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Long-term protein packaging in bio-ionic liquids: Improved catalytic activity and enhanced stability of cytochrome C against multiple stresses

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Abstract

There is a considerable interest in the use of structurally stable and catalytically active enzymes, such as cytochrome C (Cyt C), in the pharmaceutical and fine chemical industries. However, harsh process conditions, such as temperature, pH, and presence of organic solvents, are the major barriers to the effective use of enzymes in biocatalysis. Herein, we demonstrate the suitability of bio-based ionic liquids (ILs) formed by the cholinium cation and dicarboxylate-based anions as potential media for enzymes, in which remarkable enhanced activity and improved stability of Cyt C against multiple stresses were obtained. Among the several bio-ILs studied, an exceptionally high catalytic activity (> 50-fold) of Cyt C was observed in aqueous solutions of cholinium glutarate ([Ch][Glu]; 1g/mL) as compared to the commonly used phosphate buffer solutions (pH 7.2), and > 25-fold as compared to aqueous solutions of cholinium dihydrogen phosphate ([Ch][Dhp]; 0.5g/mL) —the best known IL for long term stability of Cyt C. The catalytic activity of the enzyme in presence of bio-ILs was retained against several external stimulus, such as chemical denaturants (H₂O₂ and GuHCl), and temperatures up to 120 °C. The observed enzyme activity is in agreement with its structural stability, as confirmed by UV–Vis, circular dichroism (CD), and Fourier transform infrared (FT-IR) spectroscopies. Taking advantage of the multi-ionization states of di/tri-carboxylic acids, the pH was switched from acidic to basic by the addition of the corresponding carboxylic acid and choline hydroxide, respectively. The activity was found to be maximum at a 1:1 ratio of [Ch][carboxylate], with a pH in the range from 3 to 5.5. Moreover, it was found that the bio-ILs studied herein protect the enzyme against protease digestion and allow long-term storage (at least for 21 weeks) at room temperature. An attempt by molecular docking was also made to better understand the efficacy of the investigated bio-ILs towards the enhanced activity and long term stability of Cyt C. The results showed that dicarboxylates anions interact with the active site's amino acids of the enzyme through H-bonding and electrostatic interactions, which are responsible for the observed enhancement of the catalytic activity. Finally, it is demonstrated that Cyt C can be successfully recovered from the aqueous solution of bio-ILs and reused without compromising its yield, structural integrity and catalytic activity, thereby overcoming the major limitations in the use of IL-protein systems in biocatalysis.

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Introduction

Enzymatic biocatalysis has been recognized as a key process in diverse fields of applications, including synthesis of valuable pharmaceutical intermediates and biofuels from renewable resources.^{1–3} Nowadays, there is a considerable interest in the use of structurally stable and catalytically active enzymes in food, pharmaceutical, and fine chemicals industries.⁴ However, enzymes have evolved to work in cellular environments, and are usually unstable under harsh process conditions, such as high temperature, high pressure, and presence of organic solvents. These are often the major limitations behind a more extended use of enzymes in industrial processes.⁵ Organic solvents usually have deleterious effects over enzymes, for instance by leading to the unfolding of the enzyme conformations, loss of bound water from the protein surface, and damage of the protein structure.⁶ In order to overcome these problems, some strategies have been proposed: (i) modification of the enzyme surface, thereby increasing its resistance to harsh conditions; and (ii) manipulation of the solvent environment to improve the enzymes stability and activity.⁷ Among these, the entrapment of enzymes has shown a great promise toward the improvement of their catalytic activity and stability.⁸ However, such strategy carries the risk of enzyme deactivation, particularly by shrinking and confinement of the modified enzyme during condensation and purification processes, thereby limiting its bioavailability.⁹ Interestingly, enzyme deactivation might be overcome by employing ionic liquids (ILs) as protective media.¹⁰

ILs are molten salts with unique properties, such as high ionic conductivity, high electrochemical stability, and low volatility, and are becoming popular as promising solvents for many industrial processes.^{11–13} A large number of reports are available for the processing of biomacromolecules, such as enzyme/protein and DNA in ILs.^{14–19} Nevertheless, the IL-protein systems reported up to date mainly comprise imidazolium-based ILs, which may display low biocompatibility features. Alternatively, cholinium-based ILs have been proposed in more recent years due to their enhanced biocompatible²⁰ and biodegradable²¹ nature. Various studies have shown that cholinium-based ILs are remarkable solvents to maintain the activity and stability of several proteins, including Cytochrome C (Cyt C), which is an industrially important and ubiquitous peroxidase enzyme.^{22–25} Amongst the series of cholinium-based ILs investigated, cholinium-dihydrogen phosphate ([Ch][Dhp]) has been highlighted as the most biocompatible media for Cyt C.^{26–31} Fujita et al.^{26,30,31} studied the structural stability of Cyt C in [Ch][Dhp] and showed that the secondary structure is retained at a high temperature (80 °C), and that the activity of the enzyme is kept intact even after storage at room temperature for long periods. However, no significant improvements in the catalytic activity of Cyt C was recorded by the authors.^{26,30,31} On the other hand, it has been suggested that the IL anion exerts a dominating effect on the catalytic activity and stability of enzymes.³² Recently, ILs based on anions derived from carboxylic acids and dicarboxylic acids with high hydrogen-bonding characteristics have been introduced in order to design protein friendly solvent systems.^{33–38} Towards this endeavor, cholinium-based ILs comprising anions derived from monocarboxylic acids have shown to be able to increase the activity of protease, even after storage at room temperature for 13 months.³⁵ More recently, pH switchable bio-ILs based

on dicarboxylic acids have been explored as protein friendly media for simultaneous biomass saccharification and fermentation to produce biofuels.³³

