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Introducing Biobased Ionic Liquids as the Nonaqueous Media for Enzymatic Synthesis of Phosphatidylserine

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ABSTRACT: Biobased ionic liquids with cholinium as the cation and amino acids as the anions, which could be prepared from renewable biomaterials by simple neutralization reactions, have recently been described as promising and green solvents. Herein, they were successfully used as the reaction media for enzyme-mediated transphosphatidylation of phosphatidylcholine with L-serine for phosphatidylserine synthesis for the first time. Our results indicated that the highest phosphatidylserine yield of 86.5% was achieved. Moreover, 75% original activity of the enzyme was maintained after being used for 10 batches. The present work could be considered an alternative enzymatic strategy for preparing phosphatidylserine. Additionally, the excellent results make the biobased ionic liquids more promising candidates for use as environmentally friendly solvents in biocatalysis fields.

KEYWORDS: *biobased ionic liquids, phosphatidylserine, phospholipase D, transphosphatidylation*

INTRODUCTION

Phosphatidylserine (PS), which is a phospholipids component, has many applications in functional food and pharmaceutical industries.[1,2] Recent reports have shown that PS supplemented in the diet plays an important role in preventing Alzheimer's dementia, improving memory, increasing vigilance and attention, relieving depression, and decreasing stress.[3−6]

However, because of the minor content of PS in natural form, the synthesis becomes more significant. The usual approach for preparing PS is phospholipase D (PLD)-mediated transphosphatidylation of phosphatidylcholine (PC) with L-serine. Generally, the reaction is performed in the biphasic system (i.e., water-immiscible organic solvent phase and aqueous phase)[7,8] or the purely aqueous system.[9,10] The serious drawback of these systems is that they contain a large quantity of water, which results in the undesirable hydrolysis of PC and PS. Not only does the hydrolysis consume the substrate and product, but also it gives rise to the accumulation of considerable amounts of the undesirable byproduct, phosphatidic acid (PA). Biosynthesis can be further improved with a rational selection of the reaction medium.[11] In this sense, an ideal choice would be to perform the enzymatic synthesis of PS in a green, nontoxic, and nonaqueous system.

Recent advances in ionic liquids (ILs) fields have inspired numerous studies in exploiting ILs as reaction media for biocatalysis.[12,13] Even so, the concerns have risen over the potential toxicity as well as the low biodegradability of most of the currently employed ILs.[14] To overcome these drawbacks, some ILs have already been synthesized from renewable, nontoxic biosources (choline, amino acids, etc.).[15−19]

In the present work, biobased ILs with cholinium as the cation and amino acids as the anions ([Ch][AA]) were employed as the reaction media for PS synthesis via enzymatic transphosphatidylation of PC with L-serine. The aim of this work is to show that the introduction of biobased ILs may develop an alternatively green and efficient scenario for PS preparation. As far as we know, this is the first description of [Ch][AA] ILs as the reaction media for enzymatic synthesis of PS as well.

EXPERIMENTAL PROCEDURES

Chemical and Biological Materials. PC (≥99%, from Soybean), PS (≥97%, from Soybean), PA (98%, from Soybean), and choline hydroxide ([Ch][OH], 46 wt % in H_2O) were purchased from Sigma-Aldrich (USA). L-Amino acids were obtained from Juyuan Biotechnological Co. (Shanghai, China). PLD (from *Streptomyces chromofuscus*, 60 U mg^{-1}, lyophilized powder. One unit was defined as the amount of enzyme which catalyzed 1 μmol PC to form PS per hour at 40 °C) was purchased from Asahi-kasei pharma corporation (Japan). Triple-distilled water was used for the preparation of the aqueous solutions in the synthesis of ILs. All other chemicals and reagents were of the highest purity commercially available. All the reagents were anhydrous, and all the organic solvents were dehydrated by 4 Å molecular sieves.

[Ch][AA] ILs Synthesis Procedure..[17,19] [Ch][OH] aqueous solution was added dropwise to amino acid aqueous solution of slight excess, with cooling. The mixture was stirred at about 3 °C for 48 h in the dark. Water was then removed under vacuum at 55 °C. After this step, acetonitrile/methanol (9:1, v/v) was added to precipitate the unreacted amino acid. The mixture was stirred vigorously overnight and then filtered through Celite. The solvents were evaporated under reduced pressure. Finally, the purified ionic liquid was dried in vacuum for 48 h at 70 °C and stored under moisture-free conditions until utilization. The yields of all the desired products were more than 95%.

