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*Research Article*

# Adsorption of Myoglobin on Calixarenes and Biocatalysis in Organic Media

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**Abstract** Derivatives of *p-tert*-butylcalix[4,6,8]arene carboxylic acids were used for selective adsorption of myoglobin. A mixture of myoglobin, laccase and peroxidase was used for extraction with calixarenes and only myoglobin was selectively extracted to organic media. Myoglobin and Mb $c$–calixarene exhibited pseudoactivity of peroxidase in aqueous and organic media. This protein-calixarene complex exhibited the highest specific activity of $1.37 \times 10^{-1}$ U.mg protein$^{-1}$ at initial pH 6.5 of myoglobin aqueous solution. Apparent kinetic parameters ($V'_{\max}$, $K'_{\mathrm{m}}$, $k'_{\mathrm{cat}}$ and $k'_{\mathrm{cat}}/K'_{\mathrm{m}}$) for the pseudoperoxidase activity were determined in organic media for different initial pH values of myoglobin aqueous solution by Michaelis-Menten plot. The stability of this complex was studied for different initial pH values and $t_{1/2}$ values were obtained in the range of 3.5–5.2 days. The extracted Mb $c$ in organic media was recovered into fresh aqueous solutions at alkaline pH with a recovery of pseudoperoxidase activity of over 100%.

**Keywords** calixarenes; protein adsorption; myoglobin; biocatalysis in organic media; pseudoperoxidase activity

## 1 Introduction

Specific recognition of biological molecules by synthetic compounds is a fast growing and challenging field both in academic and applied research [21]. The use of calixarenes has attracted an increasing interest in the last two decades as a powerful tool for biochemical recognition and separation of bioactive molecules such as amino acids, peptides, proteins, lectins, nucleotides, nucleosides, saccharides and steroids [21,32]. Calixarenes are macrocyclic structures which consist of phenol rings interconnected by methylene bridges in a cyclic array (Figure 1). These compounds consist of cup-shaped molecules which can form host-guest complexes with a wide range of guest molecules by introducing appropriate functionalities either at the upper or lower rim [12].

Recently, Oshima *et al.* [24,25] have reported that a calix[6]arene carboxylic acid derivative ($^{t}$Oct[6]$CH_2$COOH) exhibited high affinity for cationic proteins such as cytochrome $c$ (Cyt $c$) by promoting its extraction in organic media. They have also investigated several calix[4,6,8]arenes as protein extraction agents which revealed that large cavities of carboxylic acid derivatives of *p-tert*-octylcalix[6]arene ($^{t}$Oct[6]$CH_2$COOH) and *p-tert*-octylcalix[8]arene ($^{t}$Oct[8]$CH_2$COOH) exhibited a high extraction capacity for proteins compared with the *p-tert*-octylcalix[4]arene ($^{t}$Oct[4]$CH_2$COOH). Such extraction of Cyt $c$ was explained on the basis of a large number of lysine (Lys) residues on this protein surface whose protonated amino groups of the side chain interacted with $^{t}$Oct[6]$CH_2$COOH molecules in organic phase. Apparently, this ionic interaction would provide enough hydrophobicity for protein transfer to organic phase. Three-side chains of amino acid residues at protein surfaces could potentially participate in ionic interactions with these Cyt $c$–$^{t}$Oct[6]$CH_2$COOH molecules. However, the same authors have found that such calixarene exhibited a high selectivity for the extraction of proteins rich in Lys residues at surface since only Cyt $c$ was extracted to the organic phase when a mixture of lysozyme and Cyt $c$ was used. Besides the extraction of Cyt $c$ to organic phase by using such calixarenes, they observed a pseudoperoxidase activity of cytochrome $c$ in organic solvents by catalyzing the oxidation of 2,6-dimethoxyphenol in the presence of hydrogen

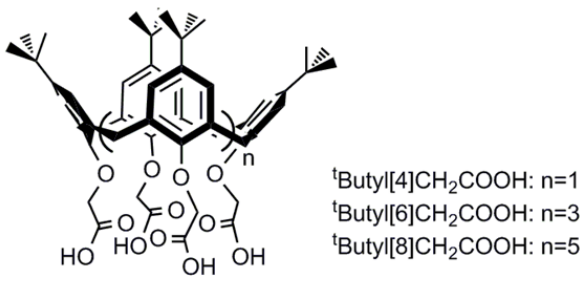



**Figure 1:** Structure of *p-tert*-butylcalix[$n$]arene acid derivatives.

peroxide [24]. Enzymes have evolved naturally to function in predominantly aqueous environment, whereby their structures result from the balance between the hydrophobic effect and other non-covalent interactions, such as hydrogen bonds and ionic contacts [26]. However, there are several advantages in the use of enzymes in organic solvents, both from the synthetic or processing point of views [31,33]. Several structural comparisons of proteins in aqueous versus non-aqueous polar media have shown that the protein fold remains generally unchanged. In fact, the observed minor structural differences between structures from organic media versus aqueous environment were found to be comparable with those found within multiple, independently determined structures in aqueous media [3,8,11,28]. Biocatalysis in non-conventional media is an important field in enzyme engineering because there are several advantages over the aqueous media such as the change in substrate specificity, selectivity and biocatalyst's stability [10,36]. On the other hand, the recovery of such proteins present in organic phase into fresh aqueous solutions has also been reported by using acidic, alkaline solutions as well as the addition of alcohols which abolished the Cyt $c$ - calixarene complex [24,25]. However, several additional parameters may play critical roles in protein calixarene interactions such as protein size, pl, hydrophilicity and the nature of amino acid residues at surface [18,35].

The aim of this work was to use an alternative method of selective protein extraction by using calixarenes in organic media. Since free native myoglobin exhibits pseudoactivity of peroxidase in aqueous medium, we decided to investigate biocatalysis of calixarene-protein complex in organic solvents in terms of pseudoactivity of peroxidase.

