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Enzymatic Synthesis of Esculin Ester in Ionic Liquids Buffered with Organic Solvents

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The enzymatic esterification of esculin catalyzed by *Candida antarctica* lipase B (Novozym 435) was carried out in ionic liquid (IL)−organic solvent mixed systems in comparison with individual systems. The reaction behaviors in IL−organic solvents were systemically evaluated using acetone as a model solvent. With organic solvents as media, the esterification rates of esculin depended mainly on its solubility in solvents; for the reactions in ILs, the reaction rates were generally low, and the anion part of the IL played a critical role in enzyme activity. Therefore, the esterification of esculin in IL−acetone mixtures made it possible to improve the solubility of esculin while the effects of ILs on lipase activity were minimized. Following the benignity of ILs to lipase activity, the anions of ILs were ranked in the order as $[Tf_2N]^-$ > $[PF_6]^-$ > $[BF_4]^-$ > $[CF_3SO_3]^-$ > $[C_4F_9SO_3]^-$ > $[TAF]^-$ > $[MDEGSO_4]^-$ > $[OctSO_4]^-$ > $[ES]^-$ = $[DMP]^-$ = $[OTs]^-$ = Cl^-. The reaction behaviors differed in different systems and largely depended on the properties of the ILs and organic solvents. In general, improvements were observed in terms of both solubility and reaction efficiency. The knowledge acquired in this work gives a better understanding of multiple interactions in IL−organic solvent systems, which provide guidance for system design and optimization.

KEYWORDS: Ionic liquids; flavonoids; esculin; enzymatic esterification; Novozym 435

INTRODUCTION

Natural flavonoids have received keen attention, and they are already used in pharmaceutical, food, and cosmetic areas due to their antioxidant activity, anticancer activity, and prevention of coronary heart disease (*1*−*3*). Unfortunately, their applications are limited by their low solubility and instability in both lipophilic and aqueous media (*4*). Many chemical and enzymatic methods have been developed to improve the properties of flavonoids. Recent research shows that the use of a biocatalyst is a favorable alternative to catalyze the acylation of flavonoids (*5*−*7*). The feasibility of enzymatic modification of commercially available flavonoid glycosides in toxic or less toxic molecular solvents has been investigated using *Candida antarctica* lipase B (CAL-B) (*6*−*10*). However, enzymatic methods applied in most organic solvents often suffer from low yields and limited productivity because of the poor solubility of flavonoid glycosides in such media. Use of polar organic solvents such as DMSO and DMF could solve the solubility problem, but they usually strip essential water from the enzyme molecules and thus deactivate the biocatalysts (*11*, *12*). Therefore, less polar organic solvents do exhibit certain advantages in terms of enzyme behavior. For example, apolar organic solvents have less influence on enzyme activity so that the reaction rates can be high (*17*).

Recently, ionic liquids (ILs) have gained attention as alternatives to organic solvents for use in synthetic biotransformations (*13*−*15*). For example, the enzymatic esterification of flavonoids (*13*), L-ascorbic acid (*16*), and geraniol (*17*) have been investigated in ionic liquids. Their potential as enzymatic reaction media arises from their specific physicochemical characteristics, such as lack of vapor pressure, properties related to hydrophobicity and polarity, good solubility for many polar and less polar organic compounds, and increased regioselectivity and enantioselectivity (*18*, *19*). Ionic liquids can improve the stability of enzymes in certain cases (*20*, *21*). However, a drawback of the reaction mediated by ILs is the lower reaction rate, most likely resulting from the lower activity coefficients of reactants and mass transfer limitations due to the higher viscosity of ILs.

With the possible use of ionic liquids on the horizon, the bottleneck of solubility could be overcome. ILs represent an alternative solution in which to dissolve polar compounds at a higher concentration, which is not always possible in conventional organic solvents. With the other concern of low reaction activity in ionic liquids, it might be possible to apply dual solvent systems where both solubility and reaction activity can be balanced. Such systems have not been well studied (*22*). Therefore, we have attempted to evaluate the possibilities of uniting the advantages of both individual systems by

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combining the better reactant solubility in ILs and the higher reaction rate in organic solvents into one system. Another aim was to achieve a better understanding of the interaction among ILs, organic solvents, reactants, products, and enzymes in order to facilitate reaction optimization and design.

