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Effect of ionic liquids, organic solvents and supercritical CO_2 pretreatment on the conformation and catalytic properties of *Candida rugosa* lipase

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ABSTRACT

The objective of this work is to assess the structure and activity of *Candida rugosa* lipase (CRL) pretreated with seventeen ionic liquids (ILs), five organic solvents and super-critical carbon dioxide (SC-CO_2). The results revealed that anion selection of ILs showed generally much greater effects on CRL esterification activity than cation choice, and CRL pretreated by ILs with strong water miscible properties showed very low esterification activity. The highest CRL activity treated with ILs [Hmim][PF6] was obtained with the value of 45078.0 U/g-protein. Furthermore, the CRL activities pretreated with five conventional organic solvents were also examined and the values increased with the log *P* decrease of organic solvents when log *P* was lower than 2.0. Finally, the CRL activities were respectively 1.2- and 1.3-fold higher over the untreated ones after pretreatment with sub- and super-critical CO_2 under the pressures of 6 MPa and 15 MPa at 40 °C for 20 min. Further analyses via FT-IR demonstrated that the high activity of CRL pretreated with ILs, organic solvents and SC-CO_2 was probably caused by the changes of CRL secondary structure. In conclusion, the results in this work will be helpful for us to choose the suitable reaction medium in CRL biocatalysis and biotransformation reactions.

1. Introduction

The yeast *Candida rugosa* has a family of functional genes codes for several isoenzymes with closely related sequences naming Lip1 to Lip 7 [1]. Recently, *C. rugosa* lipase (CRL) has been extensively demonstrated to be useful for biotransformation reactions in aqueous and non-aqueous phases due to its high activity and broad specificity [2]. Majumder and Gupta [3] reported that CRL was used to catalyze enantioselective transacetylation of benzyl alcohols with vinyl acetate, and 80% enantiomeric (R)-1-phenylethyl acetate was obtained with 38% conversion ($E = 15$) after 24 h. Ujang et al. [4] had also investigated that the kinetic resolution of ± 2-(4-chlorophenoxy) propionic acid with 1-butanol in organic solvents was catalyzed by free CRL. Kahveci and Xu [5] had further demonstrated that CRL could be employed to enrich omega-3 polyunsaturated fatty acids (PUFA) from salmon oil with a PUFA yield of 50.58% (mol.%). Simultaneously, the biodiesel synthesis was investigated by immobilized CRL under supercritical fluid conditions, and 99.99% biodiesel yield was achieved at 2 h when methanol was fed at a stepwise of 0.75 h interval [6]. Due to enzymes considerably unstable characteristics, many approaches have been employed to improve their catalytic activity and operational stability, such as genetic engineering, protein engineering, media engineering, substrate engineering, immobilization and/or process optimization [7,8].

Nowadays the enzyme-catalyzed bio-reactions in micro-/non-aqueous solvents have become one of the more exciting fields of enzymology [9,10], whose merits are to avoid water-dependent side reactions and minimize the enzymes denaturation. Organic solvents, ionic liquids (ILs), sub-/super-critical fluids, and microemulsion phase as micro/non-aqueous reaction media have been intensively described in literature [11,12]. For example, Mohile et al. [13] had studied CRL-catalysed enantioselective esterifications of butyl 2-(4-chlorophenoxy) propionate in aqueous buffer with ionic liquids as co-solvents, and found hydrophobic ionic liquids offered almost quantitative conversions with ee ≥ 99%. It was demonstrated that lipase native structure was altered to some degree, which can lead to its performance properties variance, when lipase was pretreated with ILs and organic solvents [14]. Therefore, it is very important to elucidate the correlation between lipase activity and its conformation after pretreatment by these solvents. In the past years, the correlation between lipase structure and activity required direct measurement of active-site structure

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and the effect of the reaction medium on the transition state of the reaction. But now, using Fourier transform-infrared (FT-IR) spectroscopy, it is very easy to know the changes of the non-covalent forces (hydrogen bonding, ionic, hydrophobic and van der Waals interactions) that maintain the native secondary and tertiary structures of lipases [15,16]. In our previous works, we reported the effect of conventional organic solvents and ILs on *Burkholderia cepacia* (BCL) lipase activity and its conformation structure using FT-IR and circular dichroism (CD) [14,17]. To our best knowledge, the effect of ILs pretreatment with different cation and anion structures on CRL activity and its conformation has rarely been well studied. And the understanding of the effects of sub- and super-critical CO_2 pretreatment on CRL catalytic activity and conformation has still few reports so far.