The present work involves the use of lower-rim substituted acid derivatives of *p-tert*-butylcalix[4,6,8]arenes ($^{t}$Butyl[4,6,8]$CH_2$COOH, Figure 1) for selective adsorption of myoglobin (Mb $c$) which was used as a model protein. Myoglobin is a multifunctional hemeprotein involved in several key physiological roles in animals as well as it exhibits a peroxidase-like activity in aqueous conventional medium by catalyzing the oxidation of phenolic compounds in the presence of hydrogen peroxide [4]. However, to our knowledge, there are no reports in the literature about the use of myoglobin for the oxidation of syringaldazine in the presence of hydrogen peroxide either in aqueous or organic media. Therefore, this work is concerned with the extraction of Mb $c$ with $^{t}$Butyl[4,6,8]$CH_2$COOH into organic media and the biocatalysis of Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex which catalyzed the oxidation of syringaldazine in the presence of hydrogen peroxide in organic medium. Subsequently, Mb $c$ was recovered from organic phase to fresh aqueous solution by using buffers with different pH values which apparently disrupted the non-covalent Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex.

## 2 Experimental

### 2.1 Materials

The structures and abbreviations of the extractants used in the present work are shown in Figure 1. The *p-tert*-butylcalix[$n$]arenes ($n = 4, 6$ and $8$) used as starting materials were prepared according to Gutsche's procedures [13,14,23]. These were in turn etherified with ethyl bromoacetate in the presence of anhydrous potassium carbonate in refluxing dry acetone for several hours (25–72 h), following reported procedures [1,15]. After isolation from the reaction mixture, the products were recrystallized from ethanol or ethanol-dichloromethane, yielding the corresponding esters in moderate to good yields. The acid derivatives were in turn isolated after treatment of the corresponding esters with aqueous $10\%$ tetramethylammonium hydroxide under reflux for $24$ h, followed by acidic isolation [5]. The myoglobin from horse heart (Mb $c$) and syringaldazine were obtained from Sigma Chemical Co. (USA) and all other chemicals were of analytical grade and were also purchased from Sigma Chemical Co. (USA).

### 2.2 Methods

#### *2.2.1 Adsorption of Mb c on calixarenes in organic solvents*

The Mb $c$ solutions ($14.7\ \mu$M) were prepared in appropriate buffers (i.e. 10 mM citrate, 50 mM phosphate and 10 mM glycine) with different pH values in the range of $4.5$–$9.0$ and different volumes of 2, 3 and 5 mL, as reported previously with some modifications [24,25]. Calixarenes ($^{t}$Butyl[4,6,8]$CH_2$COOH) solutions were prepared either in chloroform or dichloromethane at a concentration range of $0.1$–$3.0$ mM and different volumes of 2, 3 and 5 mL. The two phases (2, 3 and 5 mL) were mixed in stoppered glass test tubes and gently shaken for about $0.5$–$1$ h at $28\,^{\circ}$C. Phases were separated and protein concentration was measured by the absorbance of the *Soret* band peak ($407$–$410$ nm) [20], both in aqueous and organic phases (Thermo Nicolet Evolution 300) and the extractability was determined by the degree of extraction ($E = 1 - [\text{Mb } c]_{aq,eq}/[\text{Mb } c]_{aq,ini}$) [24,25].

#### *2.2.2 Protein assay*

Protein concentration in aqueous solutions was determined by Coomassie blue dye binding method by using BSA as the protein standard [29].

#### *2.2.3 Biocatalysis in aqueous medium*

*2.2.3.1 Pseudoperoxidase activity of Mb c.*

The pseudoperoxidase activity of Mb $c$ was assayed by using syringaldazine and $H_2O_2$ as substrates as described previously [9]. The assay of pseudoactivity of Mb $c$

was carried out by using 15 $\mu$L of 1 mM syringaldazine dissolved in ethanol, 3 $\mu$L of 0.8 mM $H_2O_2$, 262 $\mu$L of 50 mM phosphate buffer pH 6.5 and 50 $\mu$L of Mb *c* solutions (14.7 $\mu$M). The enzyme reaction was followed in a microtiter plate reader (Bio-Rad 680) at 550 nm due to oxidation of syringaldazine to the corresponding quinone at room temperature. The assay of pseudoperoxidase activity of Mb *c*–$^t$Butyl[6]$CH_2$COOH complex in chloroform was performed by using a reaction mixture containing 100 $\mu$L of syringaldazine 1 mM dissolved in chloroform, 3 $\mu$L of 0.8 mM $H_2O_2$, 127 $\mu$L of chloroform and 100 $\mu$L of Mb *c*–$^t$Butyl[6]$CH_2$COOH complex. One enzyme unit is defined as the amount of either Mb *c* or Mb *c*–$^t$Butyl[6]$CH_2$COOH complex required to convert one mole substrate into product per min under these experimental conditions.

*2.2.3.2 Optimum pH of pseudoperoxidase activity of Mb c.* Mb *c* (14.7 $\mu$M) was prepared in different buffers as follows: 50 mM phosphate buffer pH 6.5, 50 mM citrate buffer pH 3.5–5.5 and 10 mM glycine buffer pH 7.5–9.0. Such initial Mb *c* aqueous solutions were extracted with $^t$Butyl[6]$CH_2$COOH in organic medium and pseudoperoxidase activity of Mb *c*–$^t$Butyl[6]$CH_2$COOH complex was determined as described above.

*2.2.3.3 Kinetic parameters.*
The specific activity of Mb *c*–$^t$Butyl[6]$CH_2$COOH complex was determined by using initial aqueous solutions of Mb *c* (0.2–4.8 $\mu$M) in different buffer systems which were extracted with $^t$Butyl[6]$CH_2$COOH in organic medium, and the pseudoperoxidase activity of Mb *c*–$^t$Butyl[6]$CH_2$COOH complex was determined as described above. Apparent kinetic parameters ($V'_{max}$, $K'_{cat}$ and $K'_m$) of the reaction catalyzed by Mb *c*–$^t$Butyl[6]$CH_2$COOH complex were determined by both Michaelis-Menten and Lineweaver-Burk plots by using the software Enzyme Kinetics—SigmaPlot v.10.0 (Systat Software, Inc.). All assays were carried out in triplicates by varying the concentration of syringaldazine (0.01–0.6 mM) in the reaction medium as described previously.

