Lipase-catalyzed Synthesis of Feruloylated Lysophospholipid in Toluene-Ionic Liquids and Its Antioxidant Activity

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Abstract: In this study, Novozym 435-catalyzed interesterification of ethyl ferulate (EF) with phosphatidylcholine (PC) in a two-phase system consisting of an ionic liquid (IL) and toluene was optimized to prepare feruloylated lysophospholipids (FLPs). Optimum conditions for the interesterification process were found to be [Bmim][Tf2N]/toluene ratio of 1:1 (v/v), solvent volume of 4 mL, molecular sieves (4 Å) concentration of 80 mg/mL, reaction temperature of 55 °C, substrate molar ratio of 5:1 (PC/EF), Novozym 435 concentration of 50 mg/mL. Under these conditions, two FLPs products (1-FLP and 2-FLP) with total conversion rate of 50.79% were obtained. Because the formation of 1-FLP was significantly higher than 2-FLP, 1-FLP was purified and characterized by LC-MS and NMR. In addition, 1-FLP showed DPPH scavenging activity comparable with those of EF and BHT. Therefore, this study provides a good method for transformation of ferulic acid to improve its solubility and promote its application as functional ingredient in the food and pharmaceutical industries.

Key words: lipase, ferulic acid, phospholipids, ionic liquid, antioxidant activity

1 Introduction
Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA) is a phenolic compound naturally found in plants cell walls. Ferulic acid has attracted increasing interest because it has shown a wide range of biological activities such as antioxidant, anti-carcinogenic, antimicrobial, and anti-inflammatory activity1,2. However, because of the poor solubility in both lipophilic and hydrophilic systems, the application of FA in food, cosmetic, and pharmaceutical industries is limited. Therefore, scientists are searching for appropriate and innovative methods to obtain FA derivatives with high amphiphilicity to improve its solubility in oil and water to promote FA applications. Ferulic acid derivatives could be obtained by esterification and/or interesterification with some ingredients such as triglyceride, sugar and aliphatic alcohol3-6. Also, the obtained FA derivatives have shown therapeutic effects in the treatment of diabetes, lung, and cardiovascular diseases, as well as hepatic, neuro and photo protective effects7-9.

Incorporation of FA into phospholipids to obtain feruloylated phospholipids is an effective method to improve the solubility of FA. Phospholipids are widely applied in food, cosmetic, and pharmaceutical industries because of their emulsifying and physiological properties. Phospholipids showed miscibility with hydrophilic and hydrophobic metabolites at liquid-liquid and liquid-solid interfaces in many biological pathways10. Also, phospholipids can be self-assembled which could give the possibility to be easy manifested at the biological membrane. Hence, obtaining FA derivatives through fatty acid metabolism is more likely to improve FA solubility and function in the food and pharmaceutical industries.

Abbreviations: Novozym 435, Lipase B from Candida antarctica; EF, Ethyl ferulate; FA, Ferulic acid; FLP, Feruloylated lysophospholipid; PC, Phosphatidylcholine; [Emim][Tf2N], 1-ethyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide; [Bmim][Tf2N], 1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide; [Emim][PF6], 1-ethyl-3-methylimidazolium hexafluorophosphate; [Bmim][PF6], 1-butyl-3-methylimidazolium hexafluorophosphate; [Emim][BF4], 1-ethyl-3-methylimidazolium tetrafluoroborate; [Bmim][BF4], 1-butyl-3-methylimidazolium tetrafluoroborate; [Hmim][BF4], 1-ethyl-3-methylimidazolium tetrafluoroborate.

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incorporated with bioactive compounds such as FA\textsuperscript{11–13}. In our previous study, feruloylated lysophospholipids (FLPs) were synthesized from phosphatidylcholine and ethyl ferulate\textsuperscript{14}. In another study, 1-(4-hydroxy-3-methoxy)cinnamoyl-2-acyl-sn-glycerol-3-phosphocholine could be synthesized using chemoenzymatic method and showed high antioxidant activity\textsuperscript{15}. However, chemical synthesis of FLPs is very difficult because FA and phospholipids are sensitive to heat and oxidation. On the other hand, since the mild reaction conditions as well as no waste release, enzymatic synthesis approaches of FLPs are good compared to chemical synthesis\textsuperscript{16,17}.

