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ORIGINAL ARTICLE

Synthesis of a glutaraldehyde derivative of calix[4]arene as a cross-linker reagent for lipase immobilization

Fatih Ozcan · Ozlem Sahin · Mustafa Yilmaz

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Abstract This article describes the synthesis of a new calix[4]arene 1,3-distal glutaraldehyde derivative **4** as a cross-linker-reagent for immobilization of *Candida rugosa* lipase (CRL). *p-tert*-Butylcalix[4]arene 1,3-distal diaminoalkyl derivative (**3**) synthesized via aminolysis reaction of 5,11,17,23-*tert*-butyl-25,27-ethoxycarbonylmethoxy-26, 28-hydroxycalix[4]arene (**2**) with 1,8-diaminooctane. Compound **3** was converted to its aldehyde derivative (**4**) by the treatment with glutaraldehyde solution. **4** was used in lipase immobilization in order to see the role of calix[4]arene binding site on the lipase activity and stability. It was observed that the immobilized lipase activity was maintained at levels exceeding 95% of its original activity after 40 min.

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

Introduction

Enzymes have a wide variety of biotechnical, biomedical and pharmaceutical applications due to their high specificity, high rate of reactions, non-toxicity and water solubility, which are major advantages over inorganic catalysts. Enzyme immobilization is a method to keep enzyme molecules confined or localized in a certain defined region of space with retention of their catalytic activities. In comparison with their native form, immobilized enzymes offer several advantages, such as enhanced stability, easier product recovery and purification, the possibility of repeated usage, and continuous process technology. Different methods (such as covalent binding, electrostatic binding, hydrophobic interactions, entrapment and encapsulation) are often used for enzyme immobilization [1–5].

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 [6–9]. 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 [10]. Mainly, crown ethers forms complexes with surface ammonium groups of lysine.

Calixarenes (Fig. 1) are a class of cyclooligomers formed via a phenol-formaldehyde condensation and have generated considerable interest as useful building blocks for the synthesis of hosts for cations, anions and neutral molecules. During the last two decades, they have attracted much attention as receptors in supramolecular chemistry. 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 either at the lower rim (phenolic groups) or the upper rim (aromatic nuclei) [11, 12].

Previously, we synthesized various calix[4]arene derivatives, immobilized them onto different supporting

F. Ozcan · O. Sahin · M. Yilmaz (✉)
Department of Chemistry, Selçuk University,
42031 Konya, Turkey
e-mail: myilmaz42@yahoo.com

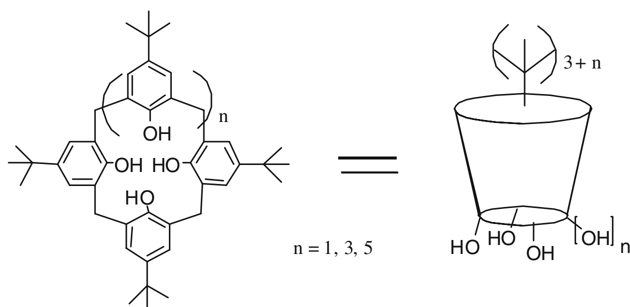


Fig. 1 Structures of calix[n]arenes

materials and tested for their molecular and enantiomeric recognition capabilities [13–16].

To our best knowledge, there exists no report on the use of calix[4]arene 1,3-distal glutaraldehyde derivative as a cross-linker-reagent for lipase immobilization. The aim of this work was to study the synthesis and the influence of calix[4]arene-as a activating agent 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 and Aldrich and used without further purification. Toluene was distilled from CaH_2 and stored over sodium wire. 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). Flash chromatography separations were performed on a Merck Silica Gel 60 (230–400 Mesh). Incubation was performed a GFL-3032 Incubator.

