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Extraction of hemoglobin with calixarenes and biocatalysis in organic media of the complex with pseudoactivity of peroxidase

Magda C. Semedo[a,b], Amin Karmali[a,b,*], Patrícia D. Barata[a,b], José V. Prata[a,b]

[a] Chemical Engineering and Biotechnology Research Center of Instituto Superior de Engenharia de Lisboa, Rua Conselheiro Emídio Navarro, 1959-007 Lisboa, Portugal
[b] Department of Chemical Engineering of Instituto Superior de Engenharia de Lisboa, Rua Conselheiro Emídio Navarro, 1959-007 Lisboa, Portugal

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ABSTRACT

The present work involves the use of *p-tert*-butylcalix[4,6,8]arene carboxylic acid derivatives (^{t}Butyl[4,6,8]CH_2COOH) for selective extraction of hemoglobin. All three calixarenes extracted hemoglobin into the organic phase, exhibiting extraction parameters higher than 0.90. Evaluation of the solvent accessible positively charged amino acid side chains of hemoglobin (PDB entry 1XZ2) revealed that there are 8 arginine, 44 lysine and 30 histidine residues on the protein surface which may be involved in the interactions with the calixarene molecules. The hemoglobin–^{t}Butyl[6]CH_2COOH complex had pseudoperoxidase activity which catalysed the oxidation of syringaldazine in the presence of hydrogen peroxide in organic medium containing chloroform. The effect of pH, protein and substrate concentrations on biocatalysis was investigated using the hemoglobin–^{t}Butyl[6]CH_2COOH complex. This complex exhibited the highest specific activity of 9.92×10^{-2} U mg protein^{-1} at an initial pH of 7.5 in organic medium. 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 from a Michaelis–Menten plot. Furthermore, the stability of the protein–calixarene complex was investigated for different initial pH values and half-life ($t_{1/2}$) values were obtained in the range of 1.96 and 2.64 days. Hemoglobin–calixarene complex present in organic medium was recovered in fresh aqueous solutions at alkaline pH, with a recovery of pseudoperoxidase activity of over 100%. These results strongly suggest that the use of calixarene derivatives is an alternative technique for protein extraction and solubilisation in organic media for biocatalysis.

1. Introduction

Calix[*n*]arenes are one of the major classes of macrocyclic host compounds in supramolecular chemistry, which exhibit molecular recognition towards a wide range of biomolecules such as amino acids, peptides and proteins, lectins, enzymes, nucleotides, saccharides and steroids [1,2]. These compounds have a cavity-shaped architecture which can form host–guest complexes with a wide range of guest molecules by addition of different functional groups either at the upper or lower rim of the calixarenes. Calixarenes are widely used in several applications namely as sensor devices, in nuclear waste treatment, as catalysts in synthetic reactions, for biocatalysis and in liquid crystals [3,4].

Recently, Oshima et al. [5,6] have demonstrated that a calix[6]arene carboxylic acid derivative (^{t}Oct[6]CH_2COOH) exhibited high affinity for cationic proteins such as cytochrome *c* (Cyt *c*) by promoting its extraction in organic media. They have also investigated other calix[4,6,8]arenes as protein extraction agents such as those with large cavities of carboxylic acid derivatives of *p-tert*-octylcalix[6]arene (^{t}Oct[6]CH_2COOH), *p-tert*-octylcalix[8]arene (^{t}Oct[8]CH_2COOH) and *p-tert*-octylcalix[4]arene (^{t}Oct[4]CH_2COOH). These authors have also reported that ^{t}Oct[6]CH_2COOH molecules strongly interact with protonated amino groups on this protein surface forming a complex between the ^{t}Oct[6]CH_2COOH and the amino groups of the side chain of the large number of lysine (Lys) residues. Besides the extraction of cytochrome *c* to organic phase by using such calixarenes, they observed pseudoperoxidase activity of Cyt *c* in organic solvents by catalyzing the oxidation of 2,6-dimethoxyphenol in the presence of hydrogen peroxide [5]. Enzymes have evolved naturally to function in a 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 [7]. However, there are several advantages in using enzymes in organic solvents, both from the synthetic and processing points of view [8,9]. Several structural comparisons of

* Corresponding author at: Chemical Engineering and Biotechnology Research Center of Instituto Superior de Engenharia de Lisboa, Rua Conselheiro Emídio Navarro, 1, 1959-007 Lisboa, Portugal. Tel.: +351 218317052; fax: +351 218317267.
E-mail address: akarmali@deq.isel.ipl.pt (A. Karmali).

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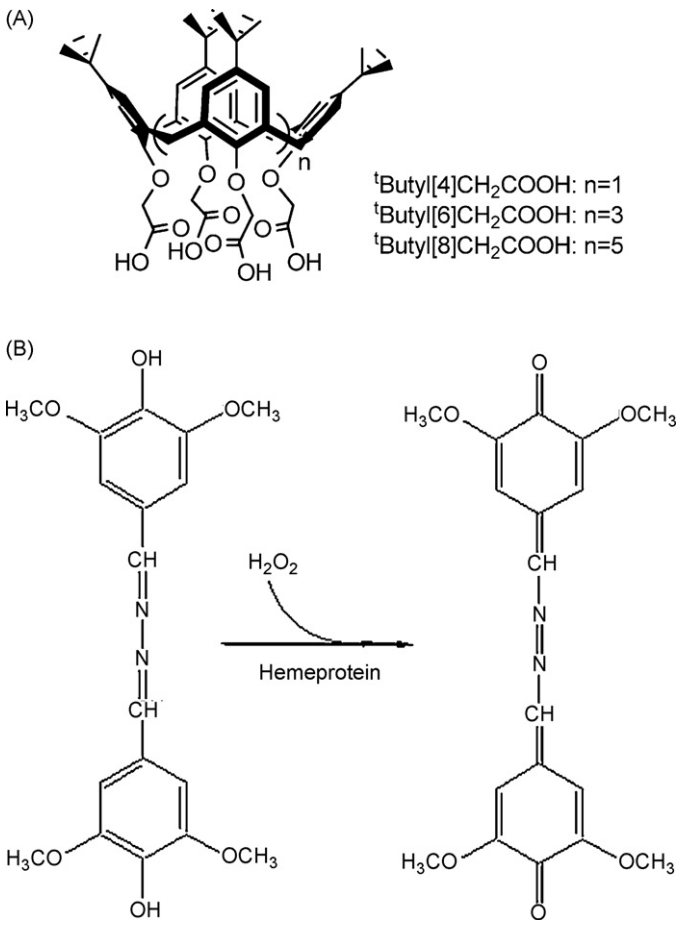