Most of the existing reports either deal with IL-protein interactions, and propose the suitability of the IL-protein systems for biotechnological applications,³⁹ or demonstrated a higher catalytic activity of enzymes in IL media without any systematic mechanistic understanding.^{40,41} Moreover, studies undertaken concerning both the thermodynamic stability and catalytic activity of enzymes in presence of ILs are scarce.^{42,43} The back extraction of the structurally stable and catalytic active enzyme from the IL solution also is not yet studied. Therefore, based on this lacuna, a comprehensive study towards the finding of efficient solvents for long-term protein packaging, while evaluating the enzyme activity and stability against multiple stresses, such as high temperature, broad pH range, presence of chemical and biological denaturants, and presence of organic solvents, was carried out in this work.

The present study reports the potential of bio-ILs for long-term protein packaging, for which an improved activity and stability of Cyt C is shown. For this purpose, a series of bio-ILs comprising the cholinium ion and di/tri-carboxylate anions (Fig 1) have been investigated. [Ch][Dhp] was also included for comparison since it is the most well documented IL regarding its compatibility with Cyt C.^{26–31} Efforts were made to assess the structural stability of the enzyme by various techniques, such as UV-Vis, circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies. Furthermore, molecular docking was carried out to better understand the reasons behind the enhanced activity and stability. To further confirm the long-term activity and stability of the enzyme under ambient conditions, Cyt C was dissolved in aqueous solutions of bio-ILs and stored for several months. Finally, the recovery of Cyt from the aqueous solutions of bio-ILs C was carried out allowing the enzyme reuse, with no significant losses on activity and stability. Therefore, the present study discloses the potential of novel bio-ILs as biocompatible media for long term packaging of proteins/enzymes, thereby overcoming the common obstacles faced in biocatalysis.

Experimental section

Materials

Cytochrome C (Cyt C) from equine heart with purity >95%, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) with purity >98%, hydrogen peroxide solution (H₂O₂) 30 % (w/w) in H₂O, choline bicarbonate ~80% pure in H₂O, cholinium dihydrogencitrate with purity >98%, cholinium bitartrate with a purity of 99%, glutaric acid with a purity of 99%, adipic acid with a purity of 99%, α -chymotrypsin from bovine pancreas, guanidine hydrochloride with a purity of >99%, absolute ethanol for HPLC, 99.8%, and phosphate buffered saline tablets (PBS) were purchased from Sigma-Aldrich. Succinic acid, propanoic acid, butanoic acid, and malonic acid with purity of >98% were acquired from Fluka, while [Ch][Dhp] was purchased from IoliTec. All other chemicals used were of analytical grade. A sodium phosphate buffer solution (10 mM) of pH 7.0 was used as a reference solvent. The water employed was double distilled, passed across a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus.

Synthesis of bio-ILs

Bio-based ILs were synthesized using a standard procedure through simple acid base reaction. 17 Equimolar solutions (1:1) of aqueous cholinium bicarbonate (80 wt% in water) and corresponding carboxylic acids were slowly mixed and stirred for 12 h at 75 °C. The mixture was then kept under stirring for 12 h. The ILs were collected and dried for 24 h in a vacuum line under nitrogen atmosphere for moisture removal. The chemical structure and purity (>98%) of the ILs were confirmed by ¹H-NMR and elemental analysis. The structure of the bio-ILs employed in the present study is shown in Fig. 1.

Peroxidase activity of Cyt C in presence of bio-ILs

The peroxidase activity of the Cyt C was measured using ABTS as substrate in the presence of H₂O₂. Cyt C catalyzes the oxidation of ABTS in the presence of H₂O₂ and produces the green-colored ABTS⁺ radical. The formation of the ABTS⁺ radical was monitored by changes in the absorption spectra at 420 nm. The absorption spectra were acquired for mixtures containing 2 μM of Cyt C, 3 mM of ABTS, 1mM of H₂O₂ and different concentrations of bio-ILs (0.25, 0.50, 0.75 and 1.00 g/mL). The reaction was initiated with the addition of H₂O₂. The reaction was subjected to 1 min of incubation with continuous measurements of absorbance changes at 420 nm. The percentage of relative activity was calculated considering the 100% of enzymatic activity in presence of PBS at pH 7.2.

Peroxidase activity of Cyt C in presence of bio-ILs under multiple stresses

Peroxidase activity of Cyt C was measured using ABTS as substrate in presence of PBS (pH 7.2), 0.5 g/mL of [Ch][Dhp], and 1.0 g/mL of the remaining ILs against multiple stresses, namely temperature, pH, and presence of chemical and biological denaturants. The activity of Cyt C was determined at 20, 40, 60, 80, 100 and 120 °C, after incubation from 30 min to 4h. In order to investigate the activity of Cyt C against chemical denaturants, Cyt C was incubated in the presence of 1 mM H₂O₂ for 15 and 30 min at 25°C. The activity of Cyt C was also determined in presence of 2, 4, and 6 M of guanidium hydrochloride (GuHCl), a commonly used chemical denaturant. To study the long storage effect, Cyt C was stored at room temperature (25 ± 2) °C in PBS and in bio-ILs aqueous solutions, and its activity was determined along regular periods, up to 21 weeks. To address the effect of pH on the enzyme activity, the pH of ILs was switched from acidic to basic by the addition of the corresponding carboxylic acid and cholinium hydroxide to the [Ch][carboxylate] IL, and then activity was determined using ABTS as substrate. The remaining activity was calculated by considering the difference of the initial activity and the activity after the completion of the reaction against various stress conditions. Relative activity was calculated considering 100% of enzyme activity in PBS.