*2.2.3.4 Stability of Mb c–$^t$Butyl[6]$CH_2$COOH complex.*
The half-life ($t_{1/2}$) of this complex was determined after Mb *c* extraction to organic phase with $^t$Butyl[6]$CH_2$COOH in $CHCl_3$, and Mb *c* (14.7 $\mu$M) was initially dissolved in 50 mM phosphate buffer pH 6.5 and 50 mM citrate buffer pH 5.5. Therefore, the pseudoactivity of peroxidase of the protein-complex was determined at suitable time intervals as described above and samples were stored at 4 °C.

*2.2.4 Recovery of Mb c from organic phase to fresh aqueous solution*

Mb *c* was recovered from organic phase to fresh aqueous solution by using buffers with different pH values (3.5–14.0) in order to disrupt the non-covalent Mb *c*–$^t$Butyl[6]$CH_2$COOH complex. Therefore, equal volumes (1 mL) of organic phase containing the protein-calixarene complex and aqueous solutions of buffer with different pH were mixed in an end-over-end mixer, for about 2 min and phases were separated by centrifugation at 10,000 ×g for 2 min. UV-Vis spectra of both phases were carried out and the degree of recovery ($E'$) was determined by $E' = [\text{Mb } c]_{aq,eq}/[\text{Mb } c]_{org,ini}$ as described in the literature [25]. Pseudoperoxidase activity of Mb *c* was assayed in both organic and aqueous phases in order to determine the recovery of pseudoactivity of Mb *c*.

*2.2.5 PDB survey of solvent accessible Arg, Lys and His residues in Mb c*

A 3D structure of Mb *c* from horse heart (PDB entry 1DWR) [2,6] was used to investigate structure solvent accessible surface which was analyzed with AREAIMOL [19] from CCP4 [7], and CCP4MG [27] was used to highlight solvent accessible arginine (Arg), lysine (Lys) and histidine (His) residues and 3D surface pictures production.

*2.2.6 FTIR analysis*

An FTIR analysis of $^t$Butyl[6]$CH_2$COOH, free Mb *c* and Mb *c*–$^t$Butyl[6]$CH_2$COOH complex, was carried out in a Bruker Vertex 70 as KBr pellets with a resolution of 2 $cm^{-1}$ in a range of 500–4000 $cm^{-1}$.

## 3 Results and discussion

Adsorption of Mb on calixarenes

Mb *c* has an Mr of 16,951 dalton, a pl value of 6.8 and has 2, 11 and 19 amino acid residues of Arg, His and Lys, respectively. Several conditions of protein extraction were tested for Mb *c* by using $^t$Butyl[4]$CH_2$COOH, $^t$Butyl[6]$CH_2$COOH and $^t$Butyl[8]$CH_2$COOH as well as two different organic solvents (i.e. $CHCl_3$ and $CH_2Cl_2$).

Our preliminary experiments were carried out with these two solvents in order to optimize protein extraction conditions in organic solvents with different log $P$ values. On the other hand, the reproducibility of protein extraction in organic phase was investigated by using different volumes (2, 3 and 5 mL) for both phases which revealed a high degree of reproducibility both in terms of protein content and pseudoactivity of peroxidase with a standard deviation of ±5.1 and 4, 3%, respectively (data not shown).

The extraction of Mb *c* at initial aqueous pH of 5.1 was investigated by using increasing concentrations of $^t$Butyl[4,6,8]$CH_2$COOH in chloroform as shown in Figure 2. As far as the degree of extraction ($E$) is concerned, the highest value of 0.92 was obtained by using $^t$Butyl[6]$CH_2$COOH at low stoichiometric ratio of calixarene to protein. However, regarding the $A_{Soret}$ of Mb *c*

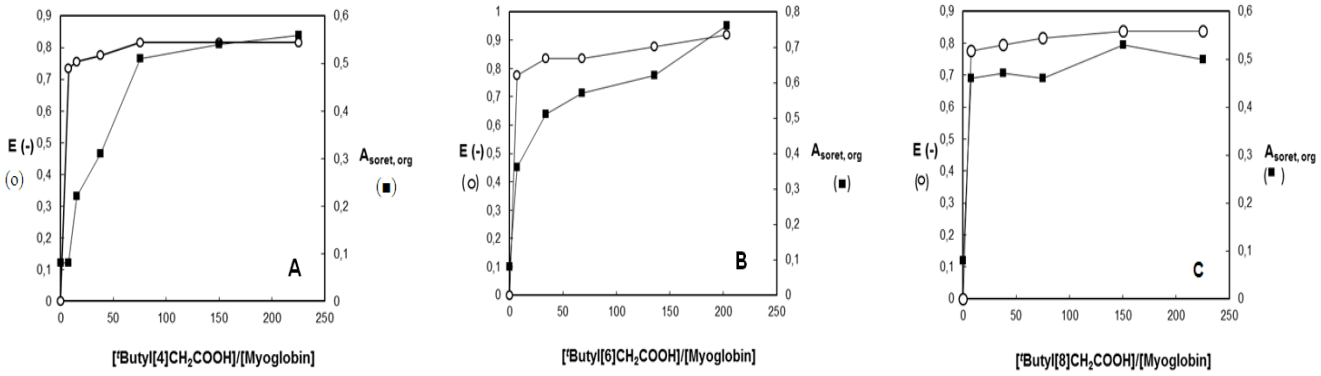



**Figure 2:** The effect of stoichiometric ratio of *p-tert*-butylcalix[$n$]arenes acid derivatives for the extraction of Mb $c$, with [Mb $c$]$_{ini}$ of 14.7 $\mu$M at pH$_{ini}$ 5.1: (a) extraction with $^{t}$Butyl[4]CH$_2$COOH; (b) extraction with $^{t}$Butyl[6]CH$_2$COOH; (c) extraction with $^{t}$Butyl[8]CH$_2$COOH. **E(-)(o);A**$_{soret,org}$(■).

