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ORIGINAL ARTICLE

Effect of the glutaraldehyde derivatives of Calix[n]arene as cross-linker reagents on lipase immobilization

Serkan Erdemir · Ozlem Sahin · Arzu Uyanik · Mustafa Yilmaz

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Abstract Synthesis of the glutaraldehyde derivatives calix[n]arene (n = 4,6,8) (Calix[n]-GA) and using as cross-linkers for immobilization of *Candida rugosa* lipase (CRL) have been discussed in this paper. The amino functional calix[n]arene derivatives (Calix[n]-NH_2) were synthesized via reduction of *dinitrile*, *hexanitrile* and *octanitrile* derivatives of calix[n]arenes. These amino functional calix[n]arene derivatives (Calix[n]-NH_2) were converted to their aldehyde derivativatives with glutaraldehyde. The calix[n]arene derivatives were used in lipase immobilization in order to see the role of calix[n]arene binding site on the lipase activitiy and stability. The activity recovery of calix[n]arene-supported lipases (Calix[n]-CRL) based on the Calix[4]-CRL, Calix[6]-CRL and Calix[8]-CRL reaches to 53.5, 66.1 and 76.4%, respectively.

Keywords Calixarene · Cross-linker · Immobilization · *Candida rugosa* lipase · Glutaraldehyde

Introduction

Lipases (E.C. 3.1.1.3) occupy a place of prominence among biocatalysts owing to their multiple and novel multifold applications in oleo-chemistry, organic synthesis, detergent formulation and nutrition [1]. In the last few years, there has been an increasing interest in the use of enzymes for the biosynthesis of molecules in organic media [2–4]. In order to use them more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity, and operational stability can be modified by immobilization. Immobilized enzymes have received considerable attention because of their advantages over unimmobilized counter parts as they improve storage, operational, thermal and conformational stabilities. They can be easily recovered for reuse [5]. As the immobilization method, covalent binding, electrostatic binding, hydrophobic interactions, entrapment and encapsulation are often used for enzyme immobilization [6–10].

The immobilization of enzymes is carried out by the formation of inter- and intra-molecular cross-linkages between the enzyme molecules by means of bifunctional reagents. Glutaraldehyde has been used as a cross linker for immobilization of enzymes in which the amino groups of a protein is expected to form a Schiff base with the glutaraldehyde [11–14]. However, in terms of stabilization, the treatment with glutaraldehyde of proteins previously adsorbed on supports bearing primary amino groups offers very good results in many cases, because it permits the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support.

Over the last decade, many crown ether activation of enzymes in organic media have been reported [15]. Mainly, crown ethers form complexes with surface ammonium groups of lysine.

Calixarenes (Fig. 1), cyclic oligomers of phenolic units linked through the ortho positions, are a fascinating class of macrocycles. They are synthetic macrocycles readily available by condensation of *p-tert*-butylphenol with formaldehyde under alkaline conditions. They have generated considerable interest as useful building blocks for the synthesis of hosts for cations, anions and neutral molecules. Due to this ability to form host–guest type

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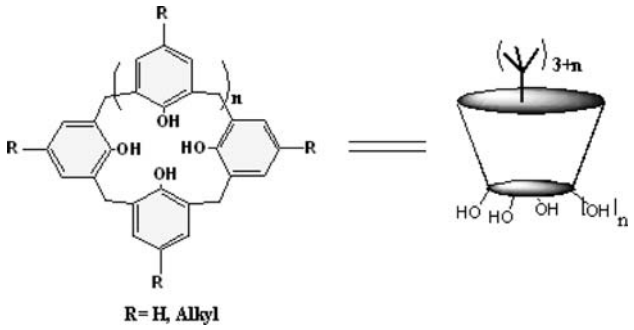


Fig. 1 The general molecular structures of calix[*n*]arenes

complexes with a variety of organic or inorganic compounds, the calixarenes have received increasing attention during the last two decades [16, 17]. The increasing interest in these compounds is stimulated by the simple large-scale synthesis of calixarenes, and the different ways in which they can be selectively functionalized from the narrow (phenolic groups) or wide rim (aromatic nuclei) [18–21].

Previously, we synthesized various calix[4]arene derivatives, immobilized them onto different supporting materials and tested for their molecular and enantiomeric recognition capabilities [22–25]. In addition, we have reported [26] the synthesis of a new calix[4]arene 1,3-distal glutaraldehyde derivative as a cross-linker-reagent for immobilization of *Candida rugosa* lipase (CRL). In this work, a series of wide-size cavity aldehyde pointed calix[n]arenes (Calix[n]-GA,n = 4,6,8) (see Scheme 1) as cross-linker-reagent for lipase immobilization (see Scheme 2) was synthesized and investigated the impact of these calix[n]arene derivatives on *Candida rugosa* lipase (CRL) activity and stability.