Synthesis

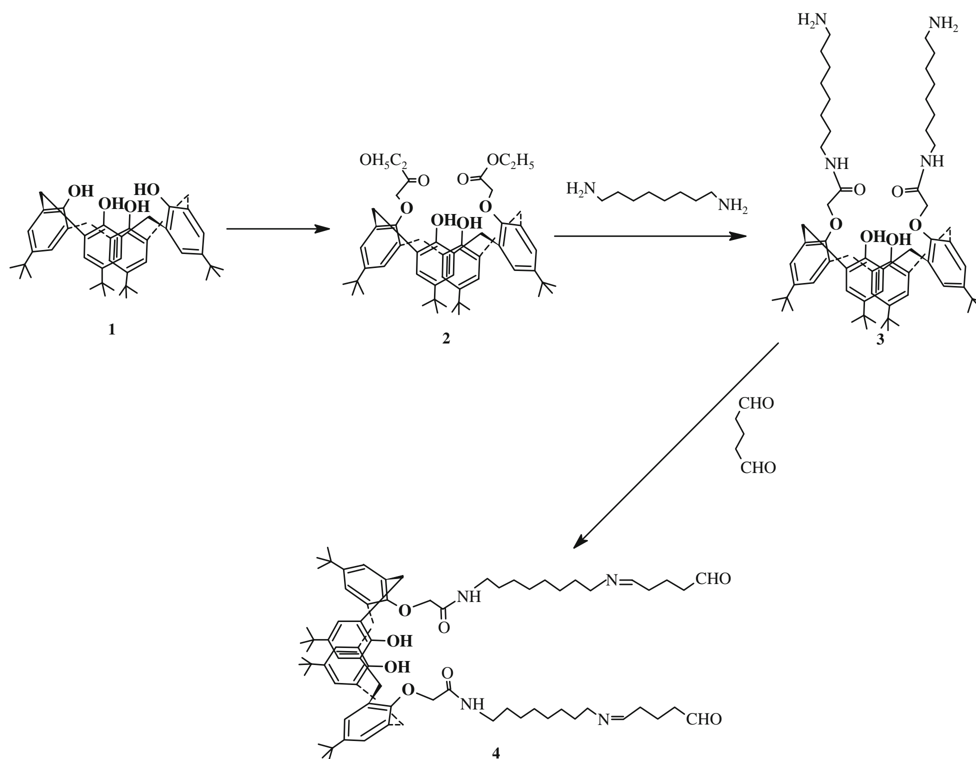
Compounds **1**, **2** were synthesized according to previously described methods [17, 18]. Compounds **3** and **4** (Schemes 1, 2) were synthesized as follows.

*Treatment of compound 2 with diaminooctane (**3**)*

1,8-Diaminooctane (17.5 mmol) was dissolved in a 1:1 CH_2Cl_2–MeOH mixture (60 mL) and added dropwise to a solution of 5,11,17,23-*tert*-butyl-25,27-ethoxycarbonylmethoxy-26,28-hydroxycalix[4]arene **2** (3.0 mmol) in CH_2Cl_2 (20 mL) with continuous stirring at room temperature for about 24 h. The reactions were monitored by TLC. After the substrate had been consumed the solvent was evaporated under reduced pressure and the residue was triturated with MeOH to give *p*-*tert*-butylcalix[4]arene derivative **3**, the crude product was purified by flash chromatography (SiO_2, EtOAc-*n*-hexane 3:1) and recrystallized from CH_2Cl_2–MeOH to give **3** as white crystals. Yield 73%; mp 143 °C; IR (KBr) 3454 and 3358 cm^{-1} (NH, OH), 1685 cm^{-1} (HN–C=O), ^{1}H NMR (400 MHz $CDCl_3$): 1.00 (18H, s, ^{t}Bu), 1.3 (18H, s, ^{t}Bu), 1.4 (24H, br s, $NHCH_2(CH_2)_6$), 1.6 (4H, t, CH_2NH_2), 2.0(4H, s, NH_2), 3.45 (4H, d, J 13.3 Hz, $ArCH_2Ar$), 3.3 (4H, t, $NHCH_2$), 4.2 (4H, d, J 13.3 Hz, $ArCH_2Ar$), 4.55 (4H, s, OCH_2), 6.9 (4H, s, ArH), 7.1 (4H, s, ArH), 7.8 (2H, s, OH), 8.8 (2H, t, NH); Anal. Calc. for $C_{64}H_{96}N_4O_6$; C, 76.80; H, 9.10; N, 5.80. Found: C, 77.5%; H, 8.58%; N, 5.93%.