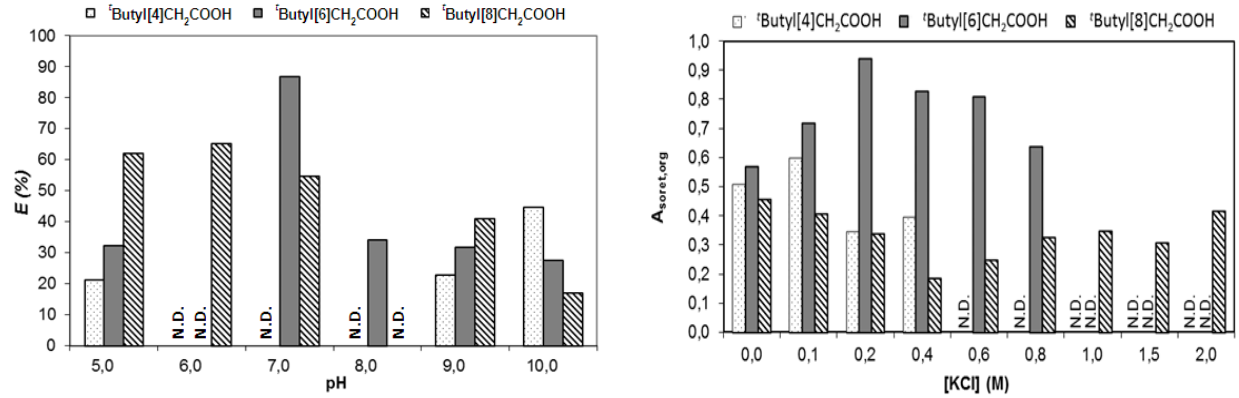
in organic phase, an increase was observed in the following order: $^{t}$Butyl[8]CH$_2$COOH > $^{t}$Butyl[6]CH$_2$COOH > $^{t}$Butyl[4]CH$_2$COOH (Figure 2). On the other hand, the stoichiometry of calixarene to protein was found to be 6.8 for Mb $c$. Besides chloroform, $CH_2Cl_2$ was also used to dissolve $^{t}$Butyl[4,6,8]CH$_2$COOH for protein extraction as shown in Figure 3(a) as a function of initial pH of Mb $c$ solution. These data strongly suggest that the best extraction conditions involved the use of pH 7.0 and 6.0 for $^{t}$Butyl[6]CH$_2$COOH and $^{t}$Butyl[8]CH$_2$COOH, respectively. Therefore, protein extraction took place in the presence of $^{t}$Butyl[4,6,8]CH$_2$COOH at pH values higher than that of the pl for Mb $c$ which suggests that protein-calixarene interactions are not limited only to ionic interactions.

According to [18], several other parameters affect protein-calixarene interactions besides electrostatic interactions, such as overall polarity of protein, surface topology, location of amino acids with charged side chains at surface and protein size. Regarding the selective extraction of myoglobin, we prepared an artificial mixture of myoglobin, laccase from *Coriolus versicolor* and horseradish peroxidase, and an extraction with calixarenes was performed as described in Materials and Methods. However, only myoglobin was extracted with calixarenes to organic media whereas other proteins (i.e. laccase and peroxidase) either remained in the aqueous phase or a precipitate was formed at the oil-water interface (data not shown). This result may be explained on the basis that both laccase and peroxidase have different pl values (i.e. 4.0 and 6.0, resp.) as well as low number of lysine residues (i.e. 5 and 6, resp.) available at protein surface compared with myoglobin [16, 22].

The effect of ionic strength of protein solution on the interaction with calixarene was investigated by using KCl in the range of 0–2.0 M (Figure 3(b)). The data presented in Figure 3(b) revealed that the highest extraction of Mb $c$ was observed at 0.2 M KCl compared with the protein solution without salt. This result strongly suggests that the presence of salt increased protein interaction with $^{t}$Butyl[4]CH$_2$COOH and $^{t}$Butyl[6]CH$_2$COOH whereas no significant increase in protein extraction was observed with $^{t}$Butyl[8]CH$_2$COOH (Figure 3(b)). On the other hand, it is well known that calix[6]arene exhibits a high affinity for potassium ion and therefore these ions may inhibit extraction of Mb $c$ with calix[6]arene. However, similar results were obtained by using sodium chloride and ammonium sulphate instead of potassium chloride in the extraction mixture (data not shown).

The effect of initial pH of Mb$c$ solutions on the degree of extraction was also investigated with $^{t}$Butyl[6]CH$_2$COOH which revealed that protein extraction occurred in a wide range of pH values (4.5–9.0) (Figure 4). These data again strongly suggest that there are several interactions involved between Mb $c$ and $^{t}$Butyl[6]CH$_2$COOH. On the other hand, protein extraction was observed at a pH value higher than its pl which points apparently that at these pH values electrostatic interactions are not involved since the protein and $^{t}$Butyl[6]CH$_2$COOH are negatively charged, and therefore other interactions may play a role in protein extraction process to organic phase.