Experimental

Materials

Candida rugosa lipase (Type VII), a commercial enzyme obtained from Sigma-chemical Co., (St. Louis, MO) was used in the immobilization. Bradford reagent, Bovine Serum Albumin 99% (BSA), *p*-nitrophenylpalmitate (*p*-NPP) and glutaraldehyde were purchased from Sigma-chemical Co., (St. Louis, MO). All starting materials and reagents used were of standard analytical grade from Fluka, Merck as well as from Aldrich and used without further purification. Other commercial grade solvents were distilled, and then stored over molecular sieves. The drying agent employed was anhydrous $MgSO_4$. All aqueous solutions were prepared with deionized water that had been passed through a Millipore milli Q Plus water purification system.

Apparatus

Melting points were determined on an Electrothermal 9100 apparatus in a sealed capillary and are uncorrected. ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer in $CDCl_3$ at room temperature unless otherwise specified. IR spectra were obtained on a Perkin Elmer 1605 FT-IR spectrometer using KBr pellets. UV-Visible spectra were obtained on a Shimadzu 160A UV-Visible recording spectrophotometer. Elemental analyses were performed using a Leco CHNS-932 analyzer. A Crison MicropH 2002 digital pH meter was used for the pH measurements. Analytical TLC was performed using Merck prepared plates (silica gel 60 F_{254} on aluminum). Morphology of the polymers before and after lipase immobilization was investigated using a scanning electronic microscope (SEM, Jeol, JSM 5310, and Japan) at 10 kV without gold sputtering.

Synthesis

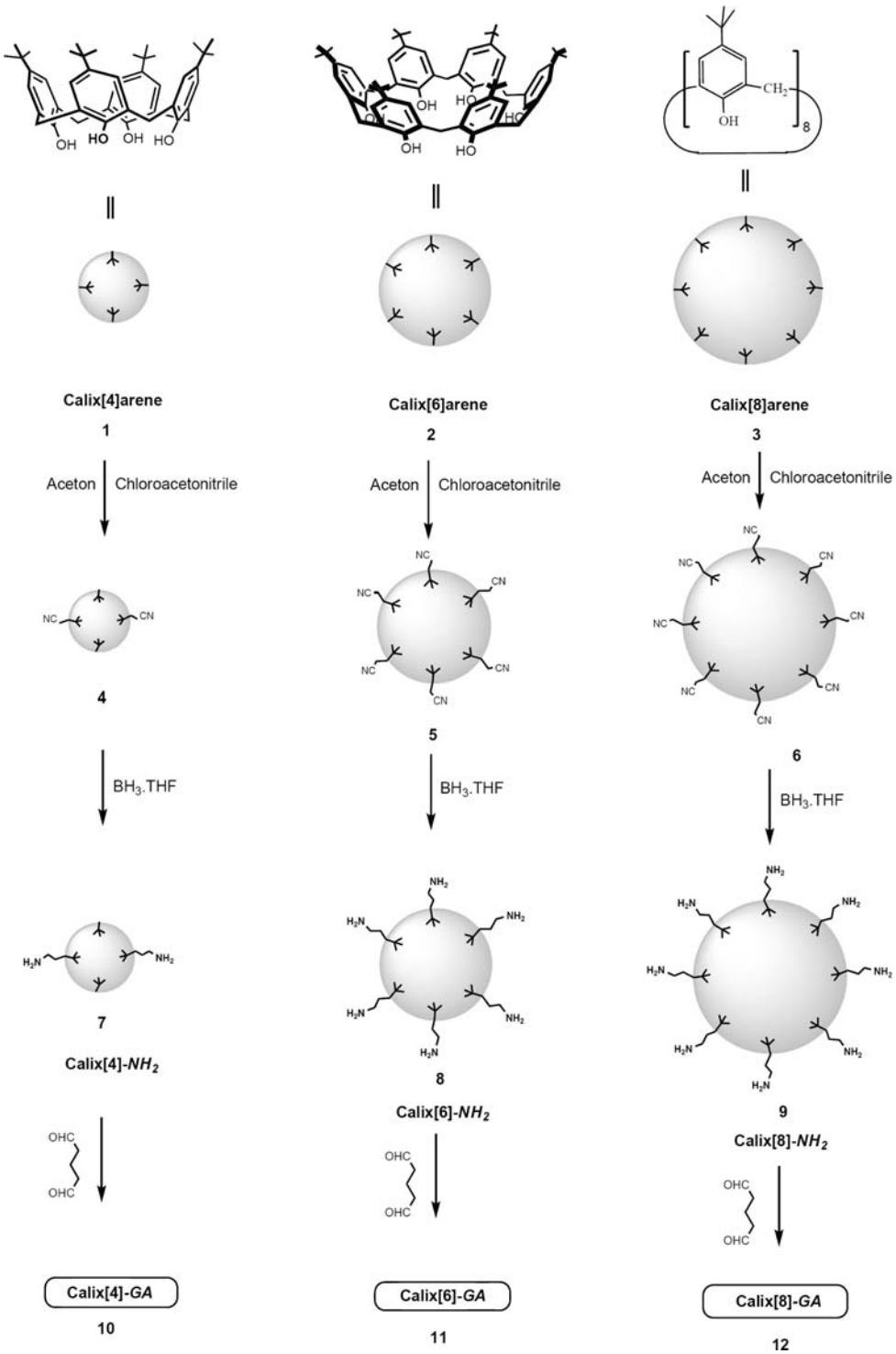
The *p-tert*-Butylcalix[n]arenes (n = 4,6,8) **1–3** and their nitrile derivatives **4–6** were synthesized according to the literature procedures [27–30].