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: 3.0 mL of [Ch][AA] ILs, 0.05 mmol PC, 0.15 mmol L-serine, 60 U PLD, and

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Table 1. Enzyme-Mediated PS Synthesis in Various [Ch][AA] ILs

Entry	ILs	Side chain of amino acid	Viscosity (mPa·s)[19]	V_0 (μmol PS/min)	PS yield (%)[a]	Time (h)	PA content[b]
1	[Ch][Gly]		121	0.64	78.1	12	-
2	[Ch][Ala]		163	0.62	76.2	15	-
3	[Ch][Val]		372	0.58	70.9	36	-
4	[Ch][Ser]		402	0.57	73.3	36	-
5	[Ch][Thr]		454	0.56	71.6	36	-
6	[Ch][Leu]		476	0.56	60.4	36	-
7	[Ch][Ile]		480	0.56	57.5	36	-
8	[Ch][Pro]		500	0.45	50.1	42	-
9	[Ch][Phe]		520	0.50	45.2	42	-
10	[Ch][His]		980	0.49	46.7	48	-
11	[Ch][Asp]		2060	0.36	84.3	60	-
12	[Ch][Glu]		2308	0.36	86.5	60	-

[a]Maximum yield. [b]PA was not detected during the sample analysis by HPLC.

0.5% water (based on the total weight of the reaction system). Samples (50 μL) were taken from the reaction mixture at specified time intervals, centrifuged to obtain the upper layer, and analyzed by HPLC.

Analysis of the Samples.[20] The sample was analyzed by a Waters e2695 HPLC system (Waters, Milford, U.S.A.) with an evaporative light scattering detector (ELSD). External standards of PC, PS, and PA were used to prepare calibration solutions at five different concentrations. Two microliter sample and 1 mL hexane−isopropane (1/1, v/v) were precisely measured and mixed thoroughly. Ten microliters of the aforementioned mixture was injected. HPLC separation was on a Lichrospher 100 Diol column (5 μm, 250 mm × 4.6 mm, Merck). Mobile phase A was *n*-hexane/2-propanol/methanol (38:1:1, v/v). Mobile phase B was 2-propanol/methanol (2:3, v/v). Each mobile phase contained 1 mM ammonium acetate. The flow rate was 1.5 mL min^{-1}, and the gradient was as follows: 0−18.7% B from 0 to 20 min, 18.7−100% B from 20 to 20.2 min, 100% B from 20.2 to 25 min, 100−0% B from 25 to 25.1 min, and 0% B from 25.1 to 30 min. The column temperature and drift pipe temperature were controlled at 25 and 65 °C, respectively, and the nitrogen pressure was controlled at 40 psi. The retention times were 2.52, 2.95, and 23.83 min for PA, PS, and PC, respectively. PS yield was calculated from the HPLC data. The average error for this assay is less than 0.5%. All the reported data are averages of experiments performed at least in duplicate.

Operational Stability (Reusability) of PLD in [Ch][Glu]. The operational stabilities of PLD in [Ch][Glu] during batch reactions were evaluated. After each batch reaction, PLD was separated by filtration using Whatman #1 filter paper, washed with isopropyl alcohol three times, and then added into the fresh reaction mixtures for the next batch.

RESULTS AND DISCUSSION

In this work, 12 [Ch][AA] ILs, i.e., [Ch][Gly], [Ch][Ala], [Ch][Val], [Ch][Ser], [Ch][Thr], [Ch][Leu], [Ch][Ile], [Ch][Pro], [Ch][Phe], [Ch][His], [Ch][Asp], and [Ch][Glu], were successfully applied to enzymatic synthesis of PS. Table 1 shows that the prepared ILs had an obvious effect on the enzymatic reaction for PS synthesis. The reaction initial rate (V_0), PS yield, and the reaction time were measured to evaluate the enzymatic reaction.