The structural changes of Mb $c$–$^{t}$Butyl[6]CH$_2$COOH complex in chloroform were investigated by UV-Vis spectroscopy as well as by FTIR compared with the free Mb $c$ in aqueous medium which revealed significant changes at *Soret* band peak as well as in Q band peaks (Figure 5(a)). Similar changes in the *Soret* band of Mb $c$ have been previously reported which was chemically modified with poly(ethylene oxide) in organic solvents [34]. From the FTIR analysis, Mb $c$–calixarene complex revealed a new strong amide I band at 1610 cm$^{-1}$ due to




(a) (b)

**Figure 3:** (a) The effect of pH and nature of calixarene (1 mM $^{t}$Butyl[4]$CH_2$COOH, $^{t}$Butyl[6]$CH_2$COOH and $^{t}$Butyl[8]$CH_2$COOH C8) on the degree of extraction ($E$) for Mb $c$. N.D.: not determined. (b) The effect of [KCl] on the extraction of Mb $c$ by using 1 mM $^{t}$Butyl[4]$CH_2$COOH, $^{t}$Butyl[6]$CH_2$COOH and $^{t}$Butyl[8]$CH_2$COOH in $CH_2Cl_2$. N.D. : not determined.

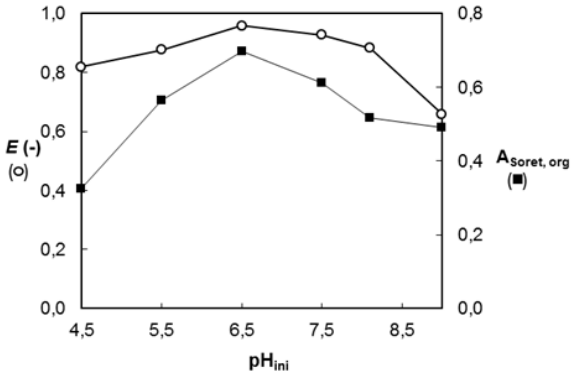



**Figure 4:** The effect of $pH_{ini}$ of aqueous solution of 14.7 $\mu$M Mb $c$ on the degree of extraction ($E$), with 3 mM $^{t}$Butyl[6]$CH_2$COOH in $CHCl_3$. **E(-)(o); $A_{soret,org}$(■).**

carbonyl group vibration (Figure 5(b)) whereas the free Mb $c$ spectrum exhibited two strong amide bands at 1655 $cm^{-1}$ (amide I) and 1541 $cm^{-1}$ (amide II). These data in FTIR spectra strongly suggest that Mb $c$ molecule was extracted to organic phase in the complex form with calixarenes due to this new absorbance band at 1610 $cm^{-1}$.

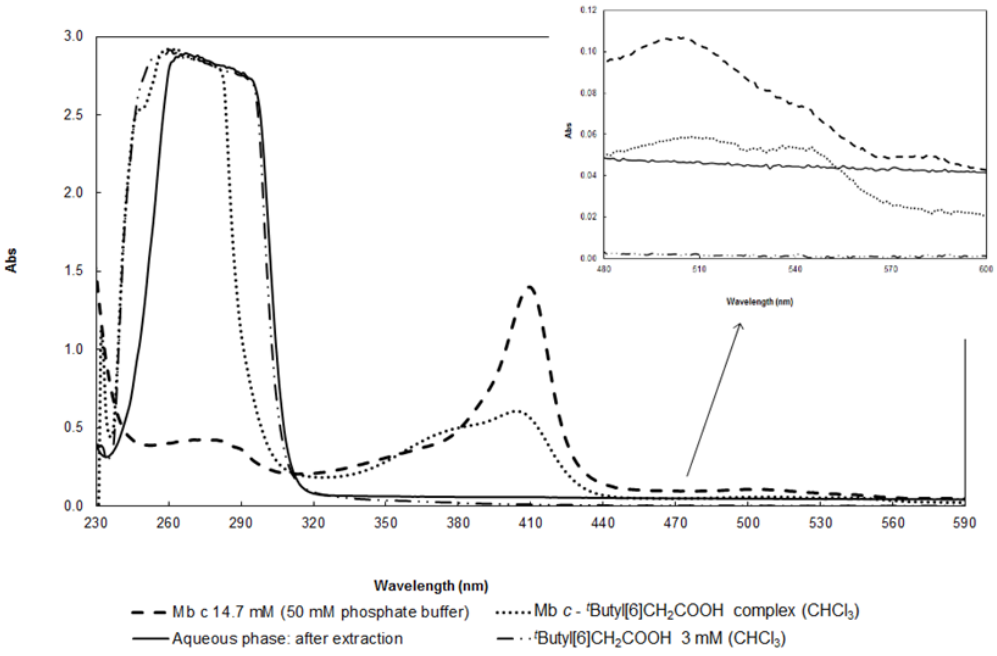
Solvent accessible positively charged amino acid side chains of Mb $c$ (PDB entry 1 DWR) revealed that all these residues (i.e. 2, 11 and 19 amino acid residues of Arg, His and Lys, resp.) are located at protein surface (data not shown) which may be involved in the interaction with calixarene molecules.

## Biocatalysis in organic solvents

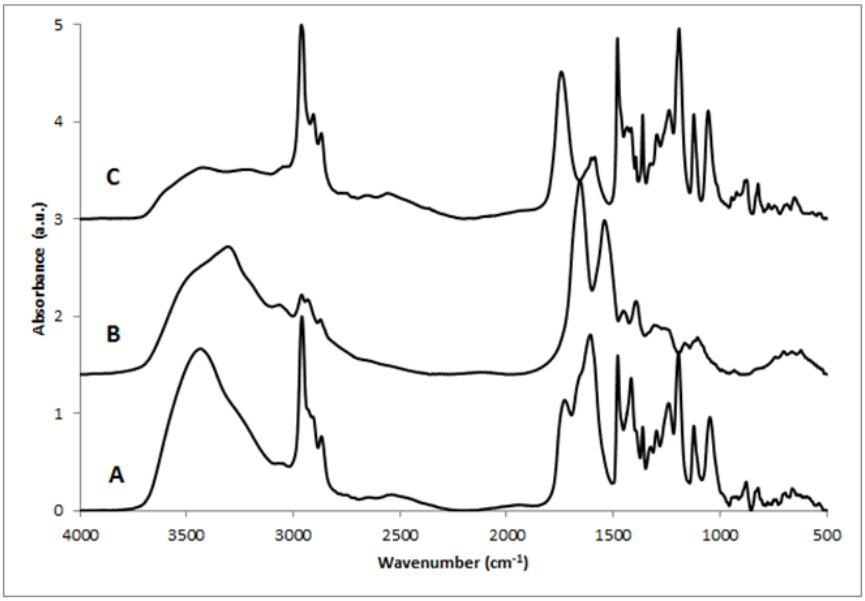
As far as biocatalysis is concerned, Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex exhibited a peroxidase-like activity in chloroform since it catalyzed the oxidation of syringaldazine in the presence of hydrogen peroxide, as shown in Figure 6(a). Moreover, there is a linear relationship between [Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex] and initial velocity of peroxidase activity (Figure 6(b)) with a specific activity of $1.37\times10^{-1}$ U.mg $protein^{-1}$ at pH 6.5. These data cannot be compared with the data on horseradish peroxidase published in the literature since the experimental conditions (i.e. solvents, substrate, concentrations and protein) are different from the ones used in the present work.