Peroxidase activity of Cyt C and protection afforded by bio-ILs against protease digestion

To appraise the effect of bio-ILs against the biological stability of Cyt C, chymotrypsin treatment was employed to hydrolyze the enzyme, thereby causing deactivation. For this purpose, Cyt C was incubated for 24 h with α-chymotrypsin (1 μM) in presence of both PBS and bio-ILs at 37 °C. The activity was measured before and after incubation using ABTS substrate. The formation of ABTS⁺ radical was monitored by changes in the absorption

spectra at 420 nm. Concentrations of 0.50 g/mL for [Ch][Dhp] and 1.0 g/mL for the remaining bio-ILs were used. The remaining activity was calculated from the difference of the activity before and after incubation of Cyt C with α -chymotrypsin.

Stability of Cyt C in presence of bio-ILs

The stability of Cyt C in presence of bio-ILs and under multiple stresses was studied by UV-vis, FTIR and CD spectroscopies. UV-Vis spectra and activity of the Cyt C in the absence and presence of various concentrations of ILs were recorded using a Shimadzu UV-1800 spectrophotometer. The spectra of Cyt C were acquired with quartz cuvettes of path length 0.1 cm. The concentration of Cyt C was 0.5 mg/mL. CD spectroscopic studies were performed using a Jasco-1500 spectrophotometer, equipped with a Peltier system for temperature control. CD calibration was performed using (1S)-(+)-10-camphorsulphonic acid (Aldrich, Milwaukee, WI), which exhibits a 34.5 M/cm molar extinction coefficient at 285 nm and 2.36 M/cm molar ellipticity (Θ) at 295 nm. The sample was pre-equilibrated at the desired temperature for 15 min and the 100 scan speed was fixed for adaptive sampling (accuracy of ± 0.01) with a response time of 1s and 1 nm bandwidth. Each sample spectrum was obtained by subtracting the appropriate blank media from the experimental spectrum and was collected by averaging three spectra. All samples were pre-equilibrated at 25 °C for 25-30 min. Far-UV and near-UV CD spectra were monitored in a cuvette with path length of 0.1 cm, with 0.5 mg/mL concentration of Cyt C. Unfortunately, due to the intrinsic absorbance of the ILs in far and near UV regions, only the region from 350 to 450 nm was investigated. However, the CD spectra in the region between 200 and 300 nm was recorded for the Cyt C extracted from aqueous solutions of bio-ILs, redissolved in PBS buffer at pH 7.2. FT-IR spectra were recorded using a Perkin Elmer Spectrum Bx spectrophotometer in the wavelength range from 1800 to 1300 cm^{-1} . For each spectrum 64 scans were made at a resolution of 2 cm^{-1} . Concentrations of Cyt C and bio-ILs were 25 mg/mL and 0.5-1.0 g/mL, respectively.

Molecular Docking

The interaction sites of CytC with the bio-ILs ions were identified using the Auto-dock vina 1.1.2 program.⁴⁴ The crystal structure of Cyt C (PDB:1hrc) was used. Auto DockTools (ADT)⁴⁵ was used to prepare the protein input files by merging non-polar hydrogen atoms, adding partial charges and atom types. Ligand (IL cation and anions) 3D atomic coordinates were computed by Gaussian 03w and ligand rigid root was generated using AutoDockTools (ADT), setting all possible rotatable bonds defined as active by torsions. The grid center at the center of mass (x-, y-, and z-axes, respectively) to cover the whole interaction surface of CytC was 40 Å $\times$ 42 Å $\times$ 58 Å. The binding model that has the lowest binding free energy was searched out from 10 different conformers for each ligand.

Recovery of Cyt C from the aqueous solution of bio-ILs

Cyt C was precipitated from the aqueous solution of bio-ILs using ice cold ethanol. The precipitated-fraction was carefully separated using centrifugation (3 min at 500 rpm) and dried under inert atmosphere. After that, Cyt C was redissolved in PBS buffer of pH 7.2, and UV-Vis, FTIR, and CD spectra were acquired as described before. Yield and concentration of Cyt C were determined by spectroscopy at 280 nm, using a calibration curve previously

established with Cyt C. The peroxidase activity of the recovered Cyt C was determined using ABTS as substrate at 420 nm.

Results and Discussion

Effect of bio-ILs and their concentration on the activity of Cyt C

The present study demonstrates the potential of cholinium-based ILs comprising anions from carboxylic acids to enhance the activity and structural stability of Cyt C under several external stresses. To know the effect of different bio-ILs having monocarboxylates, dicarboxylates and tricarboxylate as anionic constituents on the enzyme activity, the activity of Cyt C was determined using ABTS as substrate in presence of different ILs at a concentration of 0.5 g/mL. For comparison purposes, the activity was also studied in phosphate buffer solutions (PBS, 10mM) at pH 7.2, and 0.5 g/mL of [Ch][Dhp] (the best IL reported till date for Cyt C.26,29–31 Fig. 2a shows the relative activity of Cyt C in PBS and 0.5 g/mL of different bio-ILs. A ~2.5 fold increase in the enzyme activity was recorded in presence of [Ch][Dhp] as compared to its activity in PBS, which is in agreement with earlier reports.^{30,31} There is no improvement of the enzyme activity in presence of monocarboxylate-based bio-ILs, namely [Ch][But] and [Ch][Prop], compared to PBS. Nevertheless, a remarkable increased activity ranging from ~3 to 25-fold as compared to PBS, and from ~1.5 to 12-fold increased activity as compared to [Ch][Dhp], were recorded in presence of di- and tri-carboxylate-based ILs. These results indicate that the presence of more than one –COOH group is beneficial towards the improvement of the enzyme activity.