*Treatment of compound **3** with glutaraldehyde (**4**)*

A solution of compound **3** (0.1 g) in CH_2Cl_2 (5 mL) was treated with a 5 mL of 5% 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 and recrystallized from CH_2Cl_2–MeOH to give **4** as red-brown solid. Yield 54%; mp 157 °C; IR (KBr) 1723 cm^{-1} (C=O), 2783 cm^{-1} (H–C=O), ^{1}H NMR (400 MHz $CDCl_3$): 1.00 (18H, s, ^{t}Bu), 1.3 (18H, s, ^{t}Bu), 1.4–2.0 (24H, br, s, $NHCH_2(CH_2)_6$, 4H, CH_2CH_2CHO), 2.45 (4H, m CH_2CHO), 3.4 (8H, br, $ArCH_2Ar$, 4H, $NHCH_2$), 4.2 (4H, br, $ArCH_2Ar$, 4H, NCH_2),



Scheme 1 The synthetic route of preparation of calix[4]arene 1,3-distal glutaraldehyde derivative (**4**)

4.55 (4H, s, OCH_2), 6.9 (4H, s, ArH), 7.1 (4H, s, ArH), 7.2 (2H, s, N=CH), 7.9 (2H, s, OH), 8.85 (2H, t, NH), 9.75 (2H, t, CHO); Anal. Calc. for $C_{74}H_{108}N_4O_8$; C, 75.7; H, 8.5; N, 4.7. Found: C, 76.5%0; H, 8.10%; N, 4.93%.

Method of immobilization

Preparation of enzyme solution

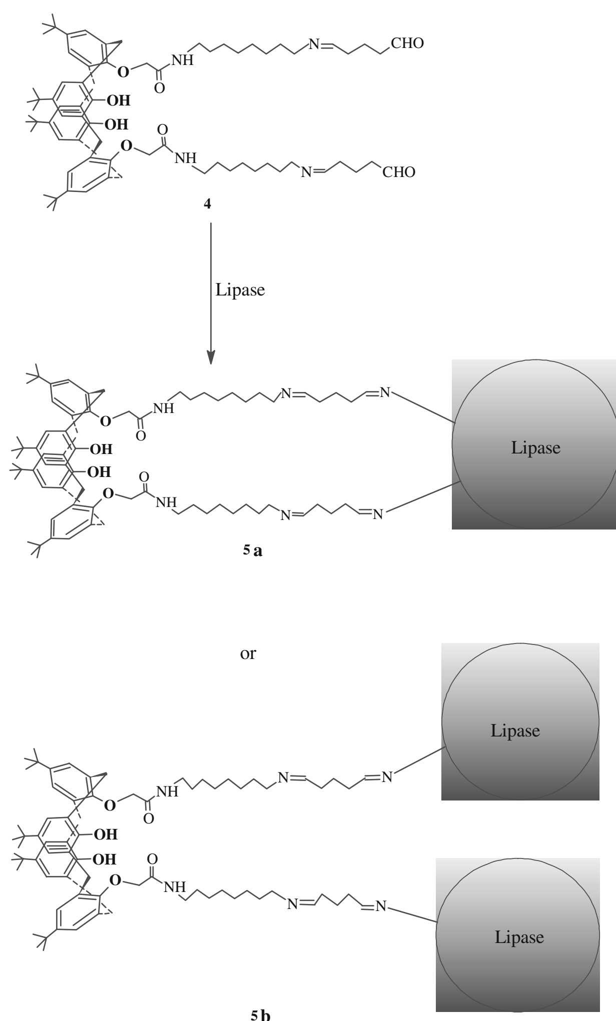
Five-hundred milligram of commercial CRL (*Candida rugosa* lipase) was dissolved in 100 mL of phosphate buffer (0.05 M, pH 7.0). The solution was centrifuged at 6000 rpm at 5 °C for 15 min.

Immobilization method

Compound **4** was suspended in 6 mL of CRL enzyme solution. The mixture was incubated at 30 °C at 100 rpm. After 5 h, the product was then washed twice thoroughly with a 0.05 M sodium phosphate buffer (pH 7.0). 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 [19], in where the initial and final concentrations of protein both in the enzyme solutions and in the washings were measured. Bovine Serum Albumin (BSA) was used as the standard to construct a calibration curve.