The effect of pH on biocatalysis in organic media is an important parameter since enzymes memorize the pH value in the last aqueous medium, and therefore the pH dependence as a function of enzyme activity is generally in agreement with that in conventional aqueous media [10, 17, 36]. Therefore, the effect of initial pH of Mb $c$ solutions on peroxidase activity was also investigated which revealed that the highest specific activity was obtained at pH 6.5 (Figure 7) which was slightly higher than the one obtained in aqueous media for Mb $c$ (data not shown). As far as pseudoactivity of peroxidase exhibited by Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex is concerned, there are no reports in the literature about this behavior in biocatalysis for this protein. Biocatalysis in non-conventional media has attracted a great interest in scientific and industrial communities for the last two decades since it is possible to obtain high yields of product compared with the aqueous media [17].

Apparent kinetic parameters ($K'_m$, $V'_{max}$ and $k'_{cat}$) were determined for the reaction catalyzed by Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex and are presented in Table 1. Again, these data cannot be compared with the data on horseradish peroxidase due to non-similarity in experimental conditions.




(a)



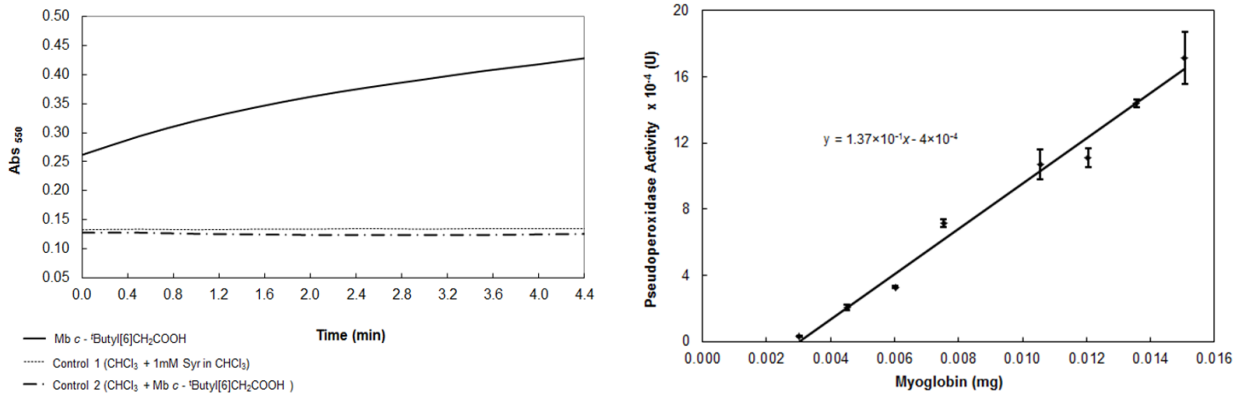

(b)

**Figure 5:** (a) Analysis of UV-Vis spectra (230–590 nm) of Mb $c$ in 50 mM phosphate buffer pH 6.5, Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex in $CHCl_3$ and Mb $c$ in aqueous phase after the extraction process with calixarene. (b) FTIR spectra of (A) Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex, (B) free Mb $c$ and (C) $^{t}$Butyl[6]$CH_2$COOH.

The stability of Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex in $CHCl_3$ was investigated by assaying its pseudoactivity at suitable time intervals as shown in Figure 8. From these data, $t_{1/2}$ values of 3.55 and 5.24 days were obtained for pH 5.5 and 6.5, respectively. Therefore, these results strongly suggest that it is possible to carry out kinetic characterization of such complex without any significant change in pseudoactivity of this protein.

Recovery of free Mb c in aqueous phase

Subsequently, Mb $c$ was recovered from organic phase to fresh aqueous solution by using buffers with different pH values (3.5–14.0) as shown in Figure 9(a). The degree of recovery ($E'$) was found to be the highest at pH 9.5 suggesting that alkaline pH was required to disrupt the Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex. However, peroxidase activity




(a) (b)

**Figure 6:** (a) Progress curve of the oxidation reaction of Syr catalyzed by Mb $c$–$^{t}$Butyl[6]$CH_2COOH$ complex ($4.5\ \mu$g protein in $CHCl_3$) in organic medium. —: Mb $c$–$^{t}$Butyl[6]$CH_2COOH$ complex; . . . . . : control 1 ($CHCl_3$ + 1 mM Syr in ethanol); – · –: control 2 (ethanol + Mb $c$–$^{t}$Butyl[6]$CH_2COOH$ in $CHCl_3$). (b) The effect of [Mb $c$] bound to $^{t}$Butyl[6]$CH_2COOH$ on initial velocity of pseudoactivity of peroxidase in organic medium.