The amino functional calix[n]arenes **(7–9)** were synthesized via reduction of nitrile derivatives of calix[n]arenes **(4–6)** by adapting known synthetic procedure [31].

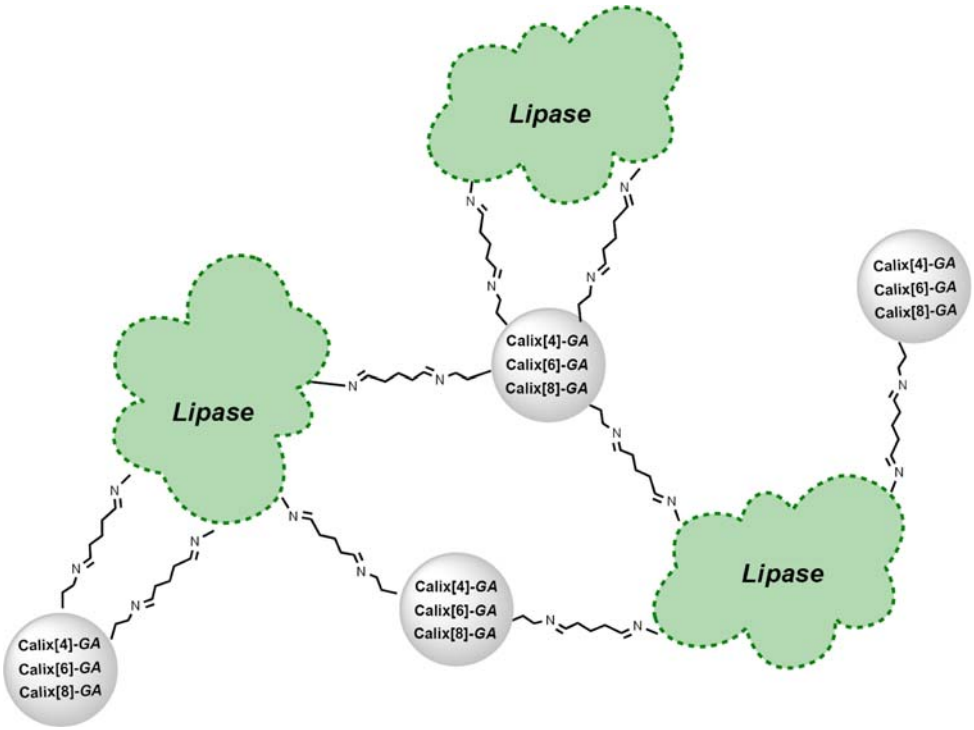
Synthesis of amino functional calix[n]arene derivatives

A solution of borane in THF (12 mL of 1 M) was added to a stirred suspension of **4, 5** and **6** (1 mmol) in THF (18 mL). The mixture was stirred for 1 h at ambient temperature, then refluxed for 5 h. After 40 min reflux, a clear solution formed. The mixture was allowed to cool to ambient temperature, and an additional aliquot (8 mL) of the borane solution added. The mixture was refluxed for an additional 12 h, and allowed to cool. A solution of HCl (60 mL of 6 N) was added, and the mixture refluxed for a further 1 h. The THF was removed on a rotary evaporator to yield a colorless suspension. The mixture was kept at 0°C for 12 h, and the solid was filtered, washed with water and dried in vacuo at ambient temperature for 24 h. For the Calix[4]-NH_2, yield; 90%, m.p; 207–210 °C (Lit., 210–211 °C). ^{1}H-NMR ($CDCI_3$) δ (ppm): 1.25 (s, 18H, Bu^t), 1.31 (s,18H, Bu^t), 2.76 (br. s, 4H, NH_2), 3.32 (t, 4H, J = 8 Hz, CH_2CH_2 N), 3.39 (d, 4H, J = 12.8 Hz, $ArCH_2Ar$), 4.08 (t, 4H, J = 8.8 Hz, OCH_2CH_2), 4.34 (d, 4H, J = 12.8 Hz, $ArCH_2Ar$), 7.00–7.10 (m, 10H, ArH, ArOH). Anal. calcd for $C_{48}H_{64}O_4N_2$: C, 78.69; H, 8.74; N, 3.83; Found: C, 78.21; H, 8.23; N; 3.54. For the Calix[6]-NH_2, yield; 82%, m.p; 210–212 °C. ^{1}H-NMR ($CDCI_3$) δ (ppm): 1.35 (s, 54H, Bu^t), 2.54 (br. s, 12H, NH_2), 3.32 (t, 12H, J = 8,1 Hz, CH_2CH_2 N), 4.21 (d, 12H,