MATERIALS AND METHODS

Materials. Commercially immobilized lipase from *Candida antarctica* B (Novozym 435) was obtained from Novozymes A/S (Bagsværd, Denmark). Palmitic acid (97%), esculin (97%), methanol, acetone, *t*-butanol, toluene, hexane, triethylamine, acetic acid, DMSO, and molecular sieves (3Å) were purchased from Sigma-Aldrich (Brøndby, Denmark). [MTOA][TAF] (methyltrioctylammonium trifluoroacetate) was supplied by Merck KGaA (Darmstadt, Germany). [MeBuPyO]·[$N(CN)_2$] (1-butyl-3-methylpyrroldinium dicyanamide) was from Io-Li-Tec (Denzlingen, Germany). The other 15 ILs were provided by Solvent Innovation Gmbh (Koln, Germany) including [BMIM]·[BF_4] (1-butyl-3-methylimidazolium tetrafluoroborate), [BMIM]·[PF_6] (1-butyl-3-methylimidazolium hexafluorophate), [DMIM]·[DMP] (1,3-dimethylimidazolium dimethylphosphate), [EMIM]·[ES] (1-ethyl-3-methylimidazolium ethylsulfate), [EMIM]·[MDEG·SO_4] (1-ethyl-3-methylimidazolium 2(2-methoxyethoxy)ethylsulfate), [EMIM]·[$OctSO_4$] (1-ethyl-3-methylimidazolium *n*-octylsulfate), [EMIM]·[OTos] (1-ethyl-3-methylimidazolium tosylate), [HMIM]·[Cl] (1-hexyl-3-methylimidazolium chloride), [OMIM]·[BF_4] (1-methyl-3-octylimidazolium tetrafluoroborate), [OMIM]·[PF_6] (1-methyl-3-octylimidazolium hexafluorophosphate), [BMIM]·[CF_3SO_3] (1-butyl-3-methylimidazolium trifluoromethanesulfonate), [MeBuPy]·[$N(CN)_2$] (1-butyl-3-methylpyridinium dicyanamide), [MeEtPy]·[$C_4F_9SO_3$] (1-ethyl-3-methylpyridinium perfluorobutanesulfonate), [MeOcPy]·[BF_4] (3-methyl-1-octylpyridinium tetrafluoroborate), and [TOMA]·[Tf_2N] (trioctylmethylammonium bis(trifluoromethylsulfonyl)imide). Basic information about the 17 ionic liquids is shown in **Table 1**. Any other chemicals were of analytical grade.

Enzymatic Esterification. The enzymatic esterification of flavonoids in ionic liquids, organic solvents, and their mixtures was carried out in glass tubes with a screw cap under stirring with magnetic bars (10 mL flat bottles, **Figure 1**). Esculin (15 mM) and palmitic acid (60 mM) were added into 2 mL of ionic liquid, organic solvent, or their mixtures (the volume ratios of ILs to organic solvents was 25/75, 50/50, or 75/25). The glass tubes with screw caps were then incubated for 2 h at 40 °C under stirring at 150 rpm until substrates were solubilized in the media. Novozym 435 in 7.5 mg/mL and 75 mg/mL activated 3 Å molecular sieves were added to the mixture. Control experiments without enzyme were also carried out in organic solvents. All reactions were run under the same conditions. Samples were withdrawn at the selected time intervals (0, 15, 24, 48, 72, and 96 h). The sample mixtures were diluted with 10× DMSO and centrifuged at 4000 rpm for 20 min to remove any particulate matter. The supernatant was used for HPLC analysis. Experiments were performed in duplicate.

Measurement of Esculin Solubility. During enzymatic esterification, palmitic acid was totally dissolved in organic solvents (*t*-butanol, hexane, toluene, and acetone) and 17 ionic liquids. The standard curves of esculin and different ILs were established. A concentration series from 0.05 to 10 mg/mL in DMSO was used, and the means of triplicate determinations were calculated. On the basis of the properties of the ILs, one elution system was used for HPLC analysis of esculin dissolved in ILs. Percentage area was used as the mass for the calculation of solubility. The measured values of the IL and flavonoid were calibrated using standard curves. All HPLC analyses were carried out in triplicate. The relative standard deviation was measured below 4.5%.

Analysis of Reaction Mixtures. The acylation reaction was monitored by HPLC analysis, which was carried out in a system (LaChrom, Merck) composed of a column (Ascentis RP C8 column, 25 cm × 4.6 mm, 5 μm, Supelco), a column oven (L-7300, Merck), an autoinjector (L-7200, Merck), a pump (L-7100, Merck), and an evaporative light-scattering detector (PL-ELS 2100, Polymer Laboratories). The various compounds were separated using the mobile phases with methanol and 10 mM triethylamine solution (TEA buffer) buffered to a pH of 4.0; the gradient of methanol to 10 mM TEA buffer was from 0/100% to 100%/0 over 15 min, 100%/0 over 10 min, from 100%/0 to 0/100% over 3 min, and 0/100% over 7 min. The run time was 35 min. The elution was performed at 40 °C with a flow-rate of 0.8 mL/min. The detector settings were evaporator, 90 °C; nebulizer, 50 °C; and gas, 1.2. Retention times at these conditions were around 10.05, 21.02, and 22.50 min for esculin, esculin ester, and palmitic acid, respectively. The quantity of product produced was calculated as a percentage: product peak area over the sum of esculin and esculin monoester peak area. Assuming the percentage area was equal to the percentage mass, the esculin and product area percentages were divided by their own molecular weights to convert them into molar percentages. All analyses were carried out in triplicate. The relative standard deviation was measured below 3.6%.

RESULTS AND DISCUSSION

Enzymatic Esterification in Organic Solvents . Esculin was enzymatically acylated with free saturated fatty acids in the traditional organic solvents. On the basis of previous studies in organic solvents, the following conditions were used: molar ratio of 1:4 (flavonoid/acyl donor), temperature of 40 °C, and the addition of 75 mg/mL molecular sieves to maintain the low water activity (*6*). Palmitic acid can completely dissolve in organic solvents and ionic liquids; therefore, it was added excessively in order to keep the reaction equilibrium shifted toward the formation of esculin ester. Esculin conversion and solubility in organic solvents as well as the solvent hydrophobicity (LogP value) for organic solvents were essential parameters to compare in the following experiments.