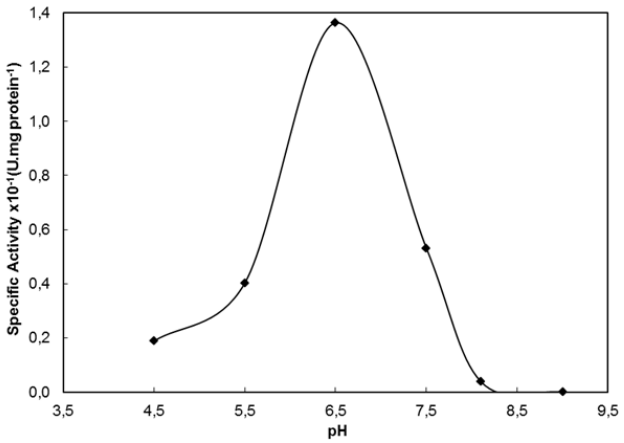



**Figure 7:** The effect of $pH_{ini}$ of aqueous solution of Mb $c$ on the specific activity of Mb $c$–$^{t}$Butyl[6]$CH_2COOH$ complex in organic medium at $pH_{ini}$ 6.5.

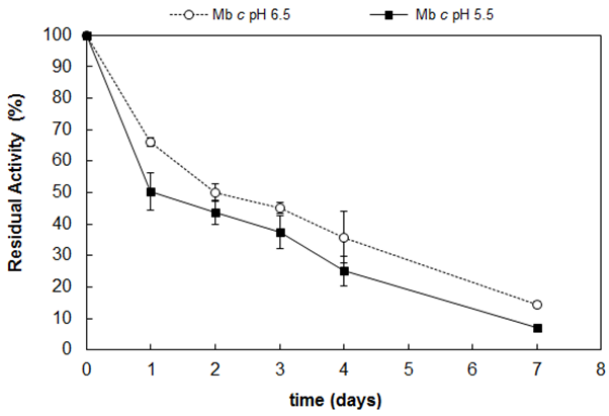



**Figure 8:** Stability of Mb $c$–$^{t}$Butyl[6]$CH_2COOH$ complex at $pH_{ini}$ 6.5 ($2.08 \times 10^{-6}$ U.mg protein$^{-1}$) and $pH_{ini}$ 5.5 ($1.75 \times 10^{-6}$ U.mg protein$^{-1}$) in $CHCl_3$ as a function of time. ○—pH 6.5 and ●—pH 5.5.

of free Mb $c$ in fresh aqueous solution exhibited the highest value at pH 10.5 and the recovery of enzyme activity was about 300% compared with the initial aqueous solution of Mb $c$. Such recoveries of pseudoperoxidase activity higher than 100% may be due to structural and conformational changes in Mb $c$ molecules during this recovery process.

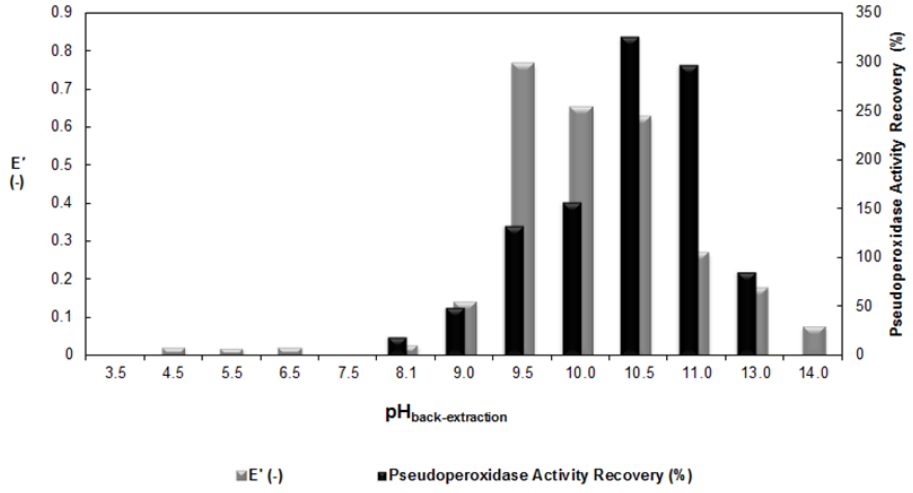
In order to investigate the recovery of free Mb $c$ into fresh aqueous solution at alkaline pH, protein and pseudoactivity assays were carried out in initial Mb $c$ aqueous solution, organic phase and in several recovery fractions as shown in Figure 9(b). These data strongly suggest that the whole Mb $c$ molecule was extracted to organic phase and free Mb $c$ was recovered into fresh aqueous medium since recovery fractions were monitored by the absorbance of the *Soret* band peak (407–410 nm) (data not shown). However, the protein recovery into fresh aqueous solution was 50.1% compared with the initial Mb $c$ aqueous solution.

In fact, we have obtained recoveries of pseudoperoxidase activity in the range of 500–600% (data not shown) under some experimental conditions and this protein extraction process could be used to increase at least 5-fold the pseudoactivity of this hemeprotein.

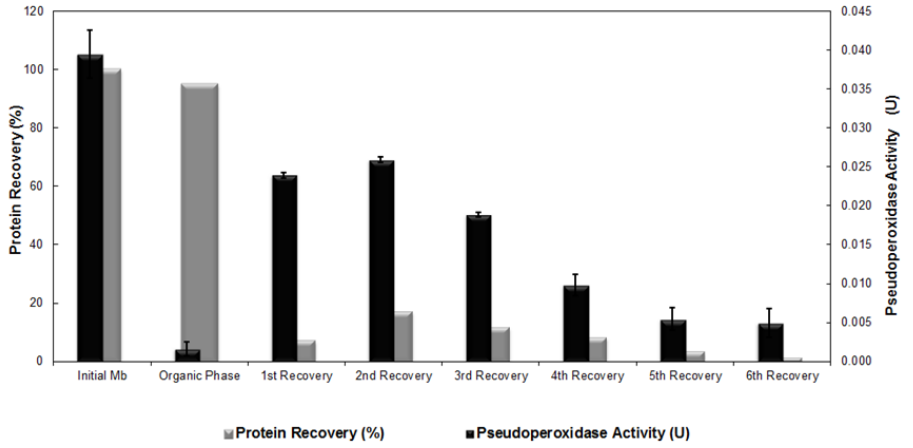
Published data have reported the recovery of cytochrome $c$ into fresh aqueous medium by using an acidic solution containing either ethanol, methanol or 1-pentanol [24, 25]. However, recent work [32] has shown that Cyt $c$ recovery into fresh aqueous medium was carried out by using a polar organic solvent in acid solutions which resulted in good recoveries of this protein. But this protein did not exhibit a pseudoperoxidase activity in aqueous medium since