Scheme 1 Synthesis of the aldehyde pointed calix[n]arene derivatives



$J = 12.8$ Hz, $ArCH_2Ar$), 4.27 (t, 12H, $J = 8.8$ Hz, OCH_2CH_2), 7.15 (s, 12H, ArH). Anal. calcd for $C_{78}H_{114}O_6N_6$: C, 76.09; H, 9.27; N, 6.83; Found: C, 76.21; H, 9.34; N; 6.54. For the Calix[8]-*NH*$_2$, yield; 89%, m.p; 234–238 °C. ^{1}H-NMR ($CDCI_3$) δ (ppm): 1.02 (s, 72H, Bu^t), 2.14 (br. s, 16H, NH_2), 3.15 (t, 16H, $J = 8{,}3$ Hz, CH_2CH_2 N), 4.21 (d, 12H, $J = 12.8$ Hz, $ArCH_2Ar$), 4.37 (t, 16H, $J = 8.4$ Hz, OCH_2CH_2), 7.09 (s, 12H, ArH). Anal. calcd for $C_{104}H_{152}O_8N_8$: C, 76.09; H, 9.27; N, 6.83; Found: C, 76.32; H, 9.21; N; 6.98.



Scheme 2 Proposed interactions of the aldehyde pointed calix[n]arene with *Candida rugosa* lipase (CRL)

Treatment of amino functional calix[n]arene derivatives with glutaraldehyde (Calix[n]-GA)

0.5 g of Calix[n]-NH_2 (n = 4,6,8) was dissolved in 10 mL of CH_2Cl_2. The solution was treated with a 10 mL of 25% aqueous glutaraldehyde solution. The mixture was stirred at room temperature for 12 h and then the organic phase was separated and washed with distilled water and then dried over magnesium sulfate. Organic solvent was evaporated under the reduced pressure and dried in a vacuum oven. The crude products were purified by flash chromatography to give 10, 11 and 12 as red-brown solids. However compound 10 was isolated purely and characterized by IR, NMR and elemental analyses, the compound 11 and 12 could not isolated as pure form in spite of several tests. These compounds were characterized by IR the carbonyl bond of aldehyde groups at around 1718–1720 cm^{-1} was observed (Fig. 2.) For the Calix[4]-GA, yield; 65%, IR (KBr) 1720 cm^{-1} (C = O). ^{1}H-NMR ($CDCI_3$) δ (ppm): 1.20 (s, 18H, Bu^t), 1.30 (s,18H, Bu^t), 2.30 (m, 4H, J = 5.6 Hz, $\underline{CH_2}CH_2CHO$), 2.45 (m, 4H, $\underline{CH_2}CHO$), 3.30 (t, 4H, J = 8 Hz, CH_2CH_2 N), 3.20 (d, 4H, J = 12.8 Hz, $ArCH_2Ar$), 4.08 (t, 4H, J = 8.8 Hz, OCH_2CH_2), 4.30 (d, 4H, J = 12.8 Hz, $ArCH_2Ar$), 1.95 (q, 4H, $CH\underline{CH_2}CH_2$), 7.00–7.10 (m, 10H, ArH, ArOH). 8.10 (s, 2H, N = CH), 9.75 (t, J = 8 Hz, 2H, CHO); Anal. calcd for $C_{48}H_{64}O_4N_2$: C, 77.5; H, 8.7; N, 3.1; Found: C, 77.9; H, 8.1; N; 3.6.

Immobilization of *Candida rugosa* lipase (CRL)

A solution of Calix[n]-GA (3 g) in DMSO (5 mL) was treated 10 mL of commercial CRL solution (0.03 g lipase/ 10 mL, 0.05 M sodium phosphate buffer pH; 7.0). The mixture was incubated at room temperature for 7 h. The reaction was terminated by adding 30 mL of 0.05 M sodium phosphate buffer pH; 7.0. After the immobilization period, the product was washed with phosphate buffer (0.05 M, pH;7) and stored at 4°C. Immobilized enzyme was analyzed for expression of bound lipase activity. The amount of immobilized protein on the support was determined by using Coomassie Brilliant Blue reagent, following Bradford's method [32], where in the initial and final concentrations of protein both in the enzyme solutions and in the washings was measured. Bovine Serum Albumin (BSA) was used as the standard to construct a calibration curve.

Activity of free and calix-supported lipases

The activity of free and immobilized lipases was measured by dissolving 0.5 g of *p*-nitrophenyl palmitate (*p*-NPP) in 100 mL of ethanol as substrate at room temperature [33]. Free lipase of 0.1 mL (or 25 mg calix-supported lipases) was added to the mixture of 1.0 mL 0.5% (w/v) *p*-NPP solution and 1 mL 0.05 M PBS buffer (pH 7.0) and incubated for 5 min at 25°C. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 to the mixture followed by