In the experiments shown in **Table 2**, acetone had the highest esculin conversion and solubility at 40 °C. DMSO was able to completely dissolve esculin, but the reaction did not occur. Esculin solubility in *t*-butanol was higher than that in toluene, but esculin conversion was slightly lower. Esculin solubility was too low in hexane, which could lead to the unnoticeable conversion. The hydrophobic and hydrophilic properties of organic solvents profoundly influence the reaction behavior in general. As esculin is a polar compound and is easy to dissolve into a hydrophilic organic solvent, the overall conversion could be complicated by both solubility and effects of solvents on enzymes. For example, DMSO could completely dissolve the compounds, but no conversion was shown. The specific interactions of DMSO with the enzyme could result in enzyme deactivation (*23*). DMF was discussed previously to cause the same effects, which inhibited enzyme activity (*24*).

The higher conversion observed in acetone could be attributable to the higher solubility of both substrates than that in both toluene and hexane. It is also possible that acetone had a profound effect on the reaction rate when placed near the boiling point, as it overcame the energy barriers, so as to make the reaction fast and efficient. Mellow et al. reported similar results in the rutin and oleic acid system with a conversion up to 70% (*12*). Therefore, acetone was selected for the evaluation IL−organic solvent mixture systems in the following investigation at 40 °C.

t-Butanol could also be a good solvent for flavonoid esterification due to the high solubility of esculin. In this

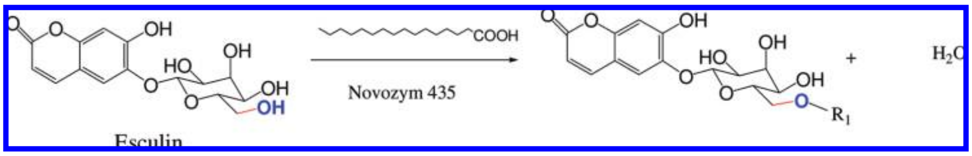
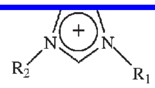
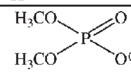
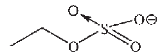
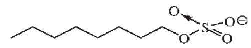
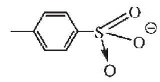
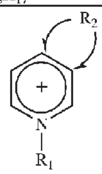
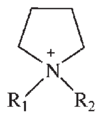


Figure 1. Scheme of the reaction between esculin and palmitic acid.

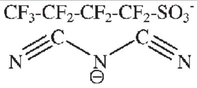
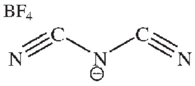
Table 1. Structure of the 17 Ionic Liquids Studied in This Article

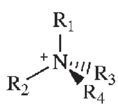
1-Alkyl-3-methylimidazolium cations 

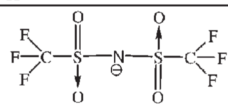
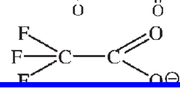
Abbreviation	R_1	R_2	X^-
[DMIM].[DMP]	CH_3	CH_3	
[EMIM].[ES]	C_2H_5	CH_3	
[EMIM].[OctSO$_4$]	C_2H_5	CH_3	
[EMIM].[OTos]	C_2H_5	CH_3	
[EMIM].[MDEGSO$_4$]	C_2H_5	CH_3	CF_3-O-CF_2-CF_2-O-CF_2-SO_3^-
[BMIM].[PF$_6$]	n-C_4H_9	CH_3	PF_6^-
[BMIM].[BF$_4$]	n-C_4H_9	CH_3	BF_4^-
[BMIM].[CF$_3$SO$_3$]	n-C_4H_9	CH_3	-CF_3-SO_3^-
[HMIM].[Cl]	n-C_6H_{13}	CH_3	Cl^-
[OMIM].[BF$_4$]	n-C_8H_{17}	CH_3	BF_4^-
[OMIM].[PF$_6$]	n-C_8H_{17}	CH_3	PF_6^-

1-Alkylpyridinium cations **1-Alkylpyrroldinium cations**

Abbreviation	R_1	R_2	X^-
[MeEtPy].[C$_4$F$_9$SO$_3$]	C_2H_5	CH_3	CF_3-CF_2-CF_2-CF_2-SO_3^-
[MeBuPy].[N(CN)$_2$]	n-C_4H_9	CH_3	
[MeOcPy].[BF$_4$]	CH_3	n-C_8H_{17}	BF_4
[MeBuPyO].[N(CN)$_2$]	n-C_4H_9	CH_3	

Alkylammonium cations 

Abbreviation	R_1	R_2,R_3,R_4	X^-
[TOMA].[Tf$_2$N]	CH_3	n-C_8H_{17}	
[TOMA].[TAF]	CH_3	n-C_8H_{17}	

experiment, the conversion of esculin with palmitic acid could only reach 26% in *t*-butanol at 40 °C (**Table 2**). If the temperature were increased, the conversion and solubility of esculin should be enhanced too. It was reported that the conversion of esculin with palmitic acid could reach 80% in *t*-butanol at 60 °C (*6*). This indicates that temperature greatly influences the reaction performance in *t*-butanol.

The stability of the enzyme is usually higher in solvents with a higher log *P* value. However, hydrophobic solvents are not always a good choice if the solubility of a hydrophilic substrate such as esculin has to be taken into account (*22*). Therefore, the selection of a solvent can be arbitrary sometimes. Safety and cost are also issues for consideration. In conclusion, we decided to take acetone for further evaluation with ionic liquids as part of the mixtures.