| Kinetic parameters | pH | | | |
|---|---|---|---|---|
| | 4.5 | 5.5 | 6.5 | 7.5 |
| $K'_{\mathrm{m}}$(mM) | $3.83\times10^{-1}\pm 1.12\times10^{-2}$ | $6.35\times10^{-1}\pm 1.56\times10^{-2}$ | $4.55\times10^{-2}\pm 2.09\times10^{-3}$ | $5.41\times10^{-2}\pm 3.23\times10^{-3}$ |
| $k'_{\mathrm{cat}}(\mathrm{s}^{-1})$ | $7.00\times10^{-3}\pm 1.03\times10^{-3}$ | $1.59\times10^{-2}\pm 2.45\times10^{-3}$ | $1.51\times10^{-2}\pm 1.14\times10^{-3}$ | $6.40\times10^{-3}\pm 6.68\times10^{-4}$ |
| $V'_{\mathrm{max}}$(U.mg protein$^{-1}$) | $2.48\times10^{-2}\pm3.66\times10^{-3}$ | $5.64\times10^{-2}\pm8.66\times10^{-3}$ | $5.33\times10^{-2}\pm4.03\times10^{-3}$ | $2.26\times10^{-2}\pm2.36\times10^{-3}$ |
| $k'_{\mathrm{cat}}/K'_{m}(\mathrm{mM}^{-1}\mathrm{s}^{-1})$ | $1.83\times10^{-2}\pm9.19\times10^{-3}$ | $2.51\times10^{-2}\pm2.50\times10^{-3}$ | $3.31\times10^{-1}\pm5.45\times10^{-3}$ | $1.18\times10^{-1}\pm2.07\times10^{-3}$ |

**Table 1:** Apparent kinetic parameters of the reaction catalyzed by Mb *c*–$^{\mathrm{t}}$Butyl[6]$CH_2$COOH complex by using Michaelis-Menten plot.




(a)




(b)

**Figure 9:** (a) Degree of back-extraction ($E'$) of Mb *c* and recovery of pseudoperoxidase activity of Mb *c* into a fresh aqueous solution. (b) Protein recovery and pseudoperoxidase activity of Mb *c* in aqueous initial solution of Mb *c* and in fresh aqueous alkaline solution. Protein concentration and peroxidase activity were determined in the aqueous initial solution of Mb *c*, organic phase after extraction with calixarene and six recovery fractions by using 0.1 M sodium carbonate pH 11.0.

its tertiary structure was fully denatured [25]. Recently, the research work on the extraction of hemoglobin by calixarene molecules and bioconversion in organic media has been published by our group [30].

The present work provides valuable scientific information in three topics as follows: selective adsorption of proteins with calixarenes to organic media, biocatalysis in organic phase and recovery of original protein into fresh aqueous solution. Regarding the first topic, the adsorption of proteins with calixarenes is dependent on several parameters such as ionic and hydrophobic interactions, pl, no. of lysine as well as arginine and histidine residues. Therefore, this type of protein adsorption is novel and, more importantly, it involves non-covalent protein adsorption on calixarene molecules which is very mild compared with covalent adsorption of proteins. On the other hand, the second topic

is of great interest to many scientists working in biocatalysis because it is possible to extract a protein from aqueous to organic phase and carry out novel reactions compared with the aqueous system with novel apparent kinetic parameters. For instance, it would be possible to carry out novel reactions catalyzed by myoglobin-calixarene complex by using novel substrates (i.e. novel aromatic compounds) in organic media. Moreover, the present biocatalyst system is novel compared with the systems proposed by [33] since myoglobin-calixarene complex is completely dissolved in organic phase. Finally, the third topic is of scientific and commercial interest since it is possible to recover about 5-fold higher activity of pseudoperoxidase of free myoglobin into fresh aqueous medium as shown in Figure 9(b).

## 4 Conclusions

The use of *p-tert*-butylcalix[4,6,8]arene carboxylic acid derivatives has been presented in this work for selective adsorption of Mb $c$ to organic phase. Mb $c$ structure (PDB entry 1DWR) revealed solvent accessible 2 Arg, 19 Lys and 11 His residues with positively charged amino acid side chains at protein surface which may be involved in the interaction with calixarene molecules. Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex revealed pseudoperoxidase activity which catalyzed the oxidation of syringaldazine in the presence of hydrogen peroxide in organic medium containing chloroform. Kinetic characterization of Mb $c$–$^{t}$Butyl[6]$CH_2$COOH complex was carried out and apparent kinetic parameters ($V'_{max}$, $K'_m$, $k'_{cat}$ and $k'_{cat}/K'_m$) for the pseudoperoxidase activity were determined in organic media for different pH values by Michaelis-Menten plot. Furthermore, the stability of the protein-calixarene complex was investigated and $t_{1/2}$ values were obtained in the range of 3.5–5.2 days. Mb $c$–calixarene complex present in organic phase was recovered in fresh aqueous solutions at alkaline pH, with a recovery of pseudoperoxidase activity of over 100%. To the authors' knowledge, there are no reports in the literature about the use of calixarene derivatives for Mb $c$ adsorption in organic media as well as biocatalysis of this complex with pseudoactivity of peroxidase in organic solvents.

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