To address the effect of the alkyl chain length of the dicarboxylate-based bio-ILs, those with the formulae [Ch][HO₂C-(CH₂)_{*n*}-COO[–]], with *n* = 1 to 4, were studied. Interestingly, Cyt C activity was enhanced with the increase of the alkyl chain length, and found to be highest when *n* = 3, i.e. with [Ch][Glu]. For a further increase in the chain length, when *n* = 4 corresponding to [Ch][Adi], a deleterious effect on the activity was observed. This decline in the enzyme activity with the increase in the anion alkyl chain length of the bio-ILs, from propanoate to butanoate and from glutarate to adipate, can be due to stronger dispersive-type interactions occurring between the bio-ILs and the enzyme polypeptide backbone, and that may destroy the structural integrity of the enzyme. Overall, [Ch][Dhp], [Ch][Dhc], [Ch][Tar], [Ch][Mal], [Ch][Suc] and [Ch][Glu] were identified as the most promising media and thus chosen for further studies.

From the previous study, it is clear that the activity of Cyt C strongly depends on the nature of the IL anion; however, it is also pertinent to know the effect of the IL concentration on the activity of Cyt C. Fig. 2b exhibits the relative activity of Cyt C at different concentrations of bio-ILs. In general, the activity of Cyt c decreases in the order: [Ch][Glu] > [Ch][Suc] > [Ch][Dhc] > [Ch][Mal] > [Ch][Tar] > [Ch][Dhp]. The activity of Cyt C increased till 0.5 g/mL of [Ch][Dhp], and started to decrease with a further increase in the concentration. On the other hand, the activity of Cyt C drastically increased with the increase in the concentration of other dicarboxylate-based bio-ILs, up to 1 g/mL, with [Ch][Glu] identified as the best bio-IL (> 50-fold activity increase than that observed in PBS).

Effect of bio-ILs and their concentration on the stability of Cyt C

The three-dimensional structure and Met80–Fe bond is essential for the Cyt C activity. The upper portion of the heme pocket, including the Met-80 ligand, is the most labile ligand, which can easily dissociate from the heme iron. Any structural alterations, namely unfolding or denaturation of proteins, are mainly responsible for the loss of activity. On the contrary, catalytic activity of Cyt C is reported to be enhanced significantly by the partial unfolding of the protein tertiary structure, as monitored by Trp59 and Fe-S(Met80).^{46,47} As discussed above, the catalytic activity of Cyt C improves substantially in presence of different bio-ILs, but whether such enhancement cause any irreversible structural changes in the protein structure is still unknown. Towards this endeavor, the stability of Cyt C in presence of different bio-ILs at different concentrations was evaluated by UV-Vis, CD and FTIR spectroscopies (Figs 3 and 4, and ESI, Figs S1-S4).

UV-vis absorption allows to infer the conformational changes of proteins in solvent media. Due to the presence of the heme prosthetic group, Cyt C shows some characteristic absorption bands. As showed in Figs. 3a and 3b, the oxidized (Fe(III)) form of Cyt C in PBS (pH 7.2) shows characteristic bands at ~ 280 nm (due to the $n-\pi^*$ transition of aromatic amino acids), an intense Soret band at ~409 nm, and a Q band at 528 and 550 nm (which reflect $\pi-\pi^*$ transitions due to porphyrin chromophore). These are in good agreement with previous reports.^{48,49} From Figs. 3a and 3b, it is evident that there is no significant shift in wavelength maxima of Cyt C in presence of different bio-ILs at concentrations of 1 g/mL, which indicates that aqueous solutions of all bio-ILs did not affect the polypeptide environment around the heme group. However, the decrease in the absorbance of the 409 nm peak indicates that there are some interactions occurring between the enzyme and bio-ILs. Similar results were observed in presence of lower concentrations of bio-ILs (Fig. S1 in the ESI). The UV-vis spectrum of Cyt C in [Ch][Glu] changed significantly below 400 nm wavelength due to the absorption of pure IL in the UV region (Fig. S2 in the ESI).