Enzymatic Esterification in Ionic Liquids. The esterification of esculin with fatty acids can also be performed in ionic liquids. because of the increase in esculin solubility and enzyme stability, enzymatic esterification in ionic liquids could be much more efficient than that in organic solvents. The enzymatic esterification of esculin in 17 different ionic liquids was carried out. Esculin solubility and conversion in ionic liquids is shown in **Table 3**. Most ionic liquids were much more polar than conventional organic solvents, but the

Table 2. Esculin Esterification with Palmitic Acid Catalyzed by Immobilized *Candida antarctica* Lipase B in Different Organic Solvents[a]

organic solvents	esculin conversion after 96 h of incubation at 40 °C (mol %)[b]	solubility (esculin g/100 g organic solvent)	solvent hydrophobicity[c] (log *P*)
DMSO	n/a	totally dissolved	−1.3
acetone	78.17	0.34	0.23
T-butanol	1.26	0.87	0.80
toluene	2.22	<0.005[d]	2.5
hexane	n/a	<0.005[d]	3.5

[a] Reaction conditions (esculin, PA = 15 mM; 60 mM; 7.5 mg/mL Novozym 435; 75 mg/mL molecular sieves; 150 rpm stirring speed rate; 2 mL of media). [b] All experiments were performed in duplicate. [c] From Degn et al. (*22*). [d] An accurate measurement of the solubility of the esculin in toluene and hexane was impossible because the solubility of esculin was below the limit of detection.

Table 3. Solubility and Conversion of Esculin in Different Ionic Liquids[a]

ionic liquids	solubility (g esculin /100 g IL)	esculin conversion (mol %)
[TOMA]·[Tf_2N]	0.91	13.99
[BMIM]·[PF_6]	0.18	5.46
[BMIM]·[BF_4]	1.97	3.81
[MeOcPy]·[BF_4]	1.91	4.96
[OMIM]·[BF_4]	6.89	4.31
[OMIM]·[PF_6]	>0.13[b]	1.16
[BMIM]·[CF_3SO_3]	5.35	0
[MeEtPy]·[$C_4F_9SO_3$]	2.55	0
[MeBuPy]·[$N(CN)_2$]	>100[b,c]	39.30
[MeBuPyO]·[$N(CN)_2$]	>100[b,c]	25.34
[MTOA]·[TAF]	21.03	0
[EMIM]·[$OctSO_4$]	40.06	0
[EMIM]·[MDEG·SO_4]	51.75	0
[EMIM]·[ES]	96.14	0
[EMIM]·[OTos]	>34.14[b]	0
[DMIM]·[DMP]	>69.89[b]	0
[HMIM]·Cl	>42.89[b]	0

[a] Reaction conditions: esculin, PA = 15 mM; 60 mM; 7.5 mg/mL Novozym 435; 75 mg/mL molecular sieves; 150 rpm stirring speed rate; 40 °C; 96 h; and 2 mL of media. [b] An accurate measurement of the solubility of esculin in ILs was impossible because the solubility of esculin was beyond the limit of detection. [c] The reaction in [MeBuPy]·[$N(CN)_2$] and [MeBuPyO]·[$N(CN)_2$] produced byproducts. The mechanism of byproduct production was not identified.

interactions with the enzyme were different. Enzymatic activity cannot be directly correlated to properties such as log *P*. The activity coefficient and viscosity may also influence the properties of the ionic liquids.

The experimental conditions for enzymatic esterification in ionic liquids were the same as those using organic solvents. **Table 3** shows that esculin conversion in most experiments in ionic liquids was below 15 mol %, which is too low for comparison with that in acetone. It was encouraging that the esculin solubility in all 17 ionic liquids was higher than that in the four organic solvents. **Figure 2** shows that esculin conversion and the reaction rate in [TOMA]·[Tf_2N] and [BMIM]·[BF_4] were considerably lower than that in acetone.

The observed esculin solubility values in ionic liquids (**Table 3**) confirmed the early predictions made by Guo et al. using the conductor-like screening model for real solvents (COSMO-RS) (*25*). According to the COSMO-RS prediction of esculin solubility in ionic liquids, ILs were categorized into three groups on the basis of basicity of the anion moiety. In this study, $[Tf_2N]^-$, $[PF_6]^-$, and $[BF_4]^-$ belong to group 3, which has low solubility and low H-bonding interaction energy. $[CF_3SO_3]^-$, $[C_4F_9SO_3]^-$, $[MDEGSO_4]^-$, $[OctSO_4]^-$, and $[ES]^-$ are part of group 2,

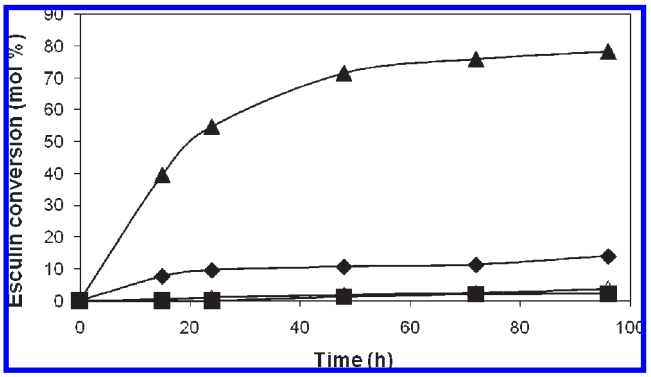


Figure 2. Esculin bioconversion in typical organic solvents and ionic liquids: (♦) TOMA·Tf_2N; (△) BMIM·BF_4 ;(▲) acetone; (■) toluene. Conditions: esculin (15 mM), palmitic acid (60 mM), molecular sieves (75 mg/mL), Novozym 435 (7.5 mg/mL), media volume 2 mL, 96 h, and 40 °C.