Fig. 1. (A) Structure of *p-tert*-butylcalix[*n*]arene acid derivatives. (B) Reaction catalysed by free Hb A and Hb A–calixarene complex with Syr and H2O2 as the substrates in aqueous and organic media, respectively.

proteins in aqueous versus non-aqueous polar media have shown that the protein folding remains generally unchanged. In fact, the observed minor structural differences between structures from organic media versus an aqueous environment were found to be comparable with those found within multiple, independently determined structures in aqueous media [10–13]. Biocatalysis in non-conventional media is an important field in enzyme engineering because there are several advantages over the aqueous media such as changes in substrate specificity, selectivity and biocatalyst stability [14,15]. On the other hand, Oshima et al. [5,6] have reported the recovery of an extracted Cyt *c* from an organic solution into fresh aqueous solutions by using acidic, alkaline solutions as well as the addition of alcohols which caused dissociation of the Cyt *c*–calixarene complex. Several additional parameters may play critical roles in protein calixarene interactions such as protein size, p*I*, hydrophilicity and the nature of amino acid residues at the protein surface [16,17].

The present study involves the selective extraction of human hemoglobin (Hb A) with ^{t}Butyl[4,6,8]CH_2COOH (Fig. 1A) in organic media, which was used as a model protein. Hemoglobin (Hb) is the major hemeprotein of red blood cells and is responsible for the transport of oxygen to the tissues as well as it exhibits peroxidase-like activity in aqueous conventional medium by catalyzing the oxidation of phenols, amines and olefins in the presence of hydrogen peroxide [18,19]. However, to our knowledge, there are no reports in the literature about the use of hemoglobin for oxidation of syringaldazine in the presence of hydrogen peroxide either in aqueous or organic media. Moreover, there are also no reports in the literature regarding the extraction of hemoglobin with calixarenes and biocatalysis of this complex in organic media. Therefore, this work is concerned with extraction of hemoglobin with ^{t}Butyl[4,6,8]CH_2COOH in organic media and biocatalysis of Hb–^{t}Butyl[6]CH_2COOH complex which catalysed the oxidation of syringaldazine in the presence of hydrogen peroxide (Fig. 1B) in organic medium. Subsequently, hemoglobin was recovered from the organic phase to fresh aqueous solution by using buffers with different pH values which apparently disrupted the Hb–^{t}Butyl[6]CH_2COOH complex.

2. Experimental

2.1. Materials

The structures and abbreviations of the calixarenes used in the present work are shown in Fig. 1A. The *p-tert*-butylcalix[*n*]arenes (*n* = 4, 6 and 8) used as starting materials were prepared according to the Gutsche's procedures [20–22]. Using previously reported methods these calixarenes were converted to esters using ethyl bromoacetate in the presence of anhydrous potassium carbonate in refluxing dry acetone for several hours (25–72 h) [23,24]. After appropriate workup, 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 workup [25]. Human hemoglobin (Hb A) for extraction experiments as well as biocatalysis was obtained from Biozyme Laboratories Limited (UK) and employed without further purification. All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Extraction of Hb A with calixarenes in organic solvents

Hb A solutions (3.9 μM) 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 [5,6]. Calixarene (^{t}Butyl[4,6,8]CH_2COOH) 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 sealed glass test tubes and gently shaken for about 0.5–1 h at 28 °C. Phases were separated and protein concentration was measured by the absorption spectrum of hemoglobin using a UV–vis spectrophotometer (Thermo Nicolet Evolution 300), in its ferrous state, is characterized by the *Soret* band peak at 407 nm (407–410 nm) [26,27], both in aqueous and organic phases and the extractability was determined by the degree of extraction: $E = 1 - [\text{Hb A}]_{aq,eq}/[\text{Hb A}]_{aq,ini}$ [5,6].

2.2.2. Protein assay

Protein concentrations in aqueous solutions were determined by Coomassie blue dye binding method by using BSA as the protein standard [28].