Assay of enzyme activity

The activity of free and immobilized lipase was measured using 0.5 g of *p*-nitrophenyl palmitate (*p*-NPP) dissolved in 100 mL of ethanol as the substrate. The increase in absorbance at 410 nm caused by the release of *p*-nitrophenol in the hydrolysis of *p*-NPP was measured spectrophotometrically. Free lipase of 0.1 mL (or 20 mg immobilized lipase) was added to a mixture of 1 mL 0.5% (w/v) *p*-NPP solution and 1 mL 0.05 M PBS buffer and incubated for 5 min at 30 °C. The reaction was terminated by adding 2 mL of 0.5 N Na_2CO_3 to the mixture followed by centrifugation (10,000 rpm for 10 min). The absorbance was measured at 410 nm by a UV–vis spectrophotometer (UV-160A, Shimadzu, Japan). The reaction rate was calculated from the slope of the absorbance versus the time curve. Molar extinction coefficient of $14.5 \times 10^3\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol (*p*-NP), which was determined from the absorbance of standard solutions of *p*-NP in the reaction medium, was used. One unit (U) of lipase activity was defined as the amount of enzyme which catalyzed the production of 1 μmole *p*-nitrophenol per minutes under the experimental conditions. Specific activity was calculated by



Scheme 2 Purposed interactions of **4** with lipase

dividing total activity (U) by amount of lipase bound to support and expressed as U/mg-protein.

Physicochemical properties of free and calix-supported lipase

Effect of pH on activity

The effect of pH on enzyme activity was determined by incubating free and immobilized enzymes separately at different pH ranging from 3.0, 4.0, 5.0 and so on up to 9.0 at 30 °C for 1 h. At the end of incubation time the residual lipase activity of each sample was determined by *p*-NPP assay as described above.

Stability assays

The stability of free and immobilized enzymes on repeated use was also examined by measuring the activity towards the hydrolysis of *p*-NPP assay. After each activity determination, the immobilized enzyme was washed with buffer solution (PBS 50 mM, pH: 7.0) and reintroduced into a fresh medium, this procedure being repeated for up to 6 cycles.

Free and immobilized lipase preparations were stored in the phosphate buffer solutions (0.05 M, pH 7.0) at 60 °C for 100 min., respectively. Samples were periodically withdrawn for activity assay. The residual activities were determined as above.

Results and discussion

Synthesis

The main focus of our research program is the design of new calixarene-based cross-linker, bearing glutaraldehyde function that has an effective binding character for the chemically and biologically important molecules. To achieve this goal, *p*-*tert*-butylcalix[4]arene (**1**) has been chosen as the precursor. The synthetic scheme for its elaboration is shown in Scheme 1. The synthesis of compounds **1**–**2** is based on previously published procedures [17, 18], while compounds **3** and **4** are reported for the first time. The synthetic route for the preparation of *p*-*tert*-butylcalix[4]arene diaminoalkylamide derivative (**3**) is described in Scheme 1; 25,27-diethoxycarbonylmethoxy-26,28-dihydroxycalix[4]arene **2** was stirred with diaminooctane in dichlorometan-methanol (1:1) at room temperature to give amide derivative of *p*-*tert*-butylcalix[4]arene **3** in 73% yield. Compound **3** was converted to its aldehyde derivative (**4**) with 5% aqueous glutaraldehyde solution in a two phase (CH_2Cl_2/water) extraction system (Scheme 1).

The new compounds **3** and **4** were characterized by a combination of IR, ^{1}H NMR, and elemental analysis. The formation of diamide derivatives of *p*-*tert*-butylcalix[4] arene **3** was confirmed by the appearance of the characteristic amide bands at about 1684 cm^{-1} in its IR spectra, and by the disappearance of ester carbonyl band at 1785 cm^{-1} in the IR spectra.

After treatment of **3** with glutaraldehyde, appeared the characteristic aldehyde proton and carbonyl bands at about 2783 and 1783 cm^{-1} respectively in the IR spectrum of **4** (Fig. 2).

The conformational characteristics of calix[4]arenes were conveniently estimated by the splitting pattern of the $ArCH_2Ar$ methylene protons in the ^{1}H NMR spectroscopy [11, 12]. ^{1}H NMR data showed that compound **3** and **4** have a cone conformation. A typical AB pattern was observed for the methylene bridge $ArCH_2Ar$ protons at 3.40–3.45 and 4.20 ppm ($J = 13.3$ Hz).