Therefore, the objectives of this work are: (1) to investigate the esterification activity of CRL pretreated with 5 conventional organic solvents with different log *P* from 0.8 to 4.5; (2) to evaluate the effect of 17 different ILs, and under sub-/super-critical CO_2 pretreatment on the CRL esterification activity; (3) to elucidate the secondary structure variance of CRL by FT-IR in the cases of above mentioned pretreatments.

2. Materials and methods

2.1. Materials

C. rugosa lipase (CRL) (lyophilized powder) with specific lipase activity ≥ 700 U/mg-protein was bought from Sigma Co. (St, Louis, MO, USA). Seventeen ILs of 1-ethyl-3-methylimidazolium tetrafluoroborate ([Emim][BF_4]), 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF_4]), 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]), 1-butyl-3-methylimidazolium trifluoromethanesulfonate [Bmim][TfO], N-octyl-3-pyridine tetrafluoroborate ([OmPy][BF_4]), 1-hexyl-3-methylimidazolium methanesulfonate ([Hmim][CH_3SO_3]), 1-ethyl-3-methylimidazolium trifluoromethanesulfonate ([Emim][TfO]), 1-octyl-3- methylimidazolium tetrafluoroborate ([Omim][BF_4]), 1-hexyl-3-methylimidazolium hexafluorophosphate ([Hmim][PF_6]), 1-ethyl-3-methylimidazolium hexafluorophosphate ([Emim][PF_6]), 1-butyl-3-methylimidazolium bis[(trifluoromethyl)sulfonyl]imide ([Bmim][Tf_2N]), 1-butyl-3-methylimidazolium methanesulfonate ([Bmim][CH_3SO_3]), 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), 1-ethyl-3-methylimidazolium chloride ([Emim][Cl]), 1-butyl-3-methylimidazolium hydroxide ([Bmim][OH]), 1-octyl-3-methylimidazolium chloride ([Omim][Cl]), and 1-octyl-3-methylimidazolium hexafluorophosphate ([Omim][PF_6]) with purifity ≥ 99.9% were purchased from Shanghai Cheng Jie Chemical Co. Ltd. (Shanghai, China). All ILs were dried to remove the water content before experiments. Organic solvents of *tert*-amyl alcohol, *tert*-butanol, petroleum ether, *n*-hexane and isooctane were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Liquid CO_2 with the purification of 99.9% was purchased from Shanxi Kewei chemical company (Shanxi, China).

2.2. CRL pretreatment with ILs

To elucidate the effect of cation and anion structures of ILs on the CRL esterification activity and its conformation, seventeen ILs with five cations and seven anions structures were employed in this work. The procedure of CRL treatment with ILs was modified according to the method by Pan et al. [14]. Generally, 40 mg CRL was placed in the seventeen different test tubes and then 0.5 mL of various ILs were added to the tubes to swell the enzyme powder. Each treatment was immersed and stirred in a rotary shaker with the rate of 200 rpm at 37 °C for 10 min. Then, the ILs was removed off with water washing and the treated CRL was used for esterification activity and its conformation assays. CRL without ILs pretreatment was used as the control.

2.3. CRL pretreatment with organic solvents

In order to evaluate the effects of log *P* of organic solvents on the CRL activity and its conformation, five conventional organic solvents with different log *P* (from 0.8 to 4.7) were tested, including *tert*-butanol (log *P* = 0.8), *tert*-amyl alcohol (log *P* = 1.3), petroleum ether (log *P* = 2.0), *n*-hexane (log *P* = 3.5) and isooctane (log *P* = 4.7). The procedure of CRL treatment with organic solvents was modified according to that in our previous work [17]. Generally, 40 mg CRL was placed in the different test tubes and then 0.5 mL of various organic solvents were added to the tubes to swell the enzyme powder. Each treatment was immersed and stirred in a rotary shaker with the rate of 200 rpm at 37 °C for 10 min. Then, the organic solvent was removed off and the treated CRL was used for esterification activity and its conformation assays. CRL without organic solvents pretreatment was used as the control.

2.4. CRL pretreatment with sub- and super-critical CO_2

The effect of SC-CO_2 on the CRL activity and its conformation was also investigated in this work. The SC-CO_2 equipment consisted mainly of a liquid CO_2 reservoir, thermostatic bath with the precision of ±1 °C, stainless steel vessel (autoclave) with the internal volume of 1 L, high pressure liquid pump, and pressure gauge with the precision of ±0.1 MPa. The equipment scheme is shown in Fig. 1.