To get more insights into the possible structural changes of Cyt C, CD spectroscopic studies were carried out in presence of different bio-ILs, at different concentrations. Figs 3c & 3d exhibit the CD spectra of Cyt C in PBS and in presence of different bio-ILs at a concentration of 1g/mL, while Fig. S3 in the ESI provides the CD spectra of Cyt C in presence of different bio-ILs at variable concentrations. Due to strong absorbance of the bio-ILs below 250 nm, only the region from 250 to 450 nm was investigated, which can yet provide insights into tertiary structural changes of in the heme vicinity. The weak spectral features in the Soret region (350-450 nm) have been assigned to charge transfer transitions between the porphyrin and heme iron, thus sensitive to changes in the axial ligands.^{50,51} As can be seen from Fig. 3b, native Cyt C exhibits an intense positive band at ~406 nm and a strong negative band around ~416 nm in PBS solutions. The negative CD band around 416 nm is related to the presence of Met 80 at the sixth coordination position of Cyt C.^{31,51} After addition of all bio-ILs there is no shift in the positive and negative bands, only the intensities of positive and negative bands are different. These non-significant changes in CD spectra suggest that incubation of Cyt C in aqueous solutions of bio-ILs does not affect the heme iron microenvironment and conformation of Cyt C.⁴⁸ Possible changes in the tertiary structure of Cyt C in presence of different bio-ILs were also investigated from the near-UV

CD spectra (250–300 nm) - data provided in the ESI, Fig S4. The tertiary structure of Cyt C is predominantly preserved by dispersive-type interactions by the side chain amino acids (Tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe)), which show characteristic bands in the mid-UV CD spectra.⁵² As can be seen in Fig. S4 in the ESI, Cyt C shows bands at ~263 nm due to Tyr, at ~282 nm due to Phe, and at ~290 nm due to Trp residues in the mid-UV CD spectra in presence of PBS buffer (pH 7.2). These bands are kept intact in presence of 0.5 g/mL of [Ch][Dhp], and may be responsible for the improvement in the catalytic activity as compared to PBS. Although there was no alteration in the Soret band in presence of other bio-ILs as confirmed by the UV-vis and far UV-CD spectra, a significant shifting of the amino acids bands in mid-UV CD spectra was observed (Fig. S4 in the ESI). This may be the reason for the improved catalytic activity in presence of dicarboxylate-based bio-ILs, as changes in the side chain amino acids residues near the active center of the enzyme may enhance the enzyme activity.⁵³ This observation was further proved by molecular docking studies, as discussed below (*vide infra*).

The changes in the secondary structure of Cyt C in presence of bio-ILs was further investigated by FTIR analysis. In FTIR spectra, the amide I band ($1600\text{--}1700\text{ cm}^{-1}$) of Cyt C is primarily related to the C=O stretching vibration of peptide backbone conformation, and amide II mainly corresponds to the N-H bending vibration and the C-N stretching vibration of the peptide backbone. Therefore, any conformational changes in the secondary structure of Cyt C causes disturbance in the amide I and amide II regions in the FTIR spectra of the enzyme.⁵⁴ Fig. S5 in the ESI provides the FTIR spectra of Cyt C in PBS and in presence of bio-ILs. In PBS, amide I and II peaks are at 1652 and 1546 cm^{-1} , respectively. In aqueous solutions containing 0.5 g/mL of bio-ILs, there is no change in the amide I band, except in case of [Ch][Suc], where a shift from 1652 cm^{-1} to 1632 cm^{-1} was observed, although with negligible changes in the amide II band (Fig. S5a in the ESI). Similarities in amide I and II peaks positions of Cyt C in presence of ILs demonstrate that the secondary structure remains almost intact in all bio-ILs at a concentration 0.5 g/mL, whereas at 1 g/mL of bio-ILs there is a substantial shifting of both amide I and amide II bands, showing that the secondary structures have undergone some structural changes (Fig. S5b in the ESI). Therefore, from FTIR results it is evident that at lower concentrations (0.5 g/mL) of bio-ILs, Cyt C keeps its native structure, whereas a perturbed secondary structure starts to prevail at higher concentrations (1.0 g/mL) of ILs. Nevertheless, such perturbation in the secondary structure did not lead to the complete unfolding or irreversible damage of Cyt C since a substantially higher catalytic activity was still observed at this higher concentration of bio-ILs.

Activity of Cyt C against H_2O_2 as oxidant, GuHCl as chemical denaturant and protease digestion in bio-ILs

The usefulness of heme peroxidases in biosensors and in immunoassays reactions is limited due to their poor stability in the presence of H_2O_2 . Cyt C is degraded by H_2O_2 because it leads to the opening of the heme porphyrin ring, rapidly inactivating Cyt C.^{55, 56} The in vivo degradation of Cyt C by H_2O_2 can interfere with respiration, accelerate aging, and enhance the metabolism of carcinogens. Therefore, it is important to protect Cyt C from adverse effects of H_2O_2 . In order to investigate the stability of Cyt C against H_2O_2 in presence of bio-ILs, allowing thus to infer the potential of ILs to act as protective solvents

over chemical denaturants, Cyt C was incubated in 1 mM H₂O₂ for 15 and 30 min at 25 °C, both in PBS and in presence of various bio-ILs aqueous solutions. Fig. 4a depicts the remaining relative activity of the enzyme in these aqueous solutions after being incubated with H₂O₂. The peroxidase activity of Cyt C in PBS retained only 70% and 48% of the initial activity after 15 min and 30 min of incubation, respectively. Similar results were obtained in presence of [Ch][Dhp]. Remarkably, the remaining activity of Cyt C in most bio-IL aqueous solutions was higher than that observed in PBS, especially in the presence of [Ch][Dhc] and [Ch][Glu], indicating the potential of these bio-ILs to protect the protein from H₂O₂ deactivation.