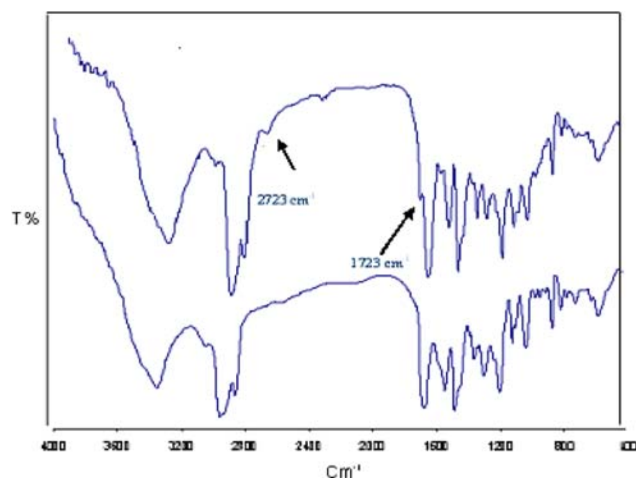


Fig. 2 IR spectrum of compounds **3** (down) and **4** (up)

Immobilization of *Candida rugosa* lipase with treatment of *p-tert*-butylcalix[4]arene derivative **4**

The main focus of our research program is the design of a new calixarene-based cross-linker, bearing 1,3-distal aldehyde function that has an effective binding and cross-linking character for the chemically and biologically important molecules. After the treatment of CRL with compound **4**, the complex was not soluble in water, possibly due to an increase in surface hydrophobicity (Scheme 2). Lipase activity was examined by using the hydrolysis of *p*-nitrophenylpalmitate (*p*-NPP) as a model reaction [19]. The hydrolysis was carried out by mixing 20 mg immobilized lipase, 1 mL 0.5% (w/v) *p*-NPP solution, and 1 mL 0.05 M PBS buffer and incubated for 5 min at 30 °C. The composition of the free and *p-tert*-butylcalix[4]arene supported CRL is reported in Table 1.

Table 1 shows the specific activities of the free and the calix-linked lipase under the optimum reaction conditions. The protein content of the insoluble product and the specific activity were 61.1% and 43.9 U/g calix-CRL, respectively. In comparison with the free enzyme, the calix-linked lipase under its optimum reaction condition retained 19.1% of the activity. In literature [20], effect of glutaraldeyde (GA) concentration on the amount of protein bound and the activity of the immobilized lipase was studied. It was observed that the increment of GA concentration from 0.5% to 10% increased the amount of immobilized protein (from 22.6 to 72.8 mg/g), while decreased the activity of the immobilized enzyme (from 54.1 to 30.3 U/mg).

After calix[4]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 enzyme hindered, and the limitations imposed by slow mass transfer of substrate or product to or from the active site of the enzyme.

Effect of pH on free and calix-linked lipases

The effect of pH on enzyme activity was determined by incubating free and calix-linked lipases separately at different pH from 3.0, 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 [19]. The activities of free and calix-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. 3). The optimum pH values were found to be 7.0 and 5.0 for free and calix-CRL enzymes, respectively. *p-tert*-butylcalix[4]arene is linked to the lipase matrix via its free aldehyde groups so that, after the immobilization, an increase occurs in the amount of the acidic groups and enzyme gains more polycationic character. A pH gradient between the domain of immobilized lipase particles and external solution occurs during the enzymic 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[4]arene units.

Effect of temperature on immobilized lipase activity

The thermal stability of free and calix-supported lipase was carried out incubating both enzymes suspended in a 0.05 M PBS buffer (pH 7.0) at the 60 °C. After this treatment, the activities were measured by *p*-NPP method. Figure 4 shows the thermal stabilities of the free and calix-linked

Table 1 The composition of the free and calix-treated lipase (Calix-CRL)

Types	Protein loading (mg/g calix)	Protein content of the insoluble product yield (%)	Lipase activity (U/g-calix)	Specific activity (U/mg protein)	Activity yield (%)
Calix-treated lipase	10.5	61.1	43.9	6.8	19.1
Free lipase	–	–	2.49[a]	35.5	100