As can be seen from Table 1, the initial reaction rates were remarkably influenced by the ILs employed. The minimum V_0 (0.36 μmol PS/min) was obtained in [Ch][Asp] and [Ch][Glu], while the maximum V_0 (0.64 μmol PS/min) was achieved when [Ch][Gly] was used as the reaction medium. It is clear that the V_0 values varied with the viscosity of the [Ch][AA] ILs used. Generally, the viscosity is one of the most important factors affecting the applications of the solvent for catalysis. It is well-known that an increase in solvent viscosity can augment the diffusion resistance of substrates, thus enhancing the mass transfer limitations and impairing the interactions between enzyme particles and substrates. Accordingly, the increase in the viscosity of the [Ch][AA] ILs resulted in the decrease of the V_0 value with the exception of [Ch][Pro].

Also, the V_0 values showed a clear dependence on the anion structures of the [Ch][AA] ILs. An increase in the number of carbon atoms in the side chain of amino acid generally resulted in a lower V_0 value (Table 1, entries 1, 2, 3, 6, and 7). Of the ILs tested, [Ch][Gly], with the anion being the simplest amino acid, displayed the highest V_0 value (0.64 μmol PS/min). In addition to the molecular size of the anion, the introduction of extra hydroxyl, phenyl ring, or carboxylic acid group

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significantly decreased the V_0 value (Table 1, entries 4, 5, 8−12). In the [Ch][AA] ILs containing hydroxyl group in the side chain of amino acid ([Ch][Ser] and [Ch][Thr]), the V_0 values were slightly lower, while the ILs with aromatic group in the anions displayed relatively lower V_0 values (i.e., 0.45, 0.50, and 0.49 μmol PS/min for [Ch][Pro], [Ch][Phe], and [Ch][His], respectively). Unfortunately, the lowest V_0 values of 0.36 μmol PS/min were recorded for [Ch][Asp] and [Ch][Glu] in which the acidic amino acids served as the anions. A reason for the substantial correlation between V_0 values and the anion structures of the [Ch][AA] ILs may be that the efficiency of the enzyme action during PS synthesis was affected by the solvent used. Particularly, the intermolecular forces of the ILs such as van der Waals, hydrogen bond, and π-stacking interactions might contribute to this effect.

Moreover, the maximal PS yields of the PLD-mediated reaction and the time they consumed in all the [Ch][AA] ILs employed are presented in Table 1 columns 6 and 7, respectively. As can be observed, although the PS yield value in [Ch][Gly] was only 78.1%, the reaction time (12 h) it needed was the shortest. By contrast, the reaction in [Ch][Glu] proceeded slowest but constantly increased up to the highest PS yield value of 86.5% after 60 h. Obviously, the lowest PS yield (45.2%) was found in [Ch][Phe].

In Table 1, we found that the PS yield has no apparent dependence on the viscosity of the [Ch][AA] ILs used. Similarly, it was shown that the PS yields showed a close correlation to the anion structures of the [Ch][AA] ILs as well. When the side chain of amino acid was alkyl group, elongation of the side chain generally led to lower PS yield (Table 1, entries 1, 2, 3, 6, and 7). The [Ch][AA] ILs containing basic amino acids as the anions displayed relatively lower PS yields, i.e., 73% and 71% for [Ch][Ser] and [Ch][Thr], respectively. The introduction of an additional carboxylic acid group to the side chain of the amino acid substantially increased the PS yields (Table 1, entries 12 and 13). Higher PS yields of 84% and 86% were exhibited in [Ch][Asp] and [Ch][Glu], respectively. To be noted was that the introduction of a phenyl ring exerted drastic effect on the PS yields (Table 1, entries 8−10). The PS yields obtained in three [Ch][AA] ILs with aromatic group were much lower. In particular, the lowest PS yield (45%) was found in [Ch][Phe]. As we all know, the maximal PS yield value was attributable to the thermodynamic equilibrium of the enzymatic transphosphatidylation of PC with L-serine for PS synthesis. Consequently, as far as the dependence of PS yield on the [Ch][AA] ILs was concerned, a speculative explanation could be that the thermodynamic equilibrium of the enzyme-mediated reaction may be affected by the ILs used. Furthermore, the [Ch][AA] ILs effects on the thermodynamic equilibrium of the reaction may be due to their specific structures, especially the anions structures.