2.2.3. Biocatalysis in aqueous medium

2.2.3.1. Assay of pseudoperoxidase activity of free Hb A. Pseudoperoxidase activity of Hb A was assayed using syringaldazine (Syr) and H_2O_2 as substrates as previously described [29,30]. The assay of pseudoactivity of Hb A was done using 15 μL of 1 mM Syr dissolved in ethanol, 3 μL of 0.8 mM H_2O_2, 262 μL of 50 mM phosphate buffer pH 6.5 and 50 μL of Hb A samples. For comparative purposes, the reaction was also done using other substrates as follows: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (1 mM),

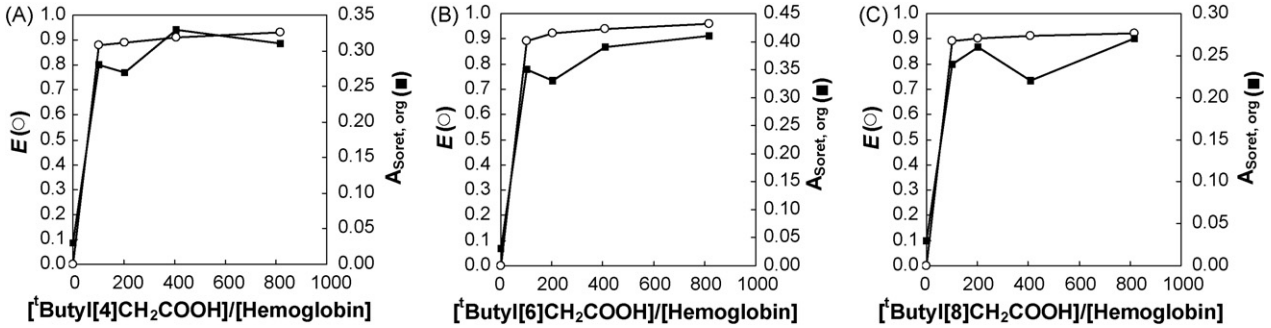

Fig. 2. The effect of stoichiometric ratio of *p-tert*-butylcalix[*n*]arenes acid derivatives on the extraction of Hb A with $[Hb\ A]_{ini}$ of 3.9 μM at pH_{ini} 5.1: (A) extraction with ^{t}Butyl[4]CH_2COOH; (B) extraction with ^{t}Butyl[6]CH_2COOH; (C) extraction with ^{t}Butyl[8]CH_2COOH.

o-dianisidine (0.19 mM) and guaiacol (10.0 mM). The different substrate oxidation reactions, catalysed by free Hb A (13.69 μg protein in 50 mM phosphate buffer pH 6.5) in aqueous medium in the presence of 3 μL of 0.8 mM H_2O_2, were determined spectrophotometrically at 550 nm (ε = 65,000 $cm^{-1}\,M^{-1}$ for Syr), 415 nm (ε = 36,000 $cm^{-1}\,M^{-1}$ for ABTS), 450 nm (ε = 8300 $cm^{-1}\,M^{-1}$ for *o*-dianisidine) and 450 nm (ε = 26,600 $cm^{-1}\,M^{-1}$ for guaiacol) using a microtiter plate reader (Bio-Rad 680) [29,30]. One enzyme unit is defined as the amount of Hb A required to convert 1 μmol of substrate into product per min under these experimental conditions.

2.2.4. Biocatalysis in organic solvents

2.2.4.1. Assay of pseudoperoxidase activity of Hb A–calixarene complex. The pseudoperoxidase activity of Hb A was assayed by using syringaldazine (Syr) and H_2O_2 as substrates as described previously [29,30]. The assay of pseudoactivity of Hb A was done using 15 μL of 1 mM Syr disssolved in chloroform, 3 μL of 0.8 mM H_2O_2, 262 μL of chloroform and 50 μL of Hb A–calixarene complex samples. The enzyme reaction was followed in a microtiter plate reader (Bio-Rad 680) at 550 nm due to oxidation of Syr to corresponding quinone at room temperature. One enzyme unit is defined as the amount of Hb A required to convert 1 μmol of substrate into product per min under these experimental conditions.

2.2.4.2. Optimum pH of pseudoperoxidase activity of Hb A–calixarene complex. Hb A (3.9 μM) 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 Hb A solutions were extracted with ^{t}Butyl[6]CH_2COOH in organic medium and pseudoperoxidase activity of Hb A–^{t}Butyl[6]CH_2COOH complex was determined as described above.

2.2.4.3. Kinetic parameters. The specific activity of Hb A–^{t}Butyl[6]CH_2COOH complex was determined by using aqueous initial solutions of Hb A (0.023–0.5 μM) in different buffer systems which was extracted with ^{t}Butyl[6]CH_2COOH in organic medium and pseudoperoxidase activity of Hb A–^{t}Butyl[6]CH_2COOH complex was determined as described above. Apparent kinetic parameters (V'_{max}, K'_{cat} and K'_{m}) of the reaction catalysed by Hb A–^{t}Butyl[6]CH_2COOH complex were determined from both Michaelis–Menten and Lineweaver-Burk plots by using the software Enzyme Kinetics—SigmaPlot v.10.0 (Systat Software, Inc.). All assays were done in triplicate by varying the concentration of syringaldazine (0.01–0.6 mM) in the reaction medium as described previously.

2.2.4.4. Stability of Hb A–^{t}Butyl[6]CH2COOH complex. The half-life ($t_{1/2}$) of this complex was determined after Hb A extraction to organic phase with ^{t}Butyl[6]CH_2COOH in $CHCl_3$, and Hb A (3.9 μM) was initially dissolved in 50 mM phosphate buffer pH 6.5 and 50 mM citrate buffer pH 5.5. Therefore, pseudoactivity of peroxidase of the protein-complex was determined at suitable time intervals as described above and samples were stored at 4 °C.

2.2.5. Recovery of Hb A from organic phase to fresh aqueous solution

After operation of a forward extraction (2 mL/2 mL) in a similar process to that described above, Hb A was recovered from organic phase to aqueous phases using back-extraction with buffers of different pH values (3.5–14.0) in order to disrupt the Hb A–^{t}Butyl[6]CH_2COOH 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 rpm for 2 min. The concentration of Hb A in the stripping solution was quantified by the absorbance of the *Soret* band in order to determine the degree of recovery (E' = $[Hb\ A]_{aq,eq}/[Hb\ A]_{org.ini}$) as described in the literature [5]. Pseudoperoxidase activity of Hb A was assayed in both organic and aqueous phases in order to determine the recovery of pseudoactivity of Hb A.

