% and the enantioselectivity (E) was 166. Immobilization led to high enantioselectivity, high conversion and fast recovery of product as compared to encapsulated free enzyme. These results confirm data in the literature[28] that indicates a considerable increase in catalytic activity of immobilized lipase by treating the support with additives. According to a published report, this phenomenon might be attributed to some distortion on the protein conformation, reducing the overall flexibility of the enzyme molecules generated from the interactions between the enzyme and the support during the immobilization.[29]

Additionally, the recovery and reusability of encapsulated lipase nanoparticles (Calix-MN-SE-Lipase) is also important for economical use of the enzyme, which is very easy due to its magnetic properties. Fig. 3. shows that the immobilized lipases still retained 28% of their conversion ratios for Calix-MN-SE-Lipase after the 5th reuse cycle. These results may be due to the inactivation of the enzyme denaturation of protein and the leakage of protein from the supports upon use. In conclusion, this (so-far unreported) long-term stability and reusability illustrates the advantage of attaching the enzymes chemically to the nanoparticles. Such stability might make the use of expensive enzymes economically viable and hence open a new horizon for enzymatic catalysis in biotechnology.

Acknowledgements

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