From a practical viewpoint, the reusability of the biocatalyst is one of essential factors for the cost-efficiency. If the biocatalyst can be reused, the economical sustainability is increased. Encouraged by the excellent experiment results above, the operational stability of PLD in [Ch][Glu] was investigated to further examine the potential of the enzyme as a catalyst for PS production. After each batch reaction, the enzyme was recovered by filtration, and the next batch was carried out with fresh substrates. Figure 1 presents the plot of the activity of the enzyme as a function of the number of reaction batches. Batch 0 corresponds to the activity of the fresh enzyme employed in the first reaction trial which was taken as 100%. It was worth noting that PLD displayed excellent operational stability, and 75% of its original activity was maintained after being used for 10 batches, highlighting the presumable cost-effectiveness of the enzyme.

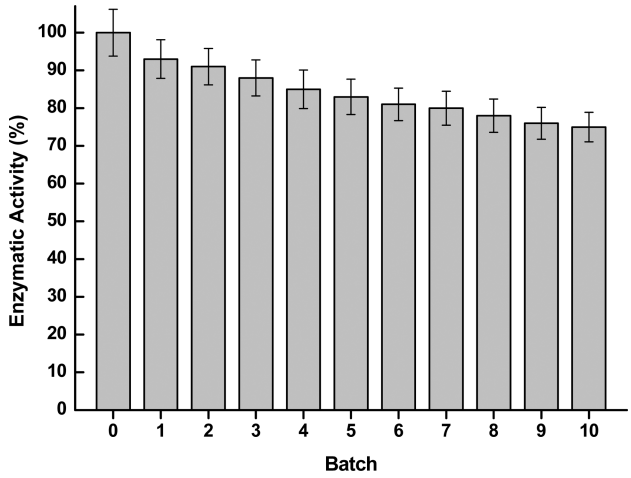

Figure 1. Operational stability of PLD in [Ch][Glu]. Reaction conditions: 3.0 mL of [Ch][Glu], 0.05 mmol PC, 0.15 mmol L-serine, 60 U PLD, 0.5% water (based on the total weight of the reaction system), 40 °C.

In general, the whole synthesis process for PS is highlighted by the application of eco-friendly solvents [Ch][AA] ILs as the reaction media. An efficient, cost-effective, and easy-to-use "green chemistry" method for the enzymatic PS preparation was provided. Additionally, the excellent results make the biobased ionic liquids promising candidates for use as environmentally friendly solvents in biocatalysis applications.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Leiros, I.; McSweeney, S.; Hough, E. The reaction mechanism of phospholipase D from *Streptomyces* sp. strain PMF snapshots along the reaction pathway reveal a pentacoordinate reaction intermediate and an unexpected final product. *J. Mol. Biol.* **2004**, *339*, 805−820.

(2) Vance, J. E.; Steenbergen, R. Metabolism and functions of phosphatidylserine. *Prog. Lipid Res.* **2005**, *44*, 207−234.

(3) Hellhammer, J.; Fries, E.; Buss, C.; Engert, V.; Tuch, A. Effects of soy lecithin phosphatidic acid and phosphatidylserine complex (PAS) on the endocrine and psychological responses to mental stress. *Stress* **2004**, *7*, 119−126.

(4) Hirayama, S.; Masuda, Y.; Rabeler, R. Effect of phosphatidylserine administration on symptoms of attention-deficit/hyperactivity disorder in children. *Agro Food Ind. Hi-Technol.* **2006**, *17*, 16−20.

