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Insights into the impact of deep eutectic solvents on horseradish peroxidase: Activity, stability and structure

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ABSTRACT

As a new type of ionic fluids that are inexpensive and environmentally friendly, deep eutectic solvents (DESs) have recently attracted a broad interest in many fields including biocatalysis. In this study, 24 DESs were prepared by mixing two cholinium salts (cholinium chloride (ChCl) and cholinium acetate (ChAc)) with four H-bond donors (HBDs) (urea, glycerol, acetamide, ethylene glycol) at three molar ratios, and their effects on the activity, stability and structure of horseradish peroxidase (HRP) have been investigated. The ChCl-based DESs have been found to be superior to the ChAc-based ones in terms of promoting the HRP activity. For the DESs composed of the same salt and same HBD, an increase in the HRP activity has been observed with a higher salt/HBD molar ratio of 1:2 < 1:1 < 2:1. Structural studies with fluorescence and circular dichroism spectroscopy have agreed well with the activity data, suggesting that DESs capable of providing the enzyme with a higher α-helix content and a slightly more relaxed tertiary structure may facilitate the HRP activity. All the 24 DESs were able to highly stabilize the enzyme. Addition of DESs may help to improve the HRP-mediated phenolic wastewater treatment. Our experiments have also supported the idea that the extensive H-bonding network throughout the DES is sufficiently strong to prevent the DES from dissociation in aqueous solution.

1. Introduction

It has always been an important issue to search for green and sustainable solvents as a reaction medium for chemical and biochemical transformations. Being considered a promising alternative to conventional organic solvents and the more advanced ionic liquids (ILs), deep eutectic solvents (DESs) have currently attracted widespread academic and industrial interests [1–3]. A DES can be easily prepared by thermal mixing an ammonium salt (such as choline chloride) with a hydrogen-bond donor (HBD, such as urea and glycerol) at a specified stoichiometric ratio or by freeze drying this mixture; the melting point of the resulting DES is lower than those of its individual components. The greenness and sustainability of this new solvent type lies in the fact that both components of the DES are inexpensive, biodegradable, non-toxic, and widely available in nature. They can be mixed easily to form the solvent with high purity without the requirement of costly labor and equipment. The formation of DESs is believed to arise from the interaction between the HBD and the salt anion through hydrogen-bonding, thus forming an extensive H-bond network throughout the solvent. Because of all these unique properties and advantages, DESs have become promising and attractive as a new type of nonaqueous solvent/co-solvent for a lot of applications including biocatalysis [2–5]. In a very recent review, Zhang et al. [3] have discussed the syntheses and properties of DESs, as well as a broad range of applications of DESs in catalysis, organic synthesis, dissolution and extraction processes, electrochemistry and material chemistry.

So far, there are only a few publications [6–12] that have reported the use of DESs, alone or as co-solvent, as media for enzymatic reactions. Although still in its infancy, the use of DESs as a solvent/co-solvent for biocatalytic applications has shown encouraging results, which have well displayed their attractive advantages over the conventional ILs, such as low cost, easy preparation with high purity, low toxicity, and high biodegradability. More importantly, DESs possess the 'designer solvent' property similar to that of conventional ILs, but with a much broader selection for design purpose: The solvent properties can be fine-tuned by selecting different DES components (i.e., cations, anions, and HBDs) with different stoichiometric proportions. In order to take advantage of this unique property to design greener solvents for biocatalytic processes, it is beneficial to have a thorough understanding as to how DESs and their compositions affect the activity, stability and structure of an enzyme. A systematic research in this matter is therefore expected to provide us with some much-needed insights into the structure–function relationship.

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In our recent study involving 24 DESs composed of two cholinium salts (choline chloride (ChCl) and choline acetate (ChAc)) combined with four hydrogen-bond donors (i.e., urea (U), acetamide (A), acetamide (A), and ethylene glycol (EG)) at three molar ratios (1:2, 1:1, 2:1) [13], we have demonstrated that both activity and stability of *Penicillium expansum* lipase (PEL) were affected by the choice of the DES components (salts and HBDs) and their molar ratios. Whether the DESs were used as a co-solvent in aqueous solution for a PEL-catalyzed hydrolytic reaction or used as a reaction medium for a biodiesel production (transesterification) reaction catalyzed by Novozym 435 (an immobilized lipase B from *Candida antarctica*), our finding was the same that the ChAc-based DESs were superior to the ChCl-based ones, while glycerol showed a better compatibility with lipase than the other three HBDs tested.

In this current study, we aim to further our investigation by examining the impacts of the above 24 DESs and their compositions on activity, stability and structure of another model enzyme, horseradish peroxidase (HRP, EC 1.11.1.7), in the hope of providing insights into the mechanistic basis about how DESs affect the enzyme functioning. Horseradish peroxidase (HRP) is an ideal model enzyme to perform our study with. This enzyme catalyzes the oxidation of a wide variety of organic compounds in the presence of hydrogen peroxide, and its catalytic mechanism has been well characterized [14], with a heme group locating at the active site responsible for the oxidation reaction. Its amino acid sequence [15] and crystal structure [16] have been determined, and it is commercially available in pure protein form.