Because of the steric hindrance of the phosphate group in the glycerol backbone of phospholipid molecular and the benzene ring in the FA molecule, there is a technical problem for incorporation of FA molecular into phospholipid molecular. Another issue is the high polarity of FA and phospholipid, which result in low solubility and mass transfer limitation of reaction system. Although solvents with higher polarity such as DMSO could increase the FA solubility and reduce the mass transfer resistance, biocatalysts in such solvents tend to be partially or completely deactivated. In addition, some organic solvents are toxic and difficult to be recovered or recycled, which limit their application in the organic synthesis reactions. Therefore, it is essential to find an appropriate and safe reaction media with good solubility for reaction substrate and avoid deactivation of biocatalyst to increase the conversions of products.

Ionic liquids (ILs) are salts consisting a mixture of cations and anions with melting points near room temperature. Ionic liquids have been used in homogeneous and heterogeneous catalysis and biocatalysis\textsuperscript{18,19}. Ionic liquids are known as “green solvents”, and their physical properties such as polarity, hydrophobicity, and hydrogen-bond basicity could be adjusted through a rational design and combination of cations and anions\textsuperscript{20}. These tunable properties are very important for biocatalytic systems. Physiochemical properties of ILs such as melting temperature, polarity, and hydrophobicity can be fine-tuned by simply changing the structure or nature of the cation or anion, which lead to new solvents\textsuperscript{21,22}. The properties and development of ILs make them valuable reaction media for production of target chemicals. Recently, using ILs as medium for the enzymatic production of FA derivatives have attracted increasing interest\textsuperscript{6,23}. However, to the best of our knowledge, there are no studies available about application of ILs as reaction media for lipase-catalyzed synthesis of feruloylated lysophospholipids (FLPs).

Therefore, the aim of the present study was to optimize biocatalytic process to prepare FLPs from ethyl ferulate (EF) and phosphatidylcholine (PC) by lipase-catalyzed synthesis in ILs as a reaction medium. Mass spectroscopy (MS) and nuclear magnetic resonance spectroscopy (NMR) were used to study the chemical structure of FLPs. Antioxidant activity of FLP product was also determined by DPPH radical scavenging assay in comparison with BHT and EF.

2 Experimental

2.1 Materials and reagents

Hen eggs were purchased from local super market, Shenyang, China. Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA, purity > 99\%) and ethyl ferulate (EF, purity >99\%) were purchased from Suzhou Chang Tong Chemical Co., Ltd. (Shanghai, China). The 4 Å 1/16 molecular sieves were purchased from UOP Co., Ltd. (Shanghai, China). Novozym 435 (lipase B from Candida antarctica, immobilized on a macroporous resin) was donated from Novozymes (Shanghai, China). [Bmim] [PF$_6$] and[Bmim] [BF$_4$] were purchased from Sigma (Shanghai, China). [Emim] [TF$_2$N], [Oimim] [PF$_6$], [Bmim] [TF$_2$N]; [Hmim] [BF$_4$]; and[Emim] [PF$_6$] were purchased from Aladdin Inc, Shanghai, China. 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) and Butylated hydroxytoluene (BHT) were purchased from Sigma (Shanghai, China). Methanol and glacial acetic acid were of chromatographic grade. All other solvents and reagents were of analytical grade.

2.2 Isolation of 1,2-diacyl Phosphatidylcholine

Egg phosphatidylcholine (PC, purity ≥ 90\%) was isolated from crude egg yolk according to a method\textsuperscript{24} with some modifications. About 300 mL egg yolk was extracted by 3-fold volume of acetone for seven times and the mixture was filtered. The solid-residue was then extracted by 3-fold volume of ethanol for six times. The obtained liquid extract was bleached by aluminium oxide for three times. The extract was filtered and the filtrate was concentrated by rotary evaporation. The purity of isolated PC was determined by HPLC (AOCS Ja 7b-91). The PC isolate was stored at −20°C for further analysis.