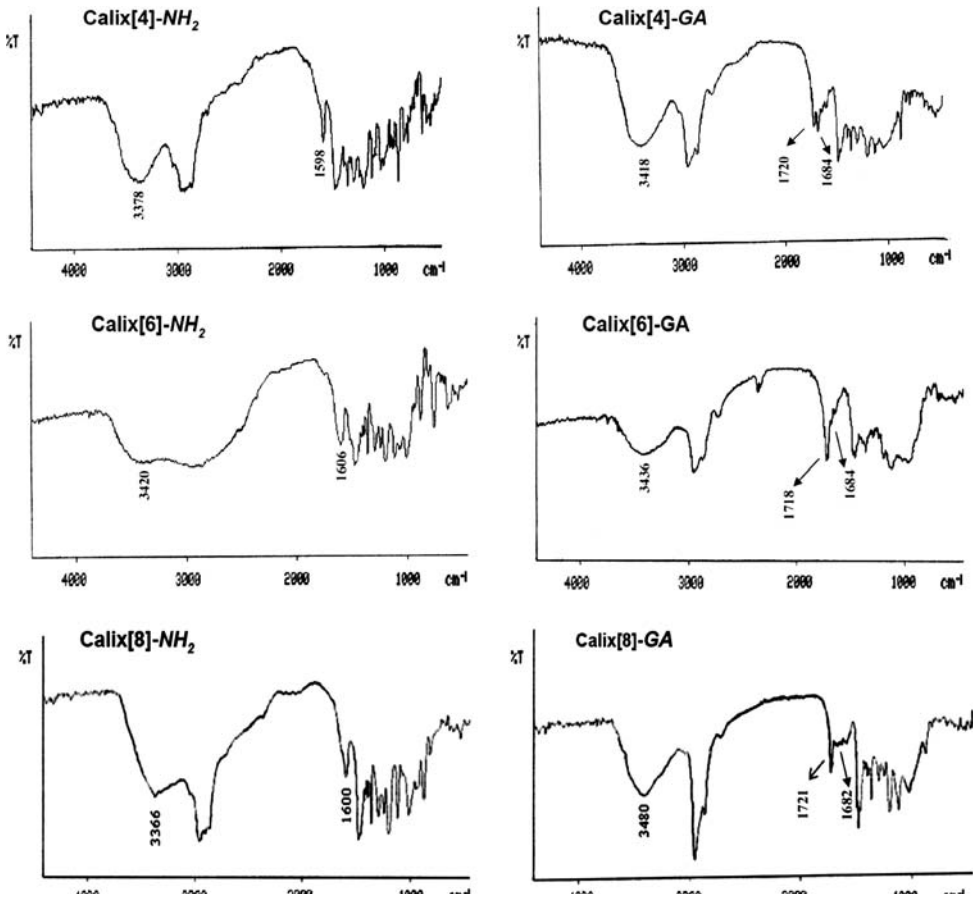


Fig. 2 FT-IR spectrums of the Calix[n]-NH_2 and Calix[n]-GA compounds

centrifugation (4,000 rpm for 5 min). The increase in the absorbance at 400 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was measured in a Shimadzu 160A UV-visible recording spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme, which liberates 1 μmol *p*-nitrophenol min^{-1} [34]. Specific activity was calculated by dividing total activity (U) by amount of lipase bound to Calix[n]-GA cross-linkers and expressed as U/mg-protein. Activity yield (%) was calculated by dividing specific activity of lipase in the immobilized preparation by specific activity of soluble lipase. All measurements were performed in triplicate and an average was taken as final result.

Effect of pH on catalytic activity of free and immobilized lipases

The optimum pH and reaction temperature of free and Calix[n]-GA supported lipases (immobilized lipases) were determined as the relative activity under the variety of pH (0.05 M phosphate buffer for pH 4.0–9.0) and temperature (25–60 °C). Relative activities were calculated as the ratio of the activity of Calix[n]-GA supported lipases after incubation to the activity at the optimum reaction pH and temperature.

Thermal stability and reusability of immobilized lipases

The thermal stability was determined by incubating the free and Calix[n]-GA supported lipases at 60 °C in phosphate buffer pH 7.0 for 120 min. To evaluate the reusability of the Calix[n]-GA supported lipases, after each reaction run, the Calix[n]-GA supported lipases was removed from the reaction medium by centrifugation (4000 rpm, 5 min, at room temperature) and washed with phosphate buffer (0.05 M, pH 7.0) to remove any residual substrate. It was then re-introduced into fresh reaction medium. This process was repeated up to 9 cycles. The activity of freshly prepared Calix[n]-GA supported lipases in the first run was defined as 100%.

Results and discussion

Synthesis

In our previous work, in order to see the role of calix[4]-arene binding site on the lipase activitiy and stability, we have described the synthesis of a new calix[4]arene 1,3-distal glutaraldehyde derivative as a cross-linker-reagent for immobilization of *Candida rugosa* lipase

(CRL)[26]. In this work the main focus of our research program is the design of a series of different-size cavity of calix[n]arene-based cross-linkers, bearing glutaraldehyde function that has an effective binding character for the chemically and biologically important molecules. To achieve this goal, we have attempted to synthesis *p-tert*-butylcalix[n]arenes (n = 4,6,8) (**1–3**) and their nitrile derivatives (**4–6**) based on previously published procedures. The *p-tert*-butylcalix[n]arene-amine derivatives (**7–9**) were synthesized with the reduction of the corresponding nitrile derivatives.