which has moderate solubility and H-bonding interaction energy. $[TAF]^-$, $[DMP]^-$, $[OTos]^-$, and Cl^- are part of group 1, which can dissolve esculin at very high concentrations and has strong H-bonding interaction energy. These results suggest that, in a solute with a structure (herein saccharide rings) that is a good H-bonding donor, the anion part of the IL or the H-bonding capability of the anion plays a decisive role in the determination of solute solubility.

On the basis of esculin solubility and enzyme activity in the ionic liquids (**Table 3**), these 17 ionic liquids can be divided into two groups: 8 less-polar ionic liquids (< 10 g esculin/100 g IL) and 9 polar ionic liquids (> 10 g esculin/100 g IL). Of the less-polar ionic liquids, although [TOMA]·[Tf_2N] was found to have relatively low esculin solubility, the use of this IL resulted in the highest conversion of the ester product. The solubility of [BMIM]·[PF_6] and [OMIM]·[PF_6] was similar, with the esculin conversion of the former being slightly higher than that of the latter. With the same anion part, [BMIM]·[BF_4], [OMIM]·[BF_4], and [MeOcPy]·[BF_4] showed similar esculin conversion, even though the esculin solubility in [OMIM]·[BF_4] was a little higher than that in the other two ionic liquids. The reactions in [MeEtPy]·[$C_4F_9SO_3$] and [BMIM]·[CF_3SO_3] did not have any enzyme activity. The esterification reaction was special in [MeBuPy]·[$N(CN)_2$] and [MeBuPyO]·[$N(CN)_2$] because some byproducts were produced during the reaction. One possible explanation for this might be that the anion part of these two special ionic liquids was able to directly react with substrates. Despite their high conversion results, the added complexity of side reactions and the need for purification made these ILs less interesting to be investigated. The remaining seven ionic liquids had very high solubility of esculin, but unfortunately, the esterification reaction did not occur in them.

The most promising ionic liquids were chosen according to their anion part, namely, $[PF_6]^-$, $[BF_4]^-$, and $[Tf_2N]^-$, as discussed before. Viscosity and thermal stability were the main physical properties for ionic liquids. For the three chosen anions, thermal stability has the following order $[PF_6]^- > [Tf_2N]^- \sim [BF_4]^-$, while viscosity follows $[PF_6]^- > [BF_4]^- > [Tf_2N]^-$(*26*). The solubility of ionic liquids in water was another important factor. The strength of H-bonding between anion and water is in the following order: $[PF_6]^- < [BF_4]^- < [Tf_2N]^-$(*26*). In the reaction used, water was one of the products, which is produced continuously. For this reason, hydrophilic ILs should be more active than the hydrophobic ones. $[Tf_2N]^-$ was more hydrophilic than the

Table 4. Esculin Conversion in Acetone Mixed with Different Ionic Liquids at Different Ratios[a]

	conversion (mol %)[b]				conversion (mol %)[c]		
ionic liquid	25% IL	50% IL	75% IL	ionic liquid	1% IL	5% IL	10% IL
[TOMA]·[Tf_2N]	78.02	51.31	48.57	[MTOA]·[TAF]	54.63	n/a	n/a
[BMIM]·[PF_6]	71.13	63.79	18.06	[EMIM]·[$OctSO_4$]	36.65	n/a	n/a
[BMIM]·[BF_4]	51.54	37.76	18.03	[EMIM]·[MDEG·SO_4]	38.34	n/a	n/a
[MeOcPy]·[BF_4]	53.50	45.47	29.61	[EMIM]·[ES]	n/a	n/a	n/a
[OMIM]·[BF_4]	26.22	22.23	12.77	[EMIM]·[OTos]	n/a	n/a	n/a
[OMIM]·[PF_6]	51.57	56.18	11.81	[DMIM]·[DMP]	n/a	n/a	n/a
[BMIM]·[CF_3SO_3]	10.77	5.20	n/a	[HMIM]·[Cl]	n/a	n/a	n/a
[MeEtPy]·[$C_4F_9SO_3$]	7.83	3.55	n/a				
[MeBuPy]·[$N(CN)_2$][d]	44.15	48.10	62.7				
[MeBuPyO]·[$N(CN)_2$][d]	36.65	40.4	39.10				

[a] Reaction conditions esculin PA = 15 mM; 60 mM; 7.5 mg/mL Novozym 435; 75 mg/mL molecular sieves; 150 rpm stirring speed rate; 40 °C; 96 or 72 h; and 2 mL of media. [b] Esculin conversion at 96 h. [c] Esculin conversion at 72 h. [d] The reactions in [MeBuPy]·[$N(CN)_2$] and [MeBuPyO]·[$N(CN)_2$] produced byproducts. The mechanism of production of the byproduct was not identified.

other two anions. Therefore, the reaction in an IL with the [Tf_2N]$^-$ anion had higher enzyme activity.