2.3 Lipase-catalyzed Synthesis of FLPs

The interesterification reaction was performed in a 10 mL screw capped vial. Phosphatidylcholine (PC, 0.25 mmol) was dissolved in toluene and Novozym 435 was added at concentration of 50 mg/mL. The mixture was then incubated at 55°C for 1.5 h under shaking. Ionic liquid was then added to hydrolyzed PC in toluene at initial liquid/toluene ratios of 3:1, 2:1, 1:1, 1:2, and 1:3 (v/v). EF of 0.05 mmol was added to the mixture. The reaction conditions, including solvent volume in range from 2 to 7 mL, molecular sieves (dried in muffle for 2 h at 350°C) in range from 0 to 120 mg/mL, and reaction temperature in range from 45 to 70°C were optimized. The interesterification reactions were carried under shaking at 250 rpm for 5 days. Samples were withdrawn at known time intervals for further analy-
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2.4 Purification and Quantitative Analysis by RP-HPLC

After interesterification reactions, the enzyme was filtered out. The reaction products were extracted by 3-fold volume of acetone, then extracted by 3-fold volume of hexane and concentrated under vacuum. The residue was separated and purified through flash column chromatography using benzene/chloroform/hexane (5:3:2, v/v/v) as the mobile phase. All experiments were performed in triplicate and the data were expressed as mean ± standard deviation.

The analysis and calculation of Feruloylated lysophospholipids (FLPs) were performed using RP-HPLC according to our previous study\(^1\) with some modifications. The temperature of Zorbax SB-C18 column (250 × 4.6 mm i.d., particle size 5 μm) was set at 30°C with a UV/DAD detector at 254 nm. The mobile phase was a binary solvent system of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile), at a flow rate of 1 mL/min. The gradient was operated as follows: 0 min: 90% A and 10% B; 0-10 min: changed to 30% A and 70% B; 10-20 min: maintained 30% A and 70% B; 20-20.5 min: changed to 90% A and 10% B. Samples of 100 μL each were withdrawn from the reaction mixture, blown by nitrogen, and diluted with methanol. The injection volume was 10 μL. The conversions of FLPs were calculated in terms of the molar percentage interesterification. The relative conversion values (% of FLP products) was calculated based on LC peak areas/total peak area. The total conversions were obtained as sum of 1-FLP and 2-FLP.

2.5 Identification of interesterification Products by LC-MS

The interesterification products were determined by HPLC-ESI-MS analysis. The mass spectrum was obtained by mass spectrometry (Agilent LC-QQQ6460, USA) with positive and negative electron spray ionization mode. The MS conditions were as follows: 3.5 kV capillary voltage; 30 V cone voltage; 40 L/Hr cone gas flow; 150°C source block temperature; 300°C dissolution temperature. The conditions of negative ion mode were: 2.9 kV capillary voltage; 30 V cone voltage; 40 L/Hr cone gas flow; 130°C source block temperature; 300°C dissolution temperature. The mass scan spectra were recorded in the range of m/z 100-800.

2.6 Identification of interesterification Products by NMR

The identification of 1-FLP was confirmed by NMR analysis which included one-dimensional NMR (\(^1\)H, \(^13\)C and DEPT) and decoupled NMR (HSQC and HMBC). \(^1\)H NMR and \(^13\)C NMR (Bruker DRX 4000 MHz NMR spectrometer, Germany) operated at 400 and 100 MHz, respectively. DMSO was used as solvent.

NMR Analysis. \(^1\)H and \(^13\)C NMR spectral data of 1-FLP were as follows: \(^1\)H NMR (400 MHz, DMSO): δ = 7.29 (s, 1H, Ar–H), 6.84 (d, 1H, Ar–H), 7.13 (dd, 1H, Ar–H), 7.60 (d, 1H, =CH–Ar), 6.48 (d, 1H, =CH(CH)= COOH), 4.16, 4.07 (dd, 2H, –CH(OH)=COOH), 4.12 (m, 2H, –CH2CH2–OH), 3.58 (m, 2H, CH2–N–), 3.82 (s, 3H, –OCH3), 3.14 (s, 9H, –CH3); \(^13\)C NMR (100 MHz, DMSO): δ = 111.5, 148.4, 149.8, 116.0, 123.6, 125.9 (Ar), 145.7 (=CH–Ar), 114.7 (–COOH), 167.3 (–COCH=), 65.7 (–CH=O–), 68.5 (–CH(OH)CH3), 66.3 (–CH2CH(OH)–), 58.9 (–CH2CH3–N–), 65.9 (–CH2CH3–N–), 56.1 (–OCH3), 53.7 (3CH3–).

ESI-MS (m/z): 434.0[M + H]+.

2.7 Determination of water content

Water content of ionic liquid and toluene was determined using coulometric Karl-Fisher analysis (915 KFTi-Touch, Metrohm) with Hydranal AG-H-methanol as solvent. A known amount of sample was put into the reaction bottle and titrated with Karl-Fischer reagent to end point and methanol sample was titrated as blank. The volume of Karl Fischer reagent for titration was recorded as V(mL), and the sample weight was recorded as m(g). The titrometric titration (T) of Karl-Fischer reagent was 4.0402 mg/mL. The water content (mg/g) was calculated as follows:

\[ w = \frac{\mbox{VT}}{m} \times 100 \]

The water content of Novozym 435, PC, and EF was determined by drying in an oven at 105°C until constant weight.

2.8 Antioxidant activity

Antioxidant activity of the 1-FLP was evaluated by free radical scavenging activity (DPPH) assay as described in previous studies\(^15,23\) with some modification. Solutions of DPPH and the test samples with different concentration were prepared in methanol. For the test, 2 mL methanolic solution of 1-FLP with different concentrations (5, 20, 30, 50, 75, 100, 150, 200, 400, and 600 μg/mL) and 2 mL methanolic solution of DPPH (0.2 mmol/L) were put into the sample tube. Positive controls, BHT and EF, were also run. The mixtures were vortexed and kept in the dark for 30 min, then the absorbance was measured at 517 nm (A0). The absorbance of methanolic solution of DPPH at the same wavelength was recorded as A0. The DPPH inhibition percentage was calculated as follows:

The inhibition (%) = \[ 1 - \frac{A - A_0}{A_0} \times 100 \]

2.9 Statistical analysis

All experiments performed in triplicate were statistically analyzed. The differences of mean values were determined
using analysis of variance (ANOVA) method and standard deviations were calculated to verify the results reliability. Significance was determined at a 95% level of probability.

3 Results and Discussion

3.1 FLPs identification

HPLC analysis was employed to identify the reaction substrates and the new products in terms of retention time, which produced fluorescence absorption under UV light (325 nm). Ferulic acid (FA) and ethyl ferulate (EF) were eluted with relative retention times of 7.384 and 11.252 min, respectively. The chromatogram for the substrates and interesterification products is shown in Fig. 1. There are two new peaks (#1 and #2) at retention time of 5.250 and 5.434 min were found after the interesterification reaction. These two peaks could be attributed to interesterification products identified as 1-feruloyl-lysophosphatidylcholine (1-FLP) and 2-feruloyl-lysophosphatidylcholine (2-FLP), respectively. The molecular structure of 1-FLP and 2-FLP products is shown in Scheme 1.