The synthetic route for the preparation of *p-tert*-butylcalix[n]arene alkylamine derivatives (**7–9**) is described in Scheme 1; *p-tert*-butylcalix[n]arene nitrile derivatives (**4–6**) were stirred with the THF–borane complex in dry THF at room temperature to give corresponding amines **7–9** in 90, 82 and 89% yields. Compunds **7–9** were converted to their aldehyde derivatives (**10–12**) with 25% aqueous glutaraldehyde solution in a two phase (CH_2Cl_2/water) extraction system (Scheme 1). The presence of aldehyde groups onto the calixarene derivatives was confirmed by FT-IR spectrum measurement. The FT-IR spectra of the **10–12** shows the appearance of a weak absorption band around at 1718–1721 cm^{-1} which is the characteristic of the carbonyl (aldehyde) groups (Fig. 2).

The structures of the calix[n]arene derivatives were identified by spectral and analytical data such as elemental analysis, FT-IR, ^1H NMR. All reduction reactions were monitored by thin layer chromatography and were checked several times by taking a small sample from the reaction mixture.

Immobilization of *Candida rugosa* lipase with treatment of calix[n]arene derivatives (**10–12**)

The main focus of our research program is the design of a new calixarene-based cross-linkers, bearing aldehyde functions that have an effective binding and cross-linking character for the chemically and biologically important molecules. After the treatment of CRL with compound **10–12**, the complexes were not soluble in water, possibly due to an increase in surface hydrophobicity.

To verify changes in the immobilized enzymatic preparation during this reaction, a SEM image of the Calix[8]-GA compound (Fig. 3a) was compared with image obtained for the immobilized lipase (Calix[8]-CRL) (Fig. 3b). The photographs were taken at 3500 magnification. After immobilization, the surface cavity of the Calix[8]-GA was filled by the rounded structure, which is presumably protein aggregate. The higher enzyme productivity exhibited by immobilized lipase (Calix[8]-CRL) might be due to proper binding or multipoint interaction of lipase functional groups with the Calix[8]-GA.

Most importantly, the immobilized lipases exhibited enzymatic activity against the *p*-NPP substrate. These results demonstrate that lipase was covalently immobilized on the GA treated calix[n]arenes successfully. The composition of the free and *p-tert*-butylcalix[n]arene supported lipases is reported in Table 1.

Table 1 shows the activity of the free and immobilized lipases. It can be seen that the amounts of bound protein are 540.8 μg/g on the Calix[4]-CRL, 570.1 μg/g on the Calix[6]-CRL, and 585.6 μg/g on the Calix[8]-CRL. The protein coupling levels seems not unusual because the Calix[8]-GA contains more functional groups which react with lipase than the others. The Calix[8]-GA has more hydrophobic surfaces than the others and in this case, lipase seem to become strongly adsorbed to hydrophobic interfaces through a large hydrophobic surface [35–38]. Therefore, the Calix[8]-CRL was found to be more efficient compared to the others (Calix[4]-CRL and Calix[6]-CRL) with respect to expression of immobilized lipase activity. The immobilized lipase retains 53.5% of the activity on Calix[4]-CRL, 66.1% on the Calix[6]-CRL and 76.4% on the Calix[8]-CRL. It demonstrates that the Calix[n]-GA supports could offer a fitting and capacious microenvironment for immobilized enzymes. This result was not surprising because cavity size of the Calix[8]arene

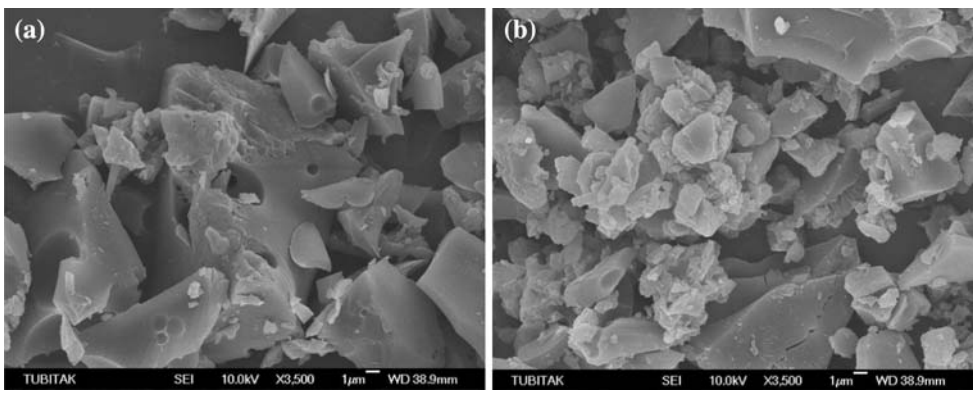


Fig. 3 SEM micrographs of the Calix[8]-GA (before immobilization) (**a**) and Calix[8]-CRL (after immobilization) (**b**)

Table 1 Yields of protein loading and activity of the calix-supported lipases

Types	Protein loading (μg/g-calix)	Protein loading yield (%)	Lipase activity (U/g-calix)	Specific activity (U/mg-protein)	Activity yield (%)
Calix[4]-CRL	540.8 ± 16	11.0 ± 0.3	10.3 ± 0.6	19.0 ± 0.5	53.5 ± 1.4
Calix[6]-CRL	570.1 ± 14	15.6 ± 0.4	13.4 ± 0.7	23.5 ± 0.8	66.1 ± 2.2
Calix[8]-CRL	585.6 ± 12	15.6 ± 0.3	15.9 ± 0.4	27.2 ± 0.6	76.4 ± 1.7
Free lipase	–[a]	–	2.49[b] ± 0.1	35.6 ± 2.1	100 ± 1.2