Besides H₂O₂, enzyme deactivation is also affected by chemical denaturants such as guanidinium chloride (GuHCl), a well-known denaturant for most of the proteins in vitro, as well as in vivo studies.^{57,58} Its deleterious action on Cyt C is quite prominent which results on a decrease in Cyt C activity and structure stability.⁵⁹ In this regard, ILs have been reported to allow its refolding by displaying a counteracting effect against the deleterious action of chaotropic agents, such as urea and GuHCl.^{60,61} Fig. 4b represents the changes in activity of Cyt C at different concentrations of GuHCl in aqueous solutions containing 0.5 g/mL of ILs. In presence of 2 M GuHCl, Cyt C still presents a higher activity, and only for higher concentrations the activity decreases. In presence of aqueous solutions of [Ch][Dhp], [Ch][Dhc], [Ch][Tar], and [Ch][Mal], Cyt C shows a marginal loss in activity, up to 6 M of GuHCl. Therefore, these bio-ILs seem suitable to protect Cyt C against the GuHCl-induced denaturation. However, bio-ILs such as [Ch][Suc] and [Ch][Glu] are not able to counteract the denatured action of GuHCl, since Cyt C was precipitated as soon as the GuHCl was added. This counteracting effect was further ascertained by CD spectra analysis. Fig. S5 in the ESI shows that Cyt C exhibits a strong positive band at ~401 nm and a strong negative band at ~416 nm in PBS. When buffer-dissolved Cyt C was treated with GuHCl the negative band consequently vanishes. These results confirm the disruption of coordination between Met80 and heme iron.⁶² Upon addition of GuHCl, only a broader positive band centered at 406 nm was observed (Fig. S6 in the ESI), indicating the conformation changes around the heme crevice. In presence of ILs, ellipticities for negative bands decrease, but are not completely destroyed as in case of PBS. This is an indication of the counteracting ability of bio-ILs, with the only exceptions of [Ch][Glu] and [Ch][Suc], against the adverse effect of GuHCl on the activity and native structure of Cyt C.

To further validate the potential of bio-ILs employed against the degradation of Cyt C by biological denaturants, α-chymotrypsin was used. As shown in Fig. 7b, in presence of [Ch][Tar], 60% of the initial activity was retained after 24 h digestion with α-chymotrypsin, whereas only 5% of activity remained in presence of PBS and [Ch][Mal]. These results indicate that [Ch][Tar] has potential utility for protecting enzyme against biological degradation.

Effect of temperature and pH on the stability and activity of Cyt C in bio-ILs

Protein stability and activity vary significantly with temperature. Therefore, we also investigated the thermal stability and activity of Cyt C in presence of bio-ILs. The activity of Cyt C was determined in presence and absence of bio-ILs at 0.5 g/mL, and at various

temperatures ranging from 20 to 120 °C (Fig. 5a). The enzyme activity increases up to 80 °C, and then decays. In presence of PBS, Cyt C is almost completely inactivated at 80 °C, while peroxidase activity was retained in presence of all bio-ILs when exposed at 100 °C for 30 min. There was a dramatic decrease in peroxidase activity at 120 °C, even in presence of the ILs. The only exception was verified with [Ch][Glu] due to a thermal-induced conformational change in the enzyme.

Fig. 5(b) shows the CD spectra of Cyt C at different temperatures. From the CD results it is clear that soret bands completely disappear at 120°C in presence of PBS, while in presence of [Ch][Glu], Cyt C exhibits both positive and negative bands. These results suggest that incubation of Cyt C in aqueous solutions of [Ch][Glu] is able to protect Cyt C against thermal denaturation. Increased thermal stability of Cyt C up to 80 °C was observed in [Ch][Dhp] by Ohno and co-workers 26,30 whereas, in our case, and up to 120 °C, Cyt C shows a ~7-fold higher activity than in presence of PBS at 20 °C. These results reveal that both the biocatalytic activity and stability of Cyt C is enhanced in [Ch][Glu], demonstrating the suitability of this bio-IL as a high temperature bio-catalytic medium.

Besides temperature, pH induced denaturation of enzymes may also be a major problem in biocatalysis when envisaging a broad range utility of IL-protein systems. Therefore, an attempt was made to investigate the effect of pH on the catalytic activity of Cyt C in presence of different bio-ILs. It can be noticed that all bio-ILs employed in this work have more than one exchangeable acidic proton and thus the pH can be modified by the addition of a base. Instead of adding mineral acids or bases which would add extra foreign ions to the IL solutions, [Ch][OH] and carboxylic acids were used to adjust the pH. Fig. 6 summarizes the effect of pH on the activity of Cyt C in presence of bio-ILs. The activity of Cyt C in presence of different bio-ILs has a maximum in the pH range 3.2-5.4, whereas below this pH range the activity markedly decreases. A strong decrease of the activity was recorded in the pH range 6.2-12.9, in agreement with previous works that demonstrated that the activity of Cyt C decreases substantially at alkaline pH.⁵⁶

Reasons behind the improved catalytic activity and enhanced stability of Cyt C in presence of bio-ILs

In order to evaluate the enhanced activity and stability of Cyt C afforded by bio-ILs at a molecular level, a molecular docking study was carried out. The binding sites of each bio-IL ion to CytC at its lowest binding energy structure were analyzed with 9 conformers. Cyt C is constituted by 104 amino acids in a small single domain with a heme group coordinated between HIS18 and MET80.^{44, 45} The lowest absolute value of affinity (kcal/mol) for each bio-IL ion to Cyt C, as well as the molecular interactions diagrams, are displayed in the ESI (Figs. S7 to S23). Moreover, best binding pose and docking affinities, and type of interaction and geometry distance (Å) are reported in the ESI (Table S1). Besides the energy scoring function, molecular docking analysis allow to identify hydrogen-bonding, electrostatic interactions and dispersive-type interactions between the bio-ILs ions and Cyt C. All bio-ILs share a common cation ([Ch]⁺), and the docking affinity values of bio-ILs anions to Cyt C follow the rank: [Dhc]⁻ > [Glu]⁻ > [Tar]⁻ > [Suc]⁻ > [Adi]⁻ > [Mal]⁻ > [But]⁻ > [Prop]⁻ > [Dhp]⁻ (ESI, Table S1).

