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Short communication

Insight into enzymatic synthesis of phosphatidylserine in deep eutectic solvents

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ABSTRACT

Deep eutectic solvents (DESs) have recently attracted a significant interest in numerous fields including biocatalysis because they are inexpensive, environmentally friendly, non-toxic, biodegradable, and enzyme compatible. Herein, these solvents were successfully used as the reaction media for enzyme-mediated transphosphatidylation of phosphatidylcholine with L-serine for the synthesis of phosphatidylserine for the first time. Enzymatic phosphatidylserine synthesis in various DESs was comprehensively investigated, employing phospholipase D. Our results indicated that >90% yield of phosphatidylserine could be achieved using choline chloride/ethylene glycol as DES. Furthermore, 81% original activity of the enzyme was maintained after being used for 10 batches. This study indicates that DESs act as potential candidates for the eco-friendly solvents in biocatalysis applications.

1. Introduction

Phosphatidylserine (PS), a type of phospholipids, can rejuvenate brain cell membranes and increase acetylcholine in the brain, the neurotransmitter that is important for memory and regaining lost information in the aging brain. Thus, it has numerous potential applications in functional food and pharmaceutical industries. It has been proved that PS supplemented in diet is beneficial for preventing Alzheimer's dementia, improving memory, increasing vigilance and attention, relieving depression, and decreasing stress. [1–3] It is a safe and potentially effective therapeutic agent.

However, in natural form PS is present in minor quantity; therefore, its goal-directed synthesis becomes more significant. Growing attention has been paid to phospholipase D (PLD)-mediated transphosphatidylation of phosphatidylcholine (PC) with L-serine for PS preparation owing to the enzyme specific selectivity. In general, the reaction is performed in the biphasic system [4,5] or the purely aqueous system [6,7]. However, serious drawback of these traditional systems is that they contain considerable amount of water, which can result in the undesirable hydrolysis of PC and PS, as well as the accumulation of the byproduct phosphatidic acid (PA). Biosynthesis can be further improved with a rational selection of the reaction medium produced from non-toxic resources. [8] In this sense, an ideal choice would be to perform the enzymatic synthesis of PS in a non-aqueous and green system instead of water or toxic and volatile organic solvents.

Over the last several years, deep eutectic solvents (DESs) have emerged as a greener alternative to conventional ionic liquids and organic solvents, attracting significant attention in numerous fields due to their unique advantages, such as nontoxic nature, biodegradability, low cost, and easy preparation with high purity [9–11]. A DES, a eutectic mixture, is generally composed of two or three inexpensive and safe components that are capable of associating with each other through hydrogen bond interactions. In most cases, the DES is prepared by mixing a quaternary ammonium salt (for example, choline chloride) with a hydrogen-bond donor (HBD, such as amines, polyols, and carboxylic acids) which has the ability to form a hydrogen bond with the halide anion of the quaternary ammonium salt. A deep eutectic mixture is formed by the disruption of the crystalline structures of the individual components, which decreases the lattice energy and leads to the generation of liquids at a given temperature. [12,13] Importantly, the formation of DES is very simple and does not need further purification. Among the emerging DES applications, its use as a solvent for biocatalytic reaction has been envisaged, and the examples covering hydrolases [14–19], lyases [20], and oxidoreductases [21–24] have been successfully reported over the last years. However, PLD-mediated reaction in DESs has not been reported till date.

Driven by the encouraging results achieved during the biocatalytic applications in DESs [14–24], in this study we attempted for the first time to introduce DESs as the reaction media for PLD-catalyzed transphosphatidylation of PC with L-serine for the synthesis of PS. The

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main objective of this study was to demonstrate that the introduction of DES may provide a promising scenario for the facile and efficient synthesis of PS. Thus, this study provides a new perspective to the understanding of DESs as promising non-toxic and green solvents.

2. Experimental

2.1. Biological and chemical materials

PC (≥99%, from soybean), PS (≥97%, from soybean), PA (98%, from soybean), and choline chloride (ChCl, ≥98%) were purchased from Sigma-Aldrich (USA). L-serine, urea, acetamide, ethylene glycol, glycerol, 1,4-butanediol, triethylene glycol, xylitol, oxalic acid, levulinic acid, malonic acid, malic acid, and citric acid (all ≥99%) were obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). PLD (from *Streptomyces chromofuscus*, 60 U mg^{-1}, lyophilized powder) was purchased from Asahi-kasei pharma corporation (Japan). *n*-Hexane, isopropanol, and methanol with chromatographic grade purity were used as the mobile phases of HPLC.

2.2. Preparation of DESs [25]

DESs were synthesized by mixing ChCl and HBDs in a defined molar ratio (see Table 1), followed by heating at 100 °C for 2–4 h, at atmospheric pressure, and under constant stirring until a stable homogeneous liquid was obtained. All the prepared DESs were allowed to cool down to room temperature and dried in a vacuum oven at 55 °C for 24 h. All the DESs were stored in sealed laboratory vials, which were kept in a desiccator.