2.2.6. PDB survey of solvent accessible Arg, Lys and His residues in Hb A

The 3D structure of Hb A from human blood (PDB entry 1XZ2) [31,32] was used to investigate the solvent accessible surface structure which was analyzed using AREAIMOL [33], CCP4 [34], and CCP4MG [35] to highlight solvent accessible arginine (Arg), lysine (Lys) and histidine (His) residues and produce 3D surface images.

3. Results and discussion

3.1. Extraction of Hb A with calixarenes in organic solvents

Hb A is a globular protein with a Mr of 64,500 Da and a p*I* at 6.8–7.1 and it has a quaternary structure, which consists of four polypeptide subunits (two α, β protomers) and each subunit contains a heme (iron-porphyrin) as the active center. This protein has 12, 38 and 44 amino acid residues of Arg, His and Lys, respectively [19,32].

Several conditions for Hb A extraction were tested by using ^{t}Butyl[4]CH_2COOH, ^{t}Butyl[6]CH_2COOH and ^{t}Butyl[8]CH_2COOH as well as two different organic solvents (*i.e.* $CHCl_3$ and CH_2Cl_2).

The extraction of Hb A at initial aqueous pH of 5.1 was investigated by using increasing concentrations of ^{t}Butyl[4,6,8]CH_2COOH (0–3 mM) in chloroform as shown in Fig. 2. The highest value of the degree of extraction (E = 0.96) was obtained by using 3 mM ^{t}Butyl[6]CH_2COOH. The analysis of A_{Soret} values in organic phase revealed that the increase was in the following order: ^{t}Butyl[6]CH_2COOH > ^{t}Butyl[4]CH_2COOH > ^{t}Butyl[8]CH_2COOH

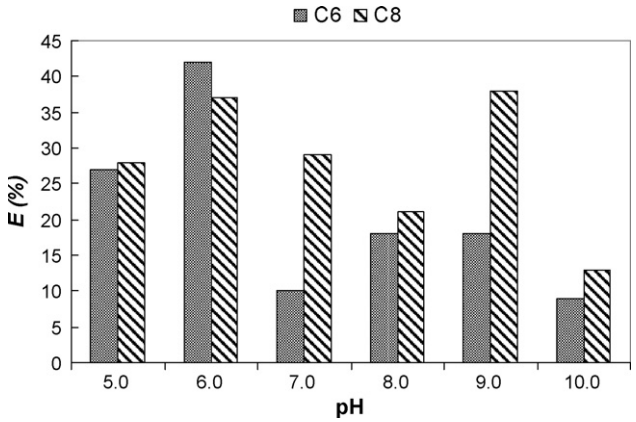

Fig. 3. The effect of pH and nature of calixarene (1 mM ^{t}Butyl[6]CH_2COOH and ^{t}Butyl[8]CH_2COOH C8) on degree of extraction (*E*) for Hb A.

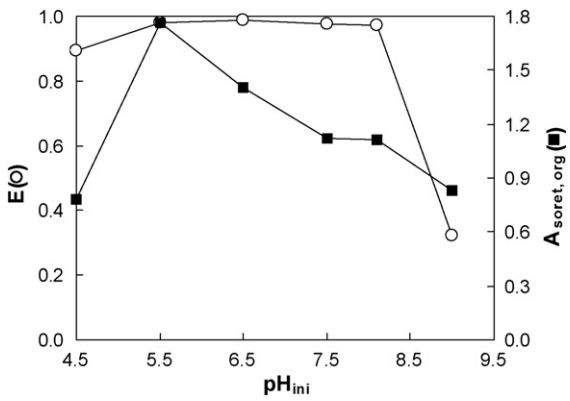

Fig. 4. The effect of pH_{ini} of aqueous solution of 3.9 μM Hb A on degree of extraction (*E*), with 3 mM ^{t}Butyl[6]CH_2COOH in $CHCl_3$.

(Fig. 2). The stoichiometry of calixarene to protein was found to be 96.8 for Hb A.

^{t}Butyl[6]CH_2COOH and ^{t}Butyl[8]CH_2COOH provided a high affinity toward Hb A in an initial pH range of 5–10 when it was extracted with CH_2Cl_2 (Fig. 3). These data strongly suggest that the best extraction conditions involved the use of pH 6.0 and 9.0 for ^{t}Butyl[6]CH_2COOH and ^{t}Butyl[8]CH_2COOH, respectively.

Hb A extraction with ^{t}Butyl[4,6,8]CH_2COOH took place at initial pH values higher than the p*I* for Hb A which suggests that protein–calixarene interactions are not limited to ionic interactions. Other parameters also 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 may be involved in the formation of the host–guest complex [16,17].

The effect of initial pH of Hb A solutions on the degree of extraction was also investigated with ^{t}Butyl[6]CH_2COOH, which revealed that protein extraction occurred in a wide range of pH values (4.5–9.0) (Fig. 4). This behavior strongly suggests that there are several interactions involved between Hb A and ^{t}Butyl[6]CH_2COOH. Additionally, protein extraction was observed at a pH value higher than its p*I* which supports the idea that at these pH values electrostatic interactions are not involved since protein and ^{t}Butyl[6]CH_2COOH are negatively charged and therefore other interactions may play a role in protein extraction process to organic phase.