According to Fig. 7a the heme group in Cyt C is adjacent to the residues HIS18 and MET80. The binding of the bio-IL anions to HIS18 appears to be the major driven force in the catalytic behaviour of Cyt C. In Fig. 7b and 7c are depicted the positions displayed by [But]⁻ and [Glu]⁻. [But]⁻ is only adjacent to the residues HIS18 of Cyt C. On the other hand, [Glu]⁻, that leads to the higher Cyt C activity (Fig. 2), is surrounded by ASN52, TRP59, TYR67 and THR78. All others anions studied present the same behaviour, binding preferentially to residues that not lead to a decrease in the Cyt C activity (Figs. S7 to S13, in the ESI). On the contrary, [Dhp]⁻ does not interact with the active site of Cyt C (Fig. 8D); rather, it interacts with the side chain amino acids of the enzyme, and thus there is no significant improvement in the enzyme catalytic activity in presence of the corresponding IL. In short, the capabilities of the dicarboxylate anions to interact with the adjacent amino acids peptide chain to the catalytically active center of Cyt C play a major role towards the substantial improvement in the catalytic activity.

Recovery of Cyt C from aqueous solutions of bio-ILs

From FTIR results (Fig. S5 in the ESI), it is clear that at high concentrations of bio-ILs (1.0 g/mL) there are strong interactions between Cyt C and bio-ILs. The question is whether such interactions may be affecting the enzyme's structure. For this purpose, Cyt C was precipitated from bio-ILs solutions by ice cold ethanol, with > 90% recovery yield of the enzyme. Cyt C was then resuspended in PBS aqueous solutions. The extracted Cyt C was characterized by UV-VIs, CD and FTIR spectroscopies. Fig. 8 shows that both the structural stability and enzymatic activity are retained after the recovery of Cyt C from the bio-ILs solutions. From FTIR spectra (Fig. 8a) it can be seen that there are no shifts in the amide I and amide II bands of Cyt C, which indicate no changes in the enzyme secondary structure. Fig. 8b shows the UV-Vis absorption spectra of Cyt C in presence and absence of different bio-ILs; the presence of the characteristic bands discussed before indicate no significant structural changes in the enzyme structure after the recovery step.

Since bio-ILs were removed in the Cyt C recovery step, they do not interfere in far UV regions, allowing thus to obtain the far-UV CD spectrum of Cyt C. Native Cyt C exhibits two negative maxima at 220 and 208 nm, shown in Fig. 8c, which are similar in all samples of the recovered enzyme. With the aim of evaluating whether enzymatic activity was affected during the recovery process, the activity of Cyt C was further studied using ABTS as substrate in novel aqueous solutions of bio-ILs. The results (Fig. 8) show that the potential of bio-ILs for the enhancement of the enzyme activity is maintained for the recovered Cyt C, further demonstrating the storage ability of bio-ILs for the enzyme.

Effect of long-term storage on the activity of Cyt C in bio-ILs

It is well known that the structural integrity of proteins is disturbed when stored at room temperature for long periods of time in aqueous buffer solutions, whereas the potential of [Ch][DhP] as potential storage media of Cyt C was demonstrated by Ohno and co-workers. 26 The authors showed that Cyt C retained more than 60% of its activity after storage for 18 months in aqueous solutions of [Ch][Dhp]; however, this IL does not exhibit significant improvements in the initial enzyme activity, as shown before. As discussed earlier, all the other bio-ILs in the present study not only lead to an improved activity of Cyt C as

compared to [Ch][Dhp], but also are able to preserve the enzyme against structural damages when stored at room temperature for 20 weeks. As can be seen from Fig. 9, Cyt C is deactivated after 1 week storage in buffer, whereas > 70% of the initial activity is kept after 21 weeks of storage at ambient conditions, showing therefore the high potential of ILs as storage media for Cyt C.

Conclusions

The novel class of bio-ILs studied here, formed by cholinium and anions derived from dicarboxylic acids, when in aqueous media, are remarkable solvents for the packaging of Cyt C, while being able to provide exceptionally high catalytic activity and enhanced stability against multiple stresses. Under the optimized conditions, an outstanding high catalytic activity (> 50 fold) of Cyt C was observed in aqueous solution of [Ch][Glu] (1g/mL), as compared to phosphate buffer solutions (pH 7.2), and ~25-fold compared to the most suited IL ([Ch][Dhp]) reported up to date. This study shows that the use of dicarboxylate-based bio-ILs can improve not only the enzymatic activity but also the stability of Cyt C, by protecting it against chemical and biological denaturants. Some bio-ILs have an incredible tendency to offset the denaturing effects of H₂O₂, GuHCl and protease on the activity and stability of Cyt C, thereby broadening the widespread utility of this enzyme in biocatalysis. The catalytic activity of the enzyme in presence of bio-ILs was retained against other external interferences, such as high temperature and pH. The enzyme activity in presence of different bio-ILs was found to be maximum in a pH range from 3 to 5.5, and in presence of [Ch][Glu] (1:1) is preserved up to 120 °C. The observed enzyme activity correlates well with the structural stability of the enzyme, as confirmed by UV–Vis, circular dichroism (CD), and Fourier transform infrared (FT-IR) spectroscopies. As confirmed by molecular docking, the capability of the dicarboxylate anions to interact with the catalytically active center of Cyt C plays a major role towards the remarkable improvement observed in the catalytic activity. The successful recovery and reuse of the enzyme from the aqueous solutions of bio-ILs was also achieved, without compromising its structural integrity and catalytic activity. Moreover, it was found that the bio-ILs studied herein allow long-term storage (~5 months) of the enzyme at room temperature, without a significant decrease of its initial activity, valuable to overcome the most commonly encountered problems in the therapeutic applications of Cyt C.