Enzymatic Esterification in IL−Acetone Mixtures. On the basis of the work presented above, it is apparent that both organic solvents and ILs possess advantages and disadvantages. The bottleneck in the esterification reaction in organic solvents was the low solubility of esculin. The main drawback of the esterification reaction in ionic liquids was the low enzyme activity. There exists one hypothesis: if the IL−organic solvent mixtures can be used in esterification, both esculin solubility and enzyme activity can be improved at the same time. In theory, the ideal mixture should have the advantages of both ionic liquids and organic solvents. The enzymatic esterification in IL−acetone mixtures and the effects of the different volume ratios in IL−organic solvent mixtures were therefore studied.

Less-Polar IL−Acetone Mixtures. The conversion of esculin in different mixtures is given in **Table 4**. Different ratios of ionic liquids were used in the media (25%, 50%, and 75% IL). Eight less-polar IL−acetone media were tested, and some of them showed potential enzyme activity and reactivity.

Esculin conversion in most IL−acetone mixtures significantly decreased with the increase in the IL ratios. The [TOMA]·[Tf_2N]−acetone mixture was the best because it had the highest conversion in the different volume percentages. In the [BMIM]·[BF_4]−, [OMIM]·[BF_4]−, and [MeOcPy]·[BF_4]−acetone mixtures, the reactions happened at a similar rate as they shared the same anion. Esculin conversion in these three IL−acetone mixtures was enhanced by the increase in esculin solubility. [BMIM]·[PF_6]− and [OMIM]·[PF_6]−acetone mixtures showed similar trends because they had the same anion. Both [BMIM]·[CF_3SO_3] and [MeEtPy]·[$C_4F_9SO_3$] had sulfonate as their anion. The reactions in [BMIM]·[CF_3SO_3]− and [MeEtPy]·[$C_4F_9SO_3$]−acetone mixtures had low esculin conversion in 25% IL−acetone mixtures, and no reaction was observed in 75% IL−acetone mixtures or 100% ILs. In general, IL−acetone mixtures maintained efficient and stable enzyme activity. The esterification reaction rate and esculin conversion increased as the acetone ratios increased. The feasibility of the use of IL−organic solvents was proved.

Polar IL−Acetone Mixtures. As shown also in **Table 4**, the reactions in seven polar ionic liquids did not have any enzyme activity after 96 h at 40 °C. The reactions in the other two special polar ionic liquids, [MeBuPy]·[$N(CN)_2$] and [MeBuPyO]·[$N(CN)_2$], had high enzyme activity. The nine polar ionic liquids were divided into two groups for the evaluation of IL−acetone mixtures with different volume ratios: seven polar ILs without enzyme activity (1%, 5%, and 10% IL in the mixture) and two polar ILs with enzyme activity (25%, 50%, and 75% IL in the mixture).

Table 4 shows that no reaction was observed after 72 h when using IL−acetone mixtures containing 5% and 10% polar ILs. For reactions in IL−acetone mixtures containing 1%, [MTOA]·[TAF], [EMIM]·[OctSO4] and [EMIM]·[MDEGSO4] had 30−50 mol % esculin conversion. One possible explanation is that these polar ILs in these concentrations cannot interact with the enzyme active site or deactivate the enzyme. Their corresponding anions were so polar that the enzyme was restrained when their anion parts appeared. The anion parts in ionic liquids are more crucial than the cation parts to affect the initial reaction rate as well as overall substrate conversion (*11*). The anion parts were compared between [PF6]$^-$ and Cl$^-$ for less polar and also polar ionic liquids. Taking [PF6]$^-$ as an example, the ionic liquid containing the anion was more compatible with the enzyme, likely due to the decreased interference with the internal hydrogen bonds of the enzyme. Another explanation of the effects of anions on enzyme activity for polar IL−organic solvent mixtures was that these polar ILs had physical or chemical characteristics similar to those of polar organic solvents such as DMSO and DMF, where their polar nature resulted in enzyme deactivation. The complete mechanism of such effects is still not full clear and requires further study.

Effects of the Different Volume Ratios in IL−Acetone Mixtures. The effects of the different IL ratios in IL−acetone mixtures were examined in this section. Only two ionic liquids ([BMIM]·[BF_4] and [TOMA]·[Tf_2N]) are shown in **Figure 3**, which demonstrates the different reaction behaviors of the systems. In contrast to the results obtained with mixtures containing the other four less polar ionic liquids, ([BMIM]·[PF_6], [OMIM]·[PF_6], [OMIM]·[BF_4], and [MeOcPy]·[BF_4]), the reaction behaviors of esculin esterification in the different IL−acetone mixtures were almost the same when the anion part of the ionic liquids was the same. The results are consistent with the literature, in which anion parts have stronger influence on initial reaction rates and substrate conversion than the cation parts (*11*).