3.2. Structural analysis of Hb A–calixarene complexes

The structural changes of Hb A–^{t}Butyl[6]CH_2COOH complex in chloroform were studied by UV–vis spectroscopy compared with the Hb A in aqueous medium. As the initial pH was lowered, the *Soret* bands were substantially blue shifted, as well as in Q band peaks (Fig. 5). These results are in agreement with previously reported UV–vis spectra [36]. In addition a PDB survey of solvent accessible positively charged amino acid side chains of Hb A (PDB entry 1XZ2) [32] revealed that 8 Arg, 30 His and 44 Lys residues are located at protein surface (Fig. 6) which may be involved in interaction with calixarene molecules.

3.3. Biocatalysis of free Hb A in aqueous medium

Free Hb A exhibited pseudoactivity of peroxidase in aqueous media when Syr, ABTS, *o*-dianisidine, pyrogallol and guaiacol were used as substrates (Fig. 7A). The pseudoactivities of free Hb A in aqueous media with each of these substrates were $1.64 \times 10^{-3} \pm 3.52 \times 10^{-5}$ U (Syr), $6.71 \times 10^{-3} \pm 9.16 \times 10^{-5}$ U (ABTS), $4.26 \times 10^{-3} \pm 9.02 \times 10^{-5}$ U (*o*-dianisidine) and $1.25 \times 10^{-3} \pm 3.41 \times 10^{-5}$ U (guaiacol) (Fig. 7A). For comparative purposes, percentages of the initial rate were determined for each substrate which are 24.4%, 100%, 63.3% and 18.6%, respectively.

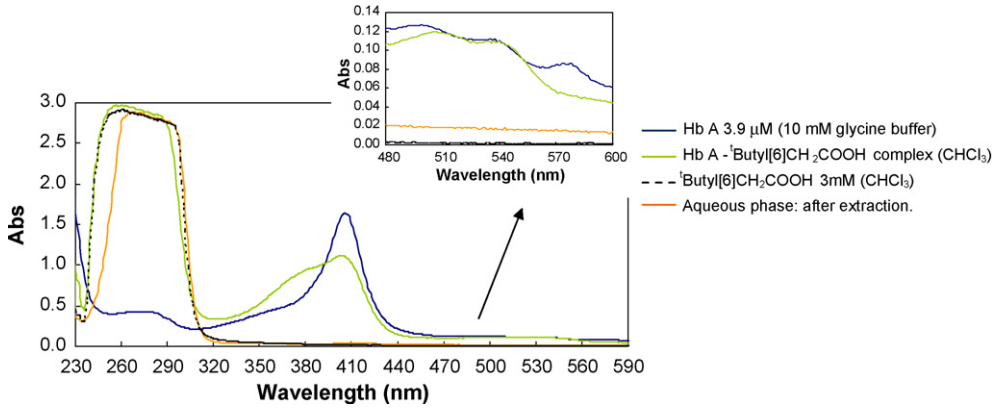

Fig. 5. Analysis of UV–vis spectra (230–590 nm) of Hb A in 10 mM glycine buffer pH 7.5, Hb A–^{t}Butyl[6]CH_2COOH complex in $CHCl_3$ and Hb A in aqueous phase after the extraction process with calixarene.