2.3. General procedure for enzymatic synthesis of PS

In a typical experiment, the enzyme-mediated reaction was performed in a batch reactor in a water bath under magnetic stirring at 40 °C. The compositions of the reaction mixtures were as follows: DES (3.0 mL), PC (0.05 mmol), L-serine (0.30 mmol), PLD (40 U), and water (0.5%) (based on the total weight of the reaction system). 50 μL aliquots of the samples were taken from the reaction mixture at specified time intervals, centrifuged to obtain the upper layer, and analyzed by HPLC.

2.4. Analysis of the samples

The samples were detected using the methods described in our previous study [26].

2.5. Operational stability of PLD in ChCl/ethylene glycol

The operational stability of PLD in ChCl/ethylene glycol during batch reactions was investigated under the optimized synthesis conditions. After each batch reaction, PLD was separated by filtration using Whatman #1 filter paper, washed with ChCl/ethylene glycol for three times, and then added into the fresh reaction mixtures for the next batch reaction.

3. Results and discussions

In this study, twelve DESs, including two amine-based DESs (HBD were urea and acetamide, respectively), five alcohol-based DESs (HBD were ethylene glycol, glycerol, 1,4-butanediol, triethylene glycol, and xylitol, respectively), and five acid-based DESs (HBD were oxalic acid, levulinic acid, malonic acid, malic acid, and citric acid, respectively), were successfully employed for the enzymatic synthesis of PS. As depicted in Table 1, the prepared DESs significantly influenced the enzymatic synthesis of PS. The reaction initial rate (V_0), yield of PS, and the reaction time were measured to evaluate the efficiency of the enzymatic reaction.

The values listed in Table 1 indicated that the initial reaction rates were remarkably affected by the DESs employed in this study. Of the tested DESs, the maximum V_0 (0.72 μmol PS/min) was achieved in ChCl/ethylene glycol; however, the minimum V_0 (0.08 μmol PS/min) was obtained in ChCl/citric acid system. Clearly, the values of V_0 varied with the viscosity of the DESs used, and the increase in the viscosity of the DESs led to a decrease in the V_0 value. These results were consistent with the study published by Gorke et al. [14] and Bubalo et al. [24], who reported that higher catalytic activity of the enzymes (lipase B from *Candida antarctica* and horseradish peroxidase, respectively) was observed in DES with lower viscosity. This may be attributed to the fact that an increase in solvent viscosity could augment the diffusion resistance of substrates, thus enhancing the mass transfer limitations and impairing the interactions between enzyme particles and substrates.

Most of the DESs exhibit relatively high viscosities except for ChCl/ethylene glycol (Table 1). The high viscosity of DES is often attributed to the presence of an extensive hydrogen bond network between each component. [9] Accordingly, the viscosity of ChCl-based DES used in this study is closely dependent on the chemical nature of HBD. Therefore, exploration of the effect of DESs with different HBDs on the enzyme activity is of significant interest and extensive research efforts are required to be devoted to the comprehensive investigation. Table 1 (entries 1 and 2) clearly showed that when the amine-based DESs served as the reaction media, higher V_0 value could be achieved in the DES with smaller molecule as HBD. This tendency was also applicable when the acid-based DESs were used as the reaction media (Table 1, entries 8–12); however, the exception (ChCl/glycerol)

Table 1
Enzyme-mediated PS synthesis in various DESs.

Entry	HBD	ChCl:HBD (molar ratio)	Viscosity (Pa·s) [25]	pH value[a]	V_0 (μmol PS/min)	PS yield (%)[b]	Time (h)
1	Urea	1:2	0.214	7.84	0.61	70.6	15
2	Acetamide	1:2	0.127	7.41	0.64	73.2	12
3	Ethylene glycol	1:2	0.025	6.02	0.72	90.3	7
4	Glycerol	1:2	0.177	4.53	0.63	92.1	12
5	1,4-Butanediol	1:4	0.047	5.76	0.70	83.4	7
6	Triethylene glycol	1:4	0.044	5.68	0.70	81.1	7
7	Xylitol	1:1	3.867	4.15	0.35	80.5	60
8	Oxalic acid	1:1	0.089	1.66	0.67	65.4	9
9	Levulinic acid	1:2	0.119	2.47	0.64	56.3	12
10	Malonic acid	1:1	0.616	1.94	0.51	52.9	30
11	Malic acid	1:1	11.475	1.98	0.20	49.1	90
12	Citric acid	1:1	45.008	4.27	0.08	35.6	120

[a] The pH of DES systems {3.0 mL of DES containing 0.05 mmol PC, 0.30 mmol L-serine, and 0.5% water (based on the total weight of the reaction system)}, measured by digital pH meter (Mettler Toledo, Switzerland).
[b] Maximum yield.