In summary, novel bio-ILs capable of enhancing both the stability and activity of Cyt C, and able to preserve the enzyme at least up to 5 months at ambient conditions, have been found. These ILs are of high interest for protein packaging aiming at overcoming some of the major limitations found in the development of robust biocatalytic processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical structure of the investigated bio-ILs.

Fig. 2.

(a) Relative activity of Cyt C in aqueous solutions containing 0.5g/mL of different bio-ILs.

(b) Relative activity of Cyt C in aqueous solutions of bio-ILs at different concentrations.

Fig. 3. (a) & (b) UV-Vis absorption spectra of Cyt C in the presence of various ILs aqueous solutions at 25 °C. (c) & (d) Near-UV CD spectra of Cyt C in the presence of various ILs aqueous solutions at 25 °C.

Fig. 4.

(a) Activity of Cyt C in PBS and aqueous bio-ILs solutions (0.5 g/mL of [Ch][Dhp] and 1 g/mL for other bio-ILs), after incubation for 15 min and 30 min with 1 mM H₂O₂ at room temperature. (b) Activity of Cyt C in buffer and bio-ILs aqueous solutions at different concentration of GuHCl. (c) Remaining activity of Cyt C after incubation in CT in presence of bio-ILs.

Fig. 5. Effect of temperature on the conformation of Cyt C: (a) relative activity of Cyt C as a function of temperature; (b) CD spectra of Cyt C at different temperatures.

$[\text{Ch}][\text{Dhp}]$ pH=1.8; RA = 80%	$\xleftarrow{1 \text{ eq. H}_3\text{PO}_4}$	$[\text{Ch}]_2[\text{Dhp}]$ pH=3.2; RA = 210%	$\xrightarrow{1 \text{ eq. [Ch][OH]}}$	$[\text{Ch}]_3[\text{Dhp}]$ pH=12.9; RA = 5%
$[\text{Ch}][\text{Dhc}]$ pH=2.7; RA = 355%	$\xleftarrow{1 \text{ eq. Citric acid}}$	$[\text{Ch}]_2[\text{Dhc}]$ pH=5.4; RA = 480%	$\xrightarrow{1 \text{ eq. [Ch][OH]}}$	$[\text{Ch}]_3[\text{Dhc}]$ pH=6.9; RA = 20%
$[\text{Ch}][\text{Suc}]$ pH=2.6 RA = 200%	$\xleftarrow{\text{Succinic acid}}$	$[\text{Ch}][\text{Suc}]$ pH=4.9; RA = 870%	$\xrightarrow{1 \text{ eq. [Ch][OH]}}$	$[\text{Ch}]_2[\text{Suc}]$ pH=7.6; RA = 7%
$[\text{Ch}][\text{Mal}]$ pH=1.1; RA = 222%	$\xleftarrow{\text{Malonic acid}}$	$[\text{Ch}][\text{Mal}]$ pH=4.6; RA = 500%	$\xrightarrow{1 \text{ eq. [Ch][OH]}}$	$[\text{Ch}]_2[\text{Mal}]$ pH=7.2; RA = 4%
$[\text{Ch}][\text{Glu}]$ pH=2.0; RA = 247%	$\xleftarrow{\text{Glutaric acid}}$	$[\text{Ch}][\text{Glu}]$ pH=5.2; RA = 2375 %	$\xrightarrow{1 \text{ eq. [Ch][OH]}}$	$[\text{Ch}]_2[\text{Glu}]$ pH=6.2; RA = 2%
$[\text{Ch}][\text{Tar}]$ pH=1.5; RA = 169%	$\xleftarrow{\text{Tartric acid}}$	$[\text{Ch}][\text{Tar}]$ pH=3.8; RA = 242%	$\xrightarrow{1 \text{ eq. [Ch][OH]}}$	$[\text{Ch}]_2[\text{Tar}]$ pH=6.2; RA = 10%

RA= Relative activity

Fig. 6.

Effect of different bio-ILs at various pH values in the activity of Cyt C.

Fig. 7. Cyt C docking pose with the lowest absolute value of affinity: (A) Cyt C heme group, (B) with [But]⁻, (C) with [Glu]⁻, and (D) with [Dhp]⁻.

Fig. 8. Structural stability and enzymatic activity of Cyt C after its recovery from aqueous solutions containing 0.75 g/mL of [Ch][Dhp] and 1.0 g/mL of the remaining bio-ILs ILs: (a) FTIR (b) UV-Vis and (c) CD spectra; and (d) Relative activity.

Fig. 9.
Activity of Cyt C in buffer and bio-ILs aqueous solutions after incubation for 2, 3 and 21 weeks at room temperature.