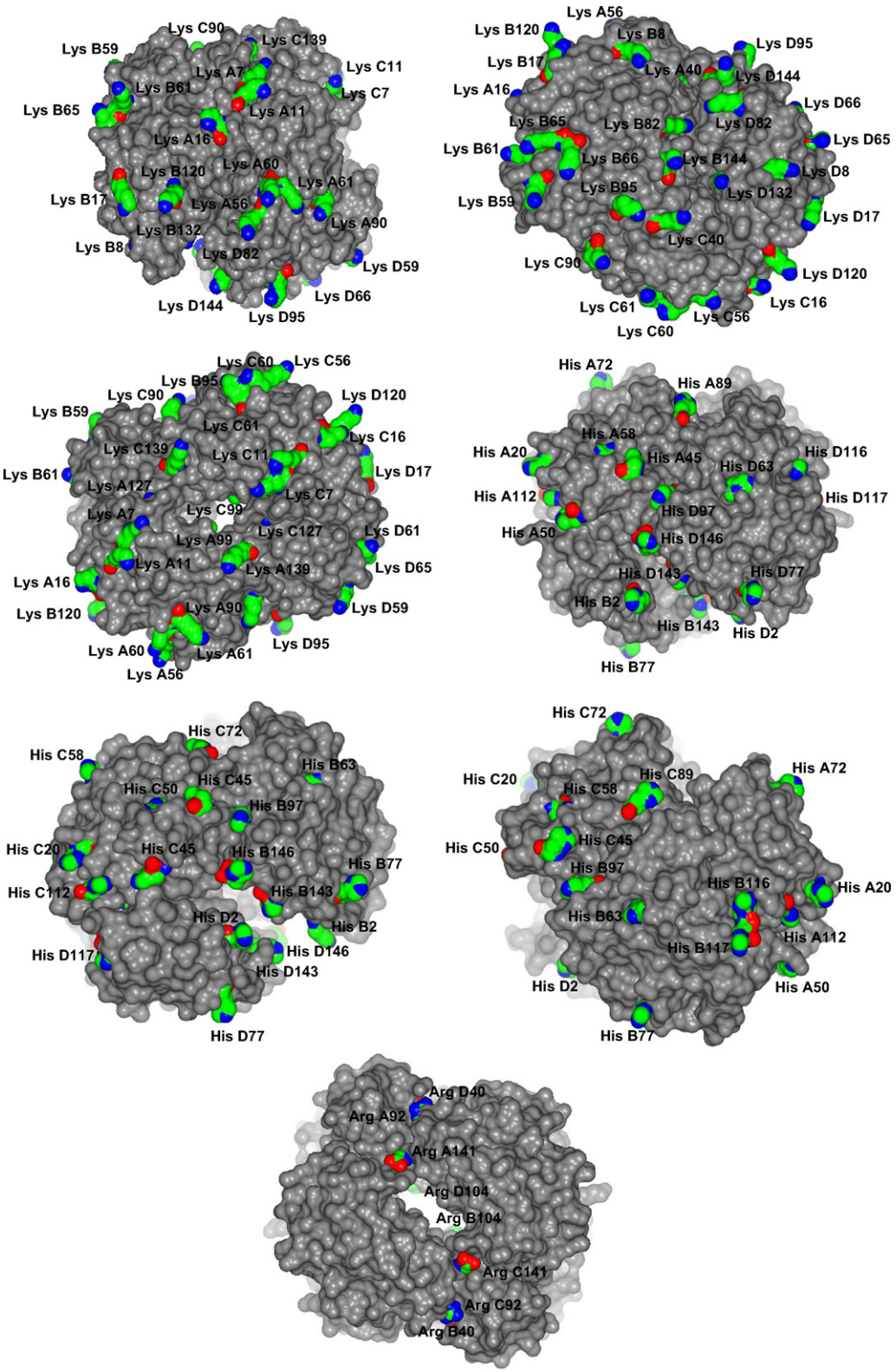

Fig. 6. View of Hb A (PDB entry 1XZ2) solvent accessible surface (grey), the α (chains A and C) and β (chains B and D) subunits, with surface Arg, Lys and His residues highlighted in their atom colors, with carbon in green, nitrogen in blue, and oxygen in red (front, back, top and bottom views of the protein molecule).

3.4. Biocatalysis in organic media by Hb A–^{t}Butyl[6]CH_2COOH complex

Since free Hb A exhibited peseudoactivity of peroxidase in aqueous medium by using typical peroxidase substrates, biocatalysis of the Hb A–^{t}Butyl[6]CH_2COOH complex was determined using organic solvents with these substrates. However, ABTS, *o*-dianisidine and pyrogallol were insoluble in chloroform whereas guaiacol was soluble in chloroform but there was no pseudoactivity of peroxidase of Hb–calixarene complex when guaiacol was used

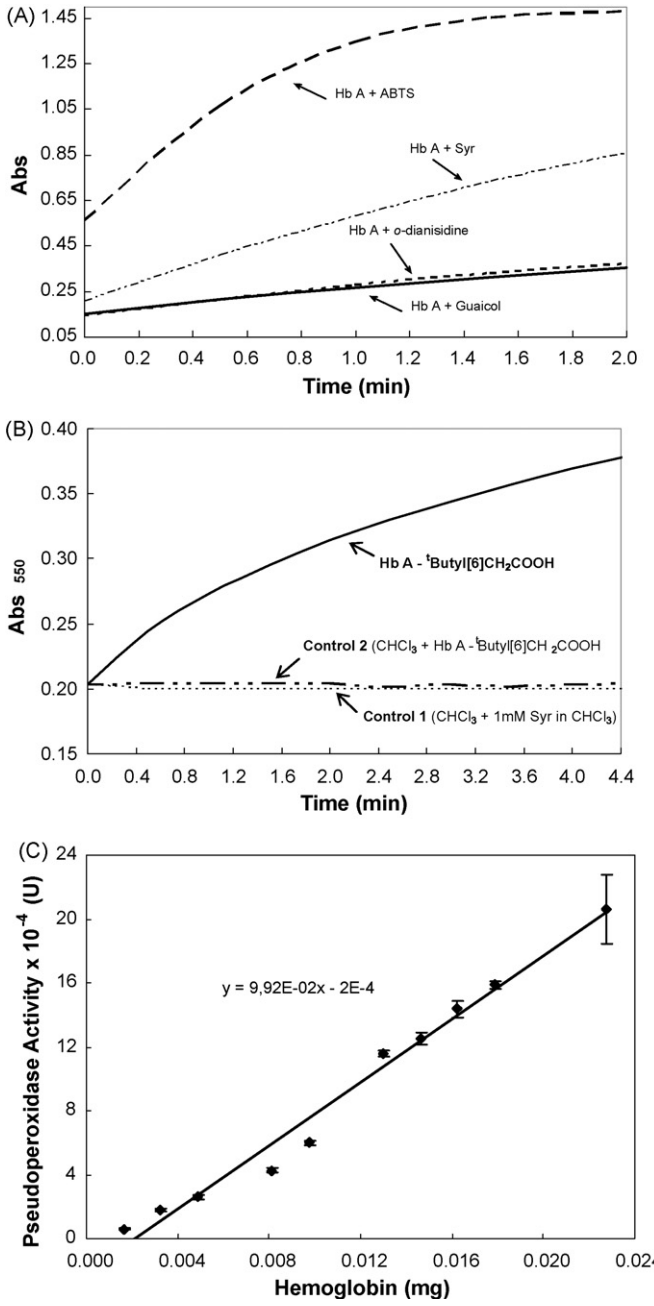

Fig. 7. (A) Progress curve of the oxidation reaction of several substrates (*i.e.* Syr, ABTS, *o*-dianisidine and guaiacol). catalysed by free Hb A (13.69 μg protein in 50 mM phosphate buffer pH 6.5) in aqueous medium in the presence of 3 μL of 0.8 mM H_2O_2 by following the absorbance of the reaction mixture at 550, 415, 450 and 450 nm, respectively as described in Sections 2.1 and 2.2. (B) Progress curve of the oxidation reaction of Syr catalysed by Hb A–^{t}Butyl[6]CH_2COOH complex (3.9 μg protein in $CHCl_3$) in organic medium. — — Hb A–^{t}Butyl[6]CH_2COOH complex; ····· Control 1 ($CHCl_3$ + 1 mM Syr in $CHCl_3$) and - - - Control 2 ($CHCl_3$ + C6—protein in $CHCl_3$). (C) The effect of [Hb A] bound to ^{t}Butyl[6]CH_2COOH on initial velocity of pseudoactivity of peroxidase in organic medium.

as the substrate (data not shown). Presumably, Hb–calixarene complex did not exhibit a high affinity for this substrate in organic medium as far as biocatalysis is concerned.