[a] Protein content of free lipase solution was 70 μg/ml

[b] Activity of the free lipase is expressed for 1 ml (0.2%)

is larger than the Calix[6]arene and Calix[4]arene. The Calix[8]-CRL could access the substrate easily than the Calix[4]-CRL and Calix[6]-CRL. As known in the literature, the larger pores and the reactive aldehyde groups in the supports would be suitable for the immobilization of enzymes (both adsorption and covalence), and also provide a good transmission for substrate and product [39]. After calix[n]arene unit immobilization, a decrease of enzyme activity is commonly observed. This could be explained by the minor modification in the three-dimensional structure of the enzyme that leads to the distortion of amino acid residues involved in catalysis, the presence of random immobilization, which causes the analyte approach to the active site of the hindered enzyme, and the limitations imposed by slow mass transfer of substrate or product to or from the active site of the enzyme [26].

Temperature and pH effect on the activity of free and immobilized lipases

The effect of pH on enzyme activity was determined by incubating free and calix-linked lipases separately at different pH from 4.0, 5.0 and so on up to 9.0 at 30 °C for 30 min. At the end of incubation time the residual lipase activity of each sample was determined by *p*-NPP assay as described [33]. The activities of free and immobilized lipases (Calix[n]-CRL) were determined at different pH values. The optimum pH values were determined from the graph of pH plotted against the percentage of relative activity (Fig. 4). The optimum pH values were found to be 7.0 and 6.0 for free and immobilized lipases (Calix[n]-CRL), respectively. A pH gradient between the domain of immobilized lipase particles and external solution occurs during the enzymatic hydrolysis reaction, resulting in the shift of the pH optimum to the acidic region under these conditions. This pH shift is possibly due to the method of immobilization, as well as secondary interactions (e.g., ionic polar interactions and hydrogen bonding) between the enzyme and calix[n]arene units.

The temperature dependence of the *p*-NPP hydrolysis reaction catalyzed by free and immobilized lipases was

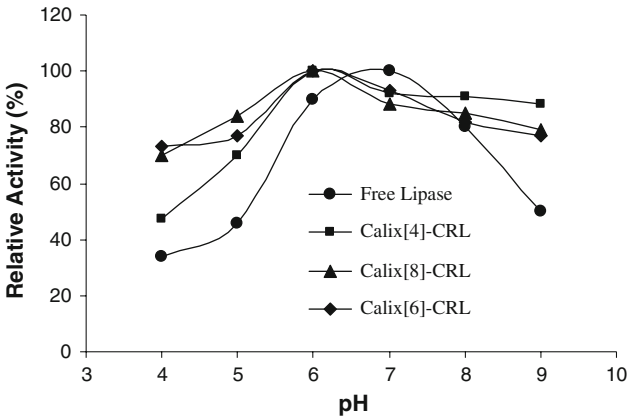


Fig. 4 Effect of pH on the activity of the free and immobilized lipases (Calix[n]-CRL)

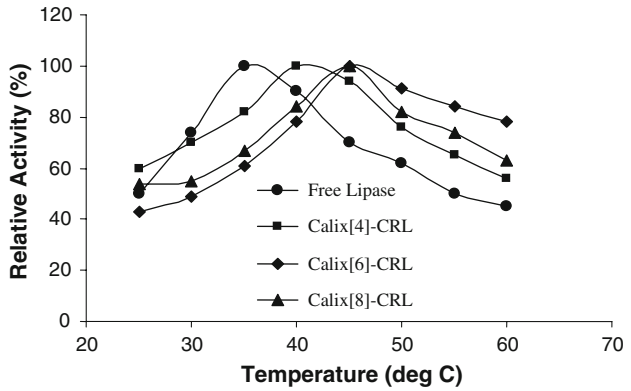


Fig. 5 Effect of temperature on the activity of the free and immobilized lipases (Calix[n]-CRL)

studied in the interval from 25 to 60 °C and the results are shown in Fig. 5. The maximum activity of the free lipase appeared at 35 °C, but the optimum temperature of the immobilized lipases (Calix[n]-CRL) was obtained around at 40–45 °C. This behavior is considered to be advantageous and can be attributed to a more rigid structure of the immobilized lipases, which prevent the split breaking of the interactions responsible for the proper globular, catalytically active structure of the lipase [40, 41]. However, it has been concluded that the immobilized lipases show

good heat resistance due to the conformational limitation on the enzyme as a result of covalent bond formation between the enzyme and the support at high temperature [42]. Alternatively, a restriction in the diffusion of the substrate at high temperature may also be responsible [43].