The same trend of the reaction in [BMIM]·[PF_6]− and [OMIM]·[PF_6]−acetone mixtures was found in [BMIM]·[BF_4]−, [OMIM]·[BF_4]− and [MeOcPy]·[BF_4]−acetone mixtures as they shared the same anion. Esculin conversion

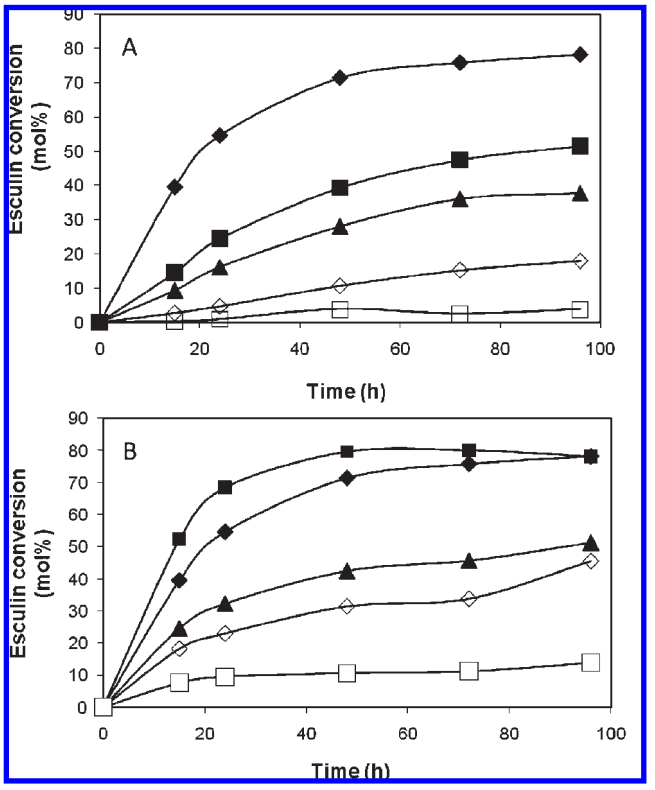


Figure 3. Esculin conversion in mixtures of ionic liquids and acetone: volume ratio of acetone to ionic liquids. (♦) 0% IL, (■) 25% IL, (▲) 50% IL, (◇) 75% IL, (□) 100% IL; (**A**) BMIM·BF_4 (**B**) TOMA·Tf_2N. Conditions: esculin (15 mM), palmitic acid (60 mM), molecular sieves (75 mg/mL), Novozym 435 (7.5 mg/mL), media volume 2 mL, 96 h, and 40 °C.

improved gradually with increases in the volume ratios of acetone in these mixtures. For 1-alkyl-3-methylimidazolium cations, increasing the alkyl chain length from butyl to octyl increased the hydrophobicity and viscosity of the ionic liquid, whereas densities and surface tension values decreased (*27*). [OMIM]·[BF_4] and [MeOcPy]·[BF_4] had the same anion and the alkyl part of the cation, but they belonged to the immidazolium-based salt and the pyridinium-based salt, respectively. In the same conditions, enzyme activity and esculin conversion in IL−organic solvent media with the immidazolium-based salt were lower than those with the pyridinium-based salt.

Esterification in [TOMA]·[Tf_2N]−acetone mixtures differed from those containing $[PF_6]^-$ and $[BF_4]^-$ anions. Esculin conversion in [TOMA]·[Tf_2N]−acetone (25% IL) media was higher than that in pure acetone and [TOMA]·[Tf_2N]−acetone (50% and 75% IL) media. The most promising media should have the advantages of both organic solvents and ionic liquids. The appropriate ratios of ILs could enhance enzyme activity and the reaction efficiency because the ILs could well interact with the enzyme and change the enzyme structure to form the acyl−enzyme intermediate (*11*). However, media with a higher ratio of IL are always more polar than organic solvents, which might inactivate the enzyme and cause lower enzymatic activity. The viscosity of ILs in the IL−organic solvent media with the higher percentage of ILs is higher than that of the organic solvents, which may affect the diffusion of substrates and products to and from the active site, finally leading to a drop in both the reaction rate and esculin conversion (*26*).

Enzymatic esterfication was examined in 17 different IL−acetone mixtures. According to their benignity to lipase

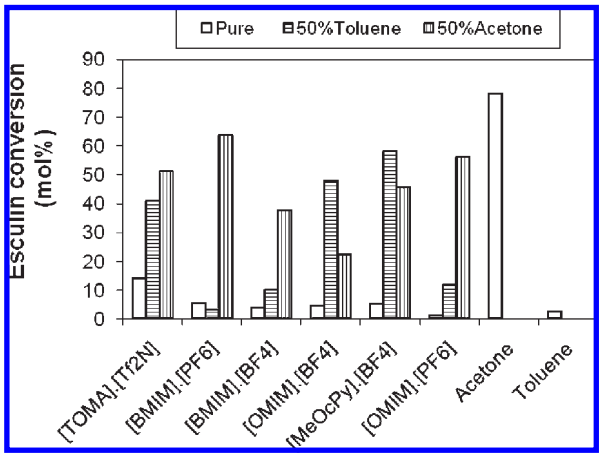


Figure 4. Esculin bioconversion in mixtures of ionic liquids with acetone and toluene. Conditions: esculin (15 mM), palmitic acid (60 mM), molecular sieves (75 mg/mL), Novozym 435 (7.5 mg/mL), media volume 2 mL, 96 h, and 40 °C.

activity, the anions of ILs were ranked in decreasing order as follows: $[Tf_2N]^-$ > $[PF_6]^-$ > $[BF_4]^-$ > $[CF_3SO_3]^-$ > $[C_4F_9SO_3]^-$ > [TAF]$^-$ > $[MDEGSO_4]^-$ > $[OctSO_4]^-$ > [ES]$^-$ = [DMP]$^-$ = [OTos]$^-$ = Cl^-. The three anions, $[Tf_2N]^-$, $[PF_6]^-$, and $[BF_4]^-$, were selected for further study.