[a] Dimension is U/mL

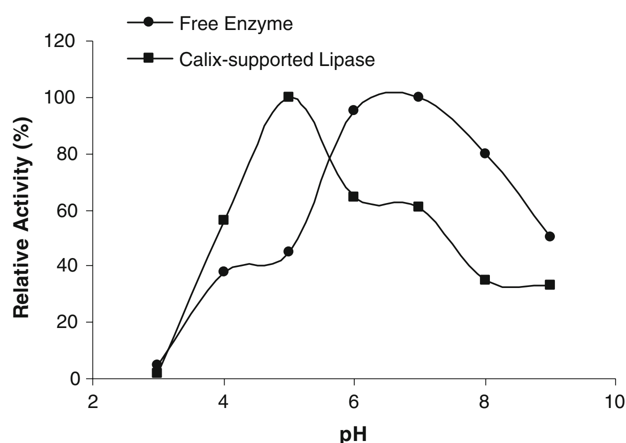


Fig. 3 Effect of pH on catalytic activity of free and immobilized lipase at 30 °C

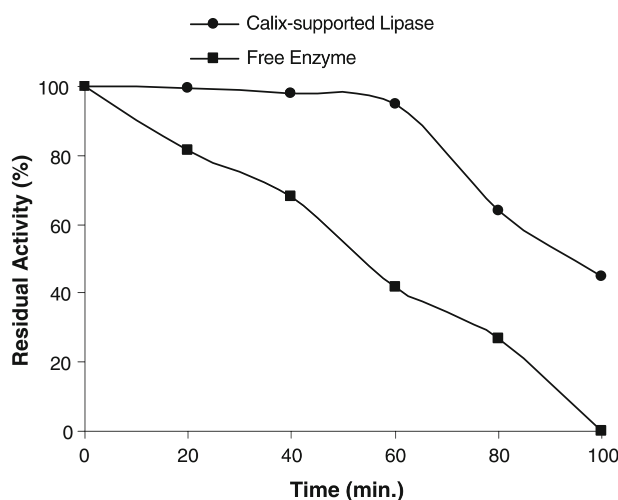


Fig. 4 Thermal stability of free and calix-supported lipase at 60 °C

lipases. Free lipase was stable only up to 35 °C, after which the catalytic activity decreased. It was found that the relative activity of the calix-supported lipase retained 95% of its activity after 40 min. At 60 °C the free lipase lost its initial activity within around 100 min. However, at the same temperature, the calix-linked lipase retained 40% of its activity.

These results demonstrated that the thermal stability of calix-supported lipase was much better than that of the free one since the formation of a crosslinked bond between the enzyme and the supports prevented the conformational transition of the enzyme at high temperatures.

Reusability of calix-supported lipase

After the treatment of CRL with compound **4**, the complex was not soluble in water due to increase crosslinked bond

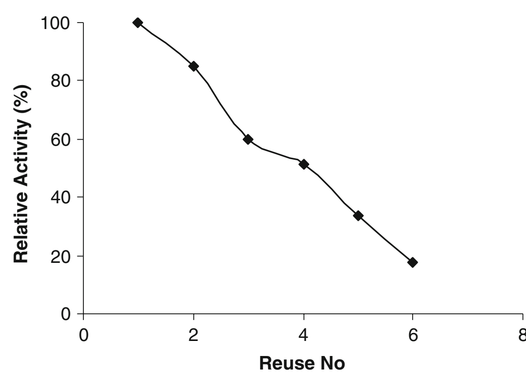


Fig. 5 Reusability of calix-supported lipase

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. 5). The relative activity was decreased to 20% after 6 uses. The leakage of protein from support's surface during stirring may be a main reason for the loss of activity. In the literature [21], activity retention (11% retention after three reuses in olive oil hydrolysis, 65% retention after four reuses in sunflower oil hydrolysis [22], and 10.5% retention after seven reuses in palm oil hydrolysis [23]) has been reported for lipases immobilized to various supports. In comparison, the activity retention of calix-linked lipase was higher.

Conclusion

In this work, fist time a calix[4]arene-based cross linker was synthesized for enzyme immobilization. The new bisamide derivative of *p-tert*-butylcalix[4]arene (**3**) was prepared by the reaction of *p-tert*-butylcalix[4]arene (**3**) with diaminooctane, and this compound was converted to its aldehyde derivative (**4**) by the treatment with glutaraldehyde solution. Compound **4** was used as a cross linker and treated with the *Candida rugosa* lipase. The immobilized compound was 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. However, thermal stability and reusability of the enzyme increased. This may have an important impact on the catalytic efficacy.

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