Thermal stability of immobilized lipases

The thermal stabilities of free and immobilized lipases (Calix[n]-CRL) were evaluated by incubating them in aqueous buffer at 60 °C. The thermal stabilities of the free and immobilized lipases are given in Fig. 6. According to Fig. 6, it can be seen that the free lipase losses its initial activity within around 100 min while the immobilized lipases retain their initial activity of about 38% for the Calix[4]-CRL, 50% for the Calix[6]-CRL and 60% for the Calix[8]-CRL after a 120 min of heat treatment. These results indicate that the thermal stability of immobilized lipases is much better than that of the free one owing to the formation of multipoint covalent bond between the enzyme and the supports which prevent the conformation transition of the enzyme at high temperature. Multipoint immobilization of multimeric enzymes may prevent subunit dissociation by inter-subunit cross-linking while simultaneously reducing conformational inactivation by intra-subunit crosslinking [44, 45]. This approach leads to a generation of multiple covalent links between the enzyme and matrix, both stabilizing the quaternary structure of the protein and increasing rigidity of the subunit structures [46]. Consequently, thermal stability of the enzyme was improved upon immobilization.

Reusability of calix-supported lipases

After the treatment of CRL with Calix[n]-NH_2, the complexes were not soluble in water due to increase crosslinked bond between the enzyme. Thus, it was used in the reusability studies, where after each run, the calix-linked lipases were washed with PBS. It was then reintroduced into fresh medium and then the enzyme activity was assayed at optimum condition. It was found that the relative activity of the immobilized lipase decreased after the second usage (Fig. 7). Figure 7 shows that the immobilized lipases were still retained 10, 30 and 45% of their original activities for Calix[4]-CRL, Calix[6]-CRL and Calix[8]-CRL after the 9th reuse, respectively. These results are due to the inactivation of the enzyme caused by the denaturation of protein and the leakage of protein from the supports upon use. In the literature [47], activity retention (11% retention after three reuses in olive oil hydrolysis, 65% retention after four reuses in sunflower oil hydrolysis [48], and 10.5% retention after seven reuses in palm oil hydrolysis [49]) has been reported for lipases immobilized to various supports. In comparison, the activity retention of calix-linked lipase was higher.

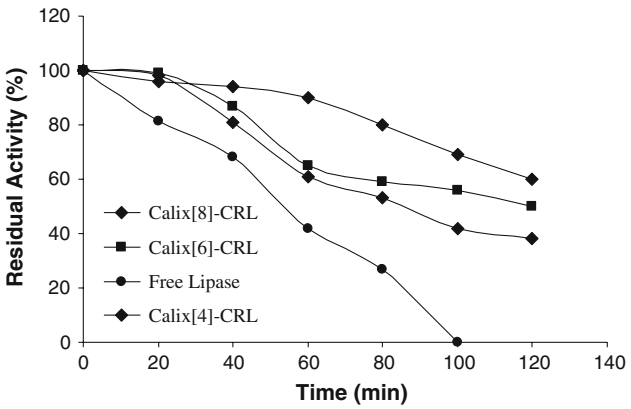


Fig. 6 Thermal stabilities of free and immobilized lipases (Calix[n]-CRL) at 60 °C

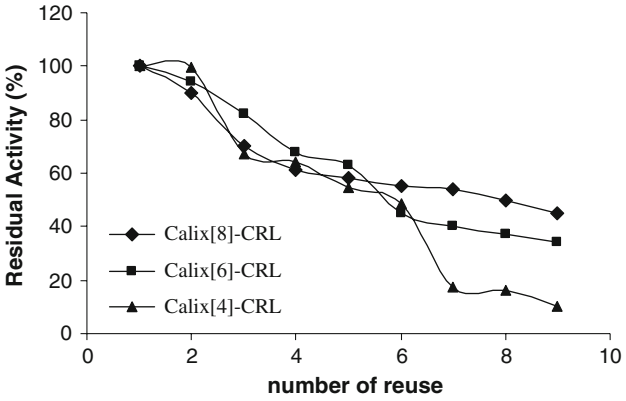


Fig. 7 Effect of repeated use on the activity of immobilized lipases (Calix[n]-CRL)

Conclusion

In this work, fist time a calix[n]arene-based cross linker was synthesized for enzyme immobilization. The amino functional calix[n]arene derivatives (Calix[n]-NH_2) were synthesized via reduction of *dinitrile*, *hexanitrile* and *octanitrile* derivatives of calix[n]arenes. These amino functional calix[n]arene derivatives (Calix[n]-NH_2) were converted to their aldehyde derivativatives with glutaraldehyde. Compounds **10**–**12** were used as a cross linker and treated with the *Candida rugosa* lipase. The immobilized compounds were not soluble in water, due to increase crosslinked bonds between the enzyme and calix[4]arene molecules. It was found that after immobilization, enzyme activity decreased. The activity ratio between the initial and intermediate stages of the enzyme was reduced by

immobilization, suggesting that there was a conformational change to the enzyme molecule due to the immobilization.

However, thermal stability and reusability of the enzyme increased. Multipoint covalent attachment of enzymes on highly activated pre-existing supports via short spacer arms and involving many residues placed on the enzyme surface promotes a rigidification of the enzyme structure of the immobilized enzyme. This should reduce any conformational change involved in enzyme inactivation and greatly increase the enzyme stability. Consequently, thermal stability of the enzyme was improved upon immobilization [50–53].

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