The Hb A–^{t}Butyl[6]CH_2COOH complex exhibits pseudoperoxidase activity in chloroform, catalyzing the oxidation of syringaldazine in the presence of hydrogen peroxide (Fig. 7B). There was a linear relationship between [Hb A–^{t}Butyl[6]CH_2COOH complex] and initial velocity of pseudoperoxidase activity (Fig. 7C) with a specific activity of 9.92×10^{-2} U mg protein^{-1} at an initial pH

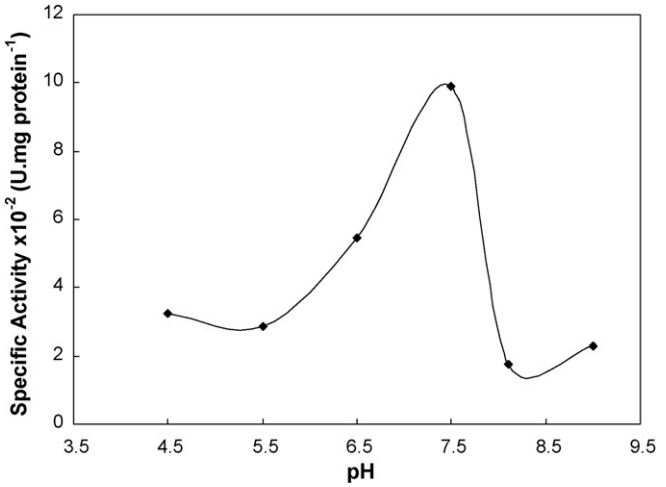

Fig. 8. The effect of pH_{ini} of aqueous solution of Hb A on the specific activity of Hb A–^{t}Butyl[6]CH_2COOH complex in organic medium.

of 7.5, which is about six-fold lower than the data obtained for Hb A dissolved in aqueous media. These data are also lower when compared with data published in the literature for horseradish peroxidase by using *o*-phenylenediamine dihydrochloride and guaiacol in toluene in the presence of H_2O_2 [37]. Enzyme activity in organic solvents depends on several parameters such as water activity, substrate–product solvation, enzyme form, the nature of the solvent and regulation of pH [8,9,38]. The pH value during enzyme pretreatment is one of the most prominent factors in biocatalysis in organic media, since enzymes have molecular memory and they are kinetically trapped in their previous conformation corresponding to the pH in the last aqueous solution. The pseudoperoxidase activity of Hb A was dependent on the initial pH at the extraction process and the highest specific activity was obtained at pH 7.5 (Fig. 8).

Apparent kinetic parameters (K'_m, V'_{max} and K'_{cat}) were determined for the reaction catalysed by Hb A–^{t}Butyl[6]CH_2COOH complex using Michaelis–Menten (Table 1) and Lineweaver–Burk plots (data not shown). The V'_{max} values obtained in this work may be compared with previously reported data for hemoglobin in dioxane (6.89×10^{-3} U mg protein^{-1}), toluene (0.496 U mg protein^{-1}) and acetonitrile (4.82×10^{-2} U mg protein^{-1}) using *o*-phenylenediamine dihydrochloride in the presence of H_2O_2 [18]. On the other hand, K'_m values (*i.e.* 2.85 mM in toluene) reported in the literature by the same group for hemoglobin, are higher than the data presented in this work.

The catalytic activity of Hb A–^{t}Butyl[6]CH_2COOH complex was low when compared with the activity of free and immobilized horseradish peroxidase in organic solvents reported in the literature [37]. Structural differences between the heme pockets of hemoglobin and conventional peroxidases are proposed as the reason for this difference in peroxidase activity [39].

The stability of Hb A–^{t}Butyl[6]CH_2COOH complex in $CHCl_3$ was investigated by assaying its pseudoactivity at suitable time intervals as shown in Fig. 9. From these data, $t_{1/2}$ values of 1.96 and 2.64 days were obtained for pH 5.5 and 6.5, respectively. Consequently, these results suggest that it is possible to perform full kinetic characterization of such a protein-complex without any significant change in pseudoactivity of this protein. As far as pseudoactivity of peroxidase exhibited by Hb A–^{t}Butyl[6]CH_2COOH complex, there are no reports in the literature about this behaviour in biocatalysis for this protein. The possibility that the heme group was extracted from the Hb A and was responsible for pseudoactivity of peroxidase was excluded because whole protein was recovered into fresh aqueous medium at alkaline pH (data not shown).

Table 1
Apparent kinetic parameters of the reaction catalysed by Hb A–^{t}Butyl[6]CH_2COOH complex by using Michaelis–Menten plot.