2. Materials and methods

2.1. Materials

Horseradish peroxidase (HRP) was purchased from Shanghai Xueman Biotechnology Co. Ltd. with a specific activity of ~250 U/mg and an RZ value of ~3.0. Choline acetate (ChAc, 99%) was purchased from ShangHai Cheng Jie Chemical Co. Ltd. 4-Aminoantipyrin (4-AAP) was purchased from Sigma–Aldrich China Inc. Choline chloride (ChCl), urea (U), glycerol (G), acetamide (A), ethylene glycol (EG), and all other reagents used were of analytical grade from Shenzhen Xinlixiang Technology Co. Ltd. The denatured HRP was prepared by heating at 100 °C for ~2 h till no activity could be detected.

2.2. Preparation of DES and DES-containing aqueous solution

An ammonium salt (ChCl or ChAc) and a hydrogen-bond donor (U, G, A or EG) were added at a molar ratio of 1:2, 1:1 or 2:1 in a beaker, mixed with a magnet stirrer at 80 °C for 1–2 h until a colorless clear liquid was formed. The resulting eutectic mixture was then dried over P_2O_5 in a desiccator at room temperature for at least 2 weeks prior to use. The DES-containing aqueous solution was prepared by dissolving a certain DES into a phosphate buffer (0.1 M, pH 7.0) at an appropriate concentration (normally up to 2.0 M), with the final pH re-adjusted to 7.0 prior to use. The DES concentration here was based on the molar concentration of the ammonium salt.

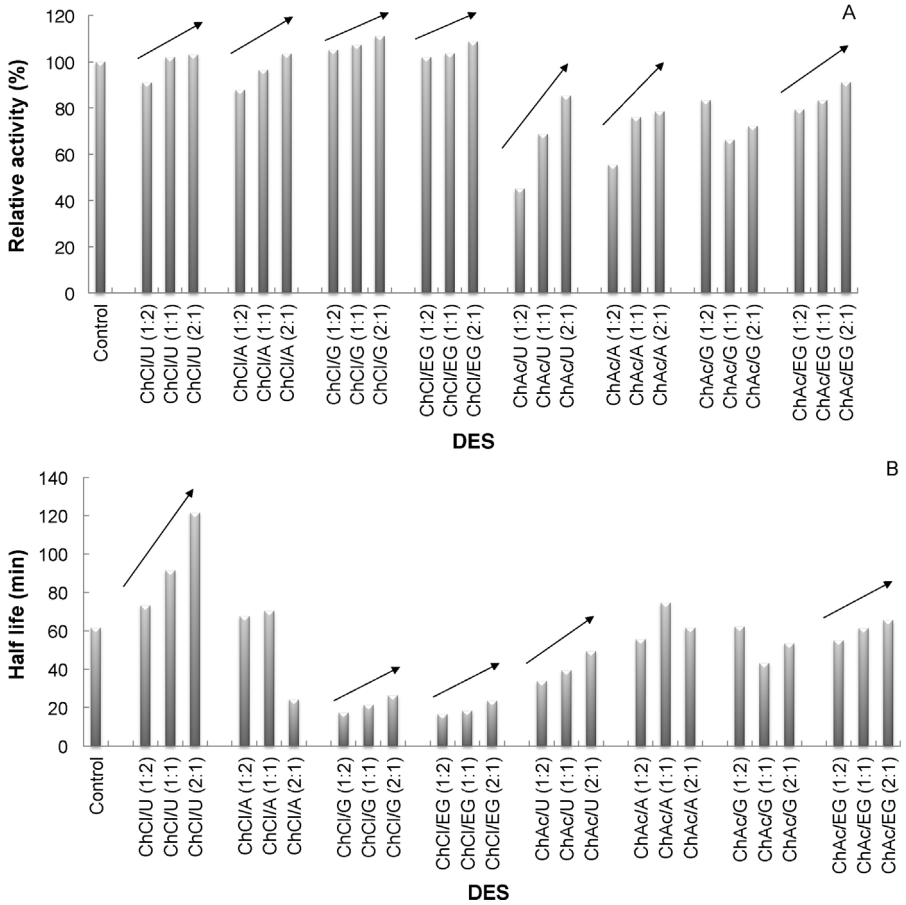

Fig. 1. HRP activity (A) and stability (B) in phosphate buffer (0.1 M, pH 7.0) containing different DESs (0.5 M). The relative activities (%) in (A) refer to the percentages of the initial reaction rates obtained by the enzyme in the DES-containing aqueous solution relative to the one obtained in the DES-free system. The half-lives (min) presented in (B) were obtained at 60 °C.