Enzymatic Esterification in Mixtures with Different Organic Solvents. Enzymatic esterification in IL−acetone mixtures performed quite well. But did the enzymatic esterification in IL−organic solvent mixtures have a similar performance with different organic solvents? Acetone, hexane, toluene, and *t*-butanol have different physical and chemical characteristics. Therefore, enzymatic esterification in different IL−organic solvent mixtures may vary. Enzymatic esterification was examined in IL−hexane, IL−*t*-butanol, and IL−toluene. Eight less-polar ionic liquids were selected on the basis of the previous results (**Table 4**) for further investigation. The volume ratios in the IL−organic solvent were 25% and 50% IL. All experimental conditions were the same as those for the IL−acetone mixtures. The results for IL−hexane and IL−*t*-butanol were disappointing in that the esculin conversions were lower than expected. Encouragingly, the enzymatic esterification in IL−toluene gave us a surprise, as some mixtures had higher rates of esculin conversion.

The effects of acetone and toluene on reaction performance were evaluated in the 50% IL−organic solvent mixtures (**Figure 4**). In these six IL−organic solvent systems, acetone significantly enhanced enzyme activity, and the reaction rate and was almost an ideal organic solvent. Toluene greatly influenced the reaction rate in some types of IL−toluene media, such as [MeOcPy]·[BF_4]−, [OMIM]·[BF_4]−, and [TOMA]·[Tf_2N]−toluene (1:1, v/v) mixtures. Esculin conversion in [MeOcPy]·[BF_4]− and [OMIM]·[BF_4]−toluene (1:1, v/v) was even higher than that in [MeOcPy]·[BF_4]− and [OMIM]·[BF_4]−acetone (1:1, v/v). Hexane and *t*-butanol partly boosted the reaction rates for their mixture systems, but the alteration was not significant or efficient. In addition, acetone and toluene were miscible with ionic liquids in contrast to *t*-butanol and hexane. *t*-Butanol and hexane mixtures were two-phase systems and immiscible with ILs. It has been reported that two-phase systems are helpful in some systems, being able to increase the solubility of products in ionic liquids and remove the product from organic solvents to the ionic liquid

layer (*24*). In this case, acetone and toluene systems were better than hexane and *t*-butanol systems. It might be that their the mass transfer behaviors influenced the mixtures.

It was clear that [MeOcPy]·[BF_4]−, [OMIM]·[BF_4]−, and [TOMA]·[Tf_2N]−organic solvent (50% IL) media had the potential for optimization. These three systems contained at least two organic solvents each with high enzyme activity, which combined the advantages of both organic solvents and ionic liquids.

Acetone is a polar and hydrophilic solvent, while toluene is a nonpolar and hydrophobic solvent. When acetone and toluene were mixed with different ILs separately, the media displayed different reaction behaviors. Over 50 mol % esculin conversion was obtained in [TOMA]·[Tf_2N]−, [BMIM]·[PF_6]−, and [OMIM]·[PF_6]−acetone (1:1, v/v) mixtures. In the IL−toluene (1:1, v/v [TOMA]·[Tf_2N]−, [OMIM]·[BF_4]−, and [MeOcPy]·[BF_4]−toluene) mixtures, esculin conversion reached over 40 mol %.

The anion part of ionic liquids played different roles in the IL−acetone and IL−toluene mixtures. ILs with the $[PF_6]^-$ anion were more hydrophobic than ILs with the $[BF_4]^-$ and $[Tf_2N]^-$ anions. When IL with $[PF_6]^-$ was mixed with acetone, esculin solubility and enzyme activity was enhanced, at the same time resulting in an increase in the rate of esterification. ILs with $[BF_4]^-$ and $[Tf_2N]^-$ anions were more hydrophilic. When mixed with toluene, it improved esculin solubility and enzyme activity.

Conclusions. The main purpose of this work was to explore the possibility of combining the higher reaction rate in conventional solvents and high solubility of esculin in ILs for enzymatic production of flavonoid esters, and to investigate the multiple interactions in IL−organic solvent systems as well as their effects on enzyme activity and reaction performance. On the basis of the experimental results, the following conclusions can be made: (1) The esterification reaction rates of esculin in organic solvents depended mainly on their individual solubility and the activity coefficients of the reactants, while for the reactions in pure ILs, the anionic part of the ILs played an important role in determining enzyme activity. (2) This work proved that it was possible to evaluate the effects of ILs on lipase activity by employing a mixed system for those that could not be distinguished in a pure IL system, which was useful for the categorization of ILs. (3) According to their reactivity to lipase activity, the anions of ILs were ranked in decreasing order as follows: $[Tf_2N]^- > [PF_6]^- > [BF_4]^- > [CF_3SO_3]^- > [C_4F_9SO_3]^- > [TAF]^- > [MDEGSO_4]^- > [OctSO_4]^- > [ES]^- = [DMP]^- = [OTs]^- = Cl^-$. ILs with $[Tf_2N]^-$ and $[BF_4]^-$ anions showed promising results. (4) This work demonstrated the feasibility of combining higher esculin conversion with high solubility of flavonoids in ILs with faster reaction rates. (5) The knowledge obtained in this work may be useful for a better understanding of the multiple interactions in IL−organic solvent systems and helpful for the design and optimization of these systems.

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