The procedure of CRL treatment with SC-CO_2 was described as follows: First of all, 200 mg CRL was placed in the catalyst basket and put into the autoclave, then CO_2 was supplied by pump into the autoclave, and then the whole system was heated until the desired pressure (6 MPa and 15 MPa) and temperature (40 °C) were achieved. Each treatment was set at 40 °C for 20 min. After depressurizing, the sub- and supercritical treated CRL was used for esterification activity and its conformation assays. CRL without pretreatment with SC-CO_2 was used as the control.

2.5. Assay of CRL esterification activity

The determination procedure of CRL esterification activity of lauric acid and dodecanol was modified according to the method in our previous work [17]. Specifically, 40 mg CRL were added into a screw capped vial, which contained 0.40 g lauric acid (0.2 M) and 0.37 g dodecanol (0.2 M) in a mixture solution of 10 mL isooctane

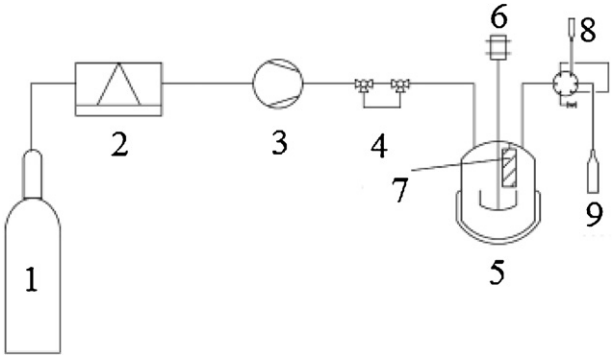

Fig. 1. The scheme of supercritical CO_2 equipment (1: CO_2 reservior; 2: thermostatic bath; 3: high pressure liquid pump; 4: reactant loop; 5: stainless vessel (autoclave); 6: high pressure gauge; 7: catalyst basket; 8: 6-way sample valve; 9: sample vessel).

solvent and 0.01 mL phosphate buffer solution (PBS). The vial was placed in a controlled temperature shaker and reacted for 30 min at 37 °C and 200 rpm. The reaction was stopped by adding 10 mL mixture solutions of ethanol and acetone (1:1, v/v) after 30 min, and the reactant was immediately titrated the unreacted FFA with 0.05 M alcoholic NaOH solution using phenolphthalein as indicator. The CRL esterification activity (U per gram protein) in this work was expressed as μm of ester formed per hour per gram protein. Protein content quantification of CRL was determined by the Bradford protein assay method using Bradford reagent from bio-rad, USA at a wavelength of 595 nm using a standard calibration curve of BSA [18]. The protein content of CRL was determined to be 5.13 wt.%.

2.6. Conformation analysis of CRL by FT-IR spectrometry

The measurement of CRL secondary structure by FT-IR spectrometry was according to the method described in our previous work [17]. FT-IR spectra were measured at 25 °C by a Vextex 70 FT-IR spectrometer (Bruker Optik GMBH, Germany) equipped with a nitrogen-cooled, mercury – cadmium – tellurium (MCT) detector, in the regions of 4000–400 cm^{-1}. Conditions were: 4 cm^{-1} spectral resolution, 20 kHz scan speed, 128 scan co-additions, and triangular apodization. By setting the microscope square diaphragm aperture to 100 μm × 100 μm, an excellent spectrum was collected within a few minutes. The spectrum acquisition (all samples were overlaid on a zinc selenide attenuated total reflectance (ATR) accessory) was from IR spectra, and the secondary structure elements, which were based on the information of amide I region and the band assignment, were manipulated using WinSpec software (LISE-Faculteĭs Universitaires Notre-Dame de la Paix, Namur, Belgium).

2.7. Data statistics analysis

All trials were conducted in triplicate and the data were analyzed by the software SAS 9.0 (SAS Institute Inc, Cary, NC, USA).

3. Results and discussion

3.1. Effect of anion and cation structures of ILs pretreatment on CRL esterification activity

The influence of the ILs anion and cation structures on CRL esterification activity is shown in Fig. 2.

As shown in Fig. 2, ILs with anion and cation structures had a significant influence on CRL esterification activities, which values were ranged from 447 U/g-protein with [Bmim][CH_3SO_3] to 45078 U/g-protein with [Hmim][PF_6]. In this work, CRL esterification activity generally decreased by the order of [PF_6]$^-$ > [BF_4]$^-$ > [TfO]$^-$ > [Tf_2N]$^-$ > [Cl]$^-$ > [OH]$^-$ > [CH_3SO_3]$^-$ apart from ILs [Emim] [Cl] when CRL was pretreated with the 17 ILs containing the above mentioned seven anions structures. As seen from Fig. 2, higher esterification activity of CRL was achieved after pretreatment with ILs containing [PF_6]$^-$