	Michaelis–Menten			
	pH			
	4.5	5.5	6.5	7.5
Kinetic parameters				
K'_m (mM)	$4.41 \times 10^{-2} \pm 5.51 \times 10^{-3}$	$9.00 \times 10^{-1} \pm 2.91 \times 10^{-2}$	$9.06 \times 10^{-1} \pm 1.62 \times 10^{-1}$	$9.25 \times 10^{-2} \pm 1.38 \times 10^{-2}$
k'_{cat} (s^{-1})	$7.11 \times 10^{-3} \pm 1.76 \times 10^{-4}$	$1.52 \times 10^{-1} \pm 1.12 \times 10^{-3}$	$1.09 \times 10^{-1} \pm 7.26 \times 10^{-4}$	$5.50 \times 10^{-2} \pm 1.85 \times 10^{-4}$
V'_{max} (U mg protein^{-1})	$6.61 \times 10^{-3} \pm 1.64 \times 10^{-3}$	$1.42 \times 10^{-1} \pm 1.04 \times 10^{-2}$	$1.01 \times 10^{-1} \pm 6.76 \times 10^{-2}$	$5.12 \times 10^{-2} \pm 1.72 \times 10^{-3}$
k'_{cat}/K'_m (mM^{-1} s^{-1})	$1.61 \times 10^{-1} \pm 1.36 \times 10^{-2}$	$1.69 \times 10^{-1} \pm 3.85 \times 10^{-2}$	$1.20 \times 10^{-1} \pm 4.48 \times 10^{-2}$	$5.95 \times 10^{-1} \pm 1.34 \times 10^{-2}$

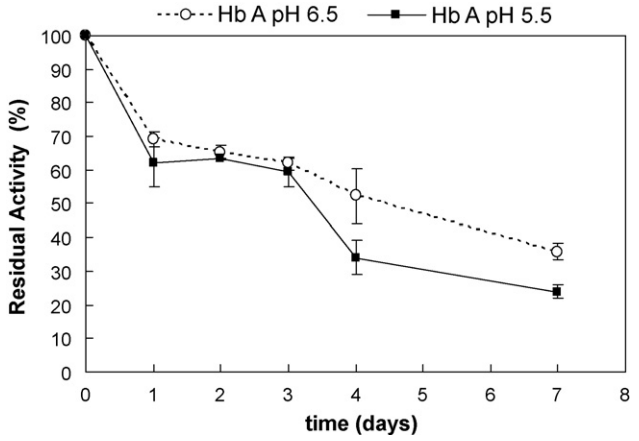

Fig. 9. Stability of Hb A–^{t}Butyl[6]CH_2COOH complex at pH_{ini} 6.5 (6.86×10^{-7} U mg protein^{-1}) and pH_{ini} 5.5 (4.34×10^{-6} U mg protein^{-1}) in $CHCl_3$ as a function of time. ○—pH 6.5 and ●—pH 5.5.

3.5. Recovery of Hb A from organic phase to fresh aqueous solution

The back-extraction of proteins from an organic phase to an aqueous stripping phase is a very important process for recovery of protein. After extraction of Hb A with ^{t}Butyl[6]CH_2COOH in chloroform, this protein was recovered by back-extraction using different pH buffers (3.5–14.0) as shown in Fig. 10. The highest degree of recovery (E') was found at pH 9.5. The data presented suggests that the pH range 7.5–9.5 was required to disrupt Hb A–^{t}Butyl[6]CH_2COOH complex. On the other hand, pseudoperoxidase activity of free Hb A in fresh aqueous solution exhibited the highest value at pH 9.0 and recovery of enzyme activity was about 150% compared with the activity of initial aqueous solution of Hb A. These results may be attributed to structural and conformational changes in Hb A molecules during this recovery process. Oshima et al. [6] have described the back-extraction of Cyt *c* into an aqueous phase using an acidic solution containing ethanol, methanol or 1-pentanol. Recently, Shimojo et al. [1] have reported that the recovery of Cyt *c* into aqueous solution can be done using a polar organic solvent in acid solutions. However, the recovered Cyt *c* did not exhibit pseudoperoxidase activity in aqueous medium since its tertiary structure was fully denatured [6].

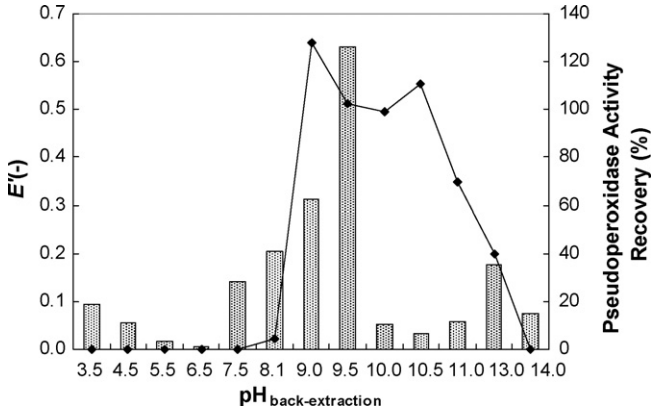

Fig. 10. Degree of back-extraction (E') of Hb A and recovery of pseudoperoxidase activity of Hb A in a fresh aqueous solution.

4. Conclusions

The use of *p-tert*-butylcalix[4,6,8]arene carboxylic acid derivatives has been presented in this work for selective extraction of Hb A into an organic phase. Hb A structure (PDB entry 1XZ2) revealed solvent accessible 8 Arg, 44 Lys and 30 His residues with positively charged amino acid side chains at protein surface which may be involved in the interaction with calixarene molecules. The Hb A–^{t}Butyl[6]CH_2COOH complex revealed pseudoperoxidase activity which catalysed the oxidation of syringaldazine in the presence of hydrogen peroxide in organic medium containing chloroform. Kinetic characterization of the Hb A–^{t}Butyl[6]CH_2COOH complex was done 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 using a Michaelis–Menten plot. Furthermore, the stability of the protein–calixarene complex was investigated and $t_{1/2}$ values were obtained in the range of 1.96–2.64 days. Hb A–calixarene complex present in the organic phase was back-extracted into 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 Hb A extraction and solubilization in organic media as well as biocatalysis of this complex with pseudoactivity of peroxidase in organic solvents.

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