The purified FLPs were identified by LC-MS. Electrospray ionization-mass spectroscopy (ESI-MS) in the positive and negative ion mode. Low energy bombardment was used to characterize the molecular structure of the products (Fig. 2). LC-MS spectrum with #3 and #4 showed major ion peaks at m/z of 193.0 and 221.2 in the negative ion mode corresponding to [M-H]⁻ ions of FA and EF, respectively. However, LC-MS spectrum of #1 and #2 peaks was appeared at m/z of 434.0 in the positive ion mode. Fragmentation of peak #1 and #2 produced a fragment ion at m/z 177.0 [M + H-OH]⁺, which was identified as fragmentation of peak #3 (FA, m/z 194) and #4 (EF, m/z 222). On the other hand, new peaks of #1 and #2 could be attributed to new products resulting from the esterification reaction, identified as 1-FLP and 2-FLP.

Because 1-FLP was formed in higher amount compared with 2-FLP, 1-FLP was characterized by NMR. The purified 1-FLP was identified by 'H and 'C NMR, using DEPT, HSQC and the HMBC spectrum. The DEPT spectrum was used to determine the carbon types in the compound, whether they are primary carbon (CH₃), secondary carbon (CH₂), tertiary carbon (CH), or quaternary carbon (C). The HSQC and HMBC spectrum were used to test the one-to-one correspondence and remote (triple) correlation between C atom in 'C NMR and H atom in 'H NMR, respectively. Relevant circumstances of HMBC (correlations of H to C) are shown in Fig. 3. According to the results of LC-MS, one-dimensional NMR ('H, 'C and DEPT) and doubled NMR (HSQC and HMBC), the molecular structure of the main product was confirmed as 1-FLP.

3.2 Effect of solvent system

According to our previous study, toluene was chosen as the best solvent medium for lipase-catalyzed synthesis of FLPs. Considering the steric hindrance of benzene ring in the FA and phosphate group in the phospholipid, two-
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step routes (hydrolysis and interesterification) was adopted to facilitate the FA molecular into PC molecular. A main problem in the interesterification reaction is the high polarity of FA and PC, which lead to lower solubility in conventional organic solvents and influence the mass transfer in the reaction system. However, the suitable ionic liquids (ILs) could not only enhance the solubility of FA at higher concentration, but also create a favorable microenvironment for the enzyme to maximize the conversions of the products.

Because of the high viscosity of ILs, which was the most drawback for application in the organic synthesis, the conversions of FLPs in the single ILs were found to be lower than 5%. However, when toluene was added to [Bmim][Tf2N], FLPs conversion increased to be 28.78% compared with 18.01% in toluene only (Table 1). This could be attributed to that the addition of toluene as co-solvent with ILs reduced the viscosity and increased the solubility of the substrate. Therefore, the two-phase system, consisting of ILs and toluene, was chosen as reaction medium for the further experiments. The high viscosity results in an increase in the resistance to mass transfer and reaction. The increase of pyridinium-ionic liquids viscosity is in agreement with the increase in alkyl side chains of cationic nitrogen. The viscosity of IL is expressed by B-coefficient (Table 1). Conversions of FLPs was also determined at different ILs/toluene ratios (Table 1). All chosen ILs belong to imidazolium-type, the conversion of FLPs varied significantly with the anions and the cations.

The FLPs conversion in [Bmim][BF4] and [Bmim][PF6] is lower because of their high viscosity or B-coefficient (Table 1). The high viscosity of ILs can be attributed to the interaction of molecules through electric charges and van der Waals forces. The viscosity of ILs was in the order [Bmim][Tf2N] > [Emim][Tf2N] > [Omim][PF6] > [Hmim][BF4]. Highest conversion rate of 28.78% was obtained with [Bmim][Tf2N] after 5 days. In the practical applications, although IL containing [Tf2N]− with stronger van der Waals forces than ([BF4]− and [PF6]−), but it showed lower viscosity. This may be attributed to the weak hydrogen bonds force of [Tf2N]−, which makes the viscosity more decrease compared with the increase caused by van der Waals forces.

Other physicochemical properties of ILs such as polarity, hydrophobicity, nucleophilicity, H-bond basicity, and ksmotropicity/chaotropicity also influence lipase activity and conversions of FLP products. In this study, log P, log S and the solvatochromic polarity scales (such as E'T and Kamlet-Taft scales) were used to quantify the polarity of ILs. The E'T Scale or Solvatochromic polarity Scale is a normalized polarity scale, which sets tetramethylsilane as 0.0 and water as 1.0 (27). The E'T, log P, and log S values of the used ILs are summarized in Table 1. From Table 1, it can be seen that the correlation between IL polarity and conversions was not clearly established for Novozym 435-catalyzed synthesis of FLPs. This result is consistent with previous studies (31, 32). Based on these results, [Bmim][Tf2N] was chosen as the optimal reaction media for Novozym.
435-catalyzed synthesis of FLPs.

3.3 Effect of molecular sieves concentration

In enzymatic reactions, minimal amount of water is necessary for the enzyme to form and maintain active conformation or the "loosening up" of the rigid structure. On the other hand, an excess of water would inhibit the interesterification reaction and promote the hydrolysis of acylated products. It was found that minimal amount of water was necessary for maintaining enzyme active in imidazolium based ILs. Moreover, the relation between water quantity and the reaction rate in ILs showed a bell shape, which was similar to behavior in common organic solvents. It was also found that the water absorbed by [Bmim][Tf$_2$N] was equivalent to that of organic solvents.

The water content in the interesterification reaction was studied and the results are shown in Fig. 4. It should be noted that water is not a product of the interesterification reaction. The Novozym 435 as catalyst was found to contain about 1.00% (w/w) water and the reaction substrates (PC and EF) provide about 2 mg of water (PC 1%–1.2%, EF 3%, w/w). However, water content was found to be 0.03 ± 0.04% (w/w) for toluene and 0.85 ± 0.14% (w/w) for [Bmim][Tf$_2$N]. The water content was needed for hydrolysis of phosphatidylcholine in the first step. However, excessive water is not preferred in the interesterification reaction in the second step. Therefore, the molecular sieves were used to control the water content during the interesterification. From Fig. 4, it can be seen that the water content decreased as 4 Å molecular sieves concentration increased. The decrease in water content increased the FLPs conversion. These results are in good agreement with findings of previous studies. However, the water content does not reflect the actual amount of water available for enzyme molecules. This because some water molecules may be attracted by solvent molecules (such as through hydrogen bonds with ILs) and molecular sieves. Novozym 435-catalyzed resulted in highest conversion rate of FLPs in [Bmim][Tf$_2$N]/toluene at molecular sieves concentration of 80 mg/mL (Fig. 4). However, conversion of FLPs decreased with the increase in molecular sieves concentration higher than 80 mg/mL. These results are in good agreement with previous studies. Therefore, 80 mg/mL was chosen as optimal molecular sieves concentration for further experiment.
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3.4 Effect of IL/toluene volume ratio

As mentioned above, [Bmim][Tf2N]/toluene was chosen as optimal reaction medium for lipase-catalyzed synthesis of FLPs. Addition of organic co-solvent to ILs system could increase the solubility of the substrate and reduce viscosity of ILs. This may be attributed to that when the organic solvent is added into ILs, the hydrogen bonds or the ion-dipole interaction between organic solvent and cation/anions of ILs would be formed, then the ionic hydrogen bonds was weaken and the ionic mobility increased, which lead to a reduction in viscosity of ILs.

The influence of varying [Bmim][Tf2N]/toluene volume ratios on the lipase-catalyzed synthesis of FLPs was investigated and the results are shown in Fig. 5. Maximum conversion rate of 28.78% was obtained at [Bmim][Tf2N]/toluene volume ratio of 1:1. However, total conversion of FLPs decreased as IL/toluene volume ratio increased from 1:1 to 3:1. In other words, solvent with higher amount of IL content leads to a decrease in conversions of FLPs, which can be attributed to the high viscosity of IL. Also, the decrease of IL content leads to a decrease in conversions of FLPs, which may be attributed to compromise of the favorable microenvironment in ILs for the enzyme compared with organic solvent. These results are in agreement with a previous study on lipase-catalyzed esterification of ferulic acid with lauril alcohol in IL-hexane mixture.

3.5 Effect of temperature

Due to the high melting point and polarity of the reaction substrates (PC and EF), the solubility of the substrates and the mass transfer are important and should be considered. Reaction temperature not only improve the rate of molecular movement and the mass transfer, but also influence the stability and activity of the lipases. Moreover, the increase of reaction temperature could reduce the viscosity of ILs and affect the thermodynamic equilibrium of lipase-catalyzed reactions. Also, choosing suitable temperature for maximum production of a targeted product is economically important. The effect of temperature in range from 45°C to 70°C on the conversion of FLPs in [Bmim][Tf2N]/toluene was studied and the results are shown in Fig. 6. The conversion of FLPs increased as the reaction temperature increased from 45°C to 55°C, which can be attributed to the reduction in viscosity of the reaction system and the increase in transfer rate of the reaction substrates. However, no further increase in conversion of FLPs was found as temperature increased from 55°C to 65°C. On the other, a decrease in conversion of FLPs was found as temperature increased from 65°C to 70°C, which can be attributed to the deactivation of Novozym 435. Therefore, the optimal reaction temperature for conversion of FLPs in the [Bmim][Tf2N]/toluene was chosen to be 55°C in further experiments.

3.6 Effect of total solvent volume

Based on the Michaelis-Menten equation, solvent volume could affect the mass transfer and the reaction rate. The effect of total [Bmim][Tf2N]/toluene volume in range from 2 to 7 mL on the conversion of FLPs was investigated and
the results are shown in Fig. 7. An increase in total conversions of FLPs products was found as solvent volume increased and maximum conversion of 50.79% was obtained at volume of 4 mL. However, the conversion rate decreased as solvent volume increased from 4 to 7 mL. This may be associated with low vapor pressure and low volatility of ILs. Therefore, solvent volume of 4 mL was chosen as optimal for further experiments.

3.7 Antioxidant activity
Antioxidant activity of 1-FLP was investigated by DPPH radical scavenging activity assay and the results are shown in Fig. 8. It was found that the DPPH scavenging activity increased significantly as the concentration of 1-FLP increased. A maximum DPPH scavenging activity of 89.26% was found at 1-FLP concentration of 150 μg/mL. Several studies have found antioxidant activity for phenolipids and could be attributed to the hydroxyl and methoxy functional groups in phenolic acid. It should also be noted that 1-FLP showed antioxidant activity close to those of EF and BHT. These results indicate that the antioxidant activity of feruloyl group in the glycerol backbone of phospholipids was maintained after interesterification. Therefore, feruloylated lysophospholipids has the potential for application in the food, cosmetic, and pharmaceutical industries.

4 Conclusion
Novozym 435-catalyzed interesterification of ethyl ferulate (EF) with phosphatidylcholine (PC) in [Bmim][Tf₂N] and toluene mixture was optimized to prepare feruloylated lysophospholipids (FLPs). Tow FLPs products (1-FLP and 2-FLP) with total conversion rate of 50.79% were obtained after optimization of reaction conditions. The optimum conditions for Novozym 435-catalyzed interesterification of EF with PC were found to be 1:1 (v/v) for [Bmim][Tf₂N]/toluene ratio, 4 mL for solvent volume, 80 mg/mL for molecular sieves (4 Å) concentration, and 55°C for temperature. 1-FLP was purified and characterized by LC-MS and NMR. Also, 1-FLP product showed DPPH scavenging activity almost equivalent to those of EF and BHT. Suitability of the prepared feruloylated lysophospholipids for applications in the food and pharmaceutical industries as well as their potential health benefits need to be investigated in future studies.

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Conflict of Interest
The authors have declared no conflict of interest.

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