(5) Hashioka, S.; Han, Y. H.; Fujii, S.; Kato, T.; Monji, A.; Utsumi, H.; Sawada, M.; Nakanishi, H.; Kanba, S. Phosphatidylserine and

DOI: 10.1021/jf505296k
J. Agric. Food Chem. 2015, 63, 1558−1561

phosphatidylcholine-containing liposomes inhibit amyloid β and interferon-γ-induced microglial activation. *Free Radical Biol. Med.* **2007**, *42*, 945−954.
(6) Vaisman, N.; Kaysar, N.; Zaruk-Adasha, Y.; Pelled, D.; Brichon, G.; Zwingelstein, G.; Bodennec, J. Correlation between changes in blood fatty acid composition and visual sustained attention performance in children with inattention: Effect of dietary n-3 fatty acids containing phospholipids. *Am. J. Clin. Nutr.* **2008**, *87*, 1170−1180.
(7) Hosokawa, M.; Shimatani, T.; Kanada, T.; Inoue, Y.; Takahashi, K. Conversion to docosahexaenoic acid-containing phosphatidylserine from squid skin lecithin by phospholipase D-mediated transphosphatidylation. *J. Agric. Food Chem.* **2000**, *48*, 4550−4554.
(8) Zhang, Y. N.; Lu, F. P.; Chen, G. Q.; Li, Y.; Wang, J. L. Expression, purification, and characterization of phosphatidylserine synthase from *Escherichia coli* K_{12} in *Bacillus subtilis*. *J. Agric. Food Chem.* **2009**, *57*, 122−126.
(9) Dittrich, N.; Ulbrich-Hofmann, R. Transphosphatidylation by immobilized phospholipase D in aqueous media. *Biotechnol. Appl. Biochem.* **2001**, *34*, 189−194.
(10) Iwasaki, Y.; Mizumoto, Y.; Okada, T.; Yamamoto, T.; Tsutsumi, K.; Yamane, T. An aqueous suspension system for phospholipase D-mediated synthesis of PS without toxic organic solvent. *J. Am. Oil Chem. Soc.* **2003**, *80*, 653−657.
(11) Klibanov, A. M. Improving enzymes by using them in organic solvents. *Nature* **2001**, *409*, 241−246.
(12) van Rantwijk, F.; Sheldon, R. A. Biocatalysis in ionic liquids. *Chem. Rev.* **2007**, *107*, 2757−2785.
(13) Tavares, A. P. M.; Rodriguez, O.; Macedo, E. A. New generations of ionic liquids applied to enzymatic biocatalysis. In *Ionic liquids—New aspects for the future*; Kadokawa, J. I., Ed.; InTech: Rijeka, Croatia, 2013; pp 537−556.
(14) Petkovic, M.; Seddon, K. R.; Rebelo, L. P.; Silva Pereira, C. Ionic liquids: A pathway to environmental acceptability. *Chem. Soc. Rev.* **2011**, *40*, 1383−1403.
(15) Fukumoto, K.; Yoshizawa, M.; Ohno, H. Room temperature ionic liquids from 20 natural amino acids. *J. Am. Chem. Soc.* **2005**, *127*, 2398−2399.
(16) Hu, S.; Jiang, T.; Zhang, Z.; Zhu, A.; Han, B.; Song, J.; Xie, Y.; Li, W. Functional ionic liquid from biorenewable materials: Synthesis and application as a catalyst in direct aldol reactions. *Tetrahedron Lett.* **2007**, *48*, 5613−5617.
(17) Moriel, P.; Garcia-Suarez, E. J.; Martinez, M.; Garcia, A. B.; Montes-Moran, M. A.; Calvino-Casilda, V.; Banares, M. A. Synthesis, characterization, and catalytic activity of ionic liquids based on biosources. *Tetrahedron Lett.* **2010**, *51*, 4877−4881.
(18) Petkovic, M.; Ferguson, J. L.; Gunaratne, H. Q.; Ferreira, R.; Leitao, M. C.; Seddon, K. R.; Rebelo, L. P.; Silva Pereira, C. Novel biocompatible cholinium-based ionic liquids—Toxicity and biodegradability. *Green Chem.* **2010**, *12*, 643−649.
(19) Liu, Q. P.; Hou, X. D.; Li, N.; Zong, M. H. Ionic liquids from renewable biomaterials: Synthesis, characterization, and application in the pretreatment of biomass. *Green Chem.* **2012**, *14*, 304−307.
(20) Duan, Z. Q.; Hu, F. Highly efficient synthesis of phosphatidylserine in the eco-friendly solvent γ-valerolactone. *Green Chem.* **2012**, *14*, 1581−1583.

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J. Agric. Food Chem. 2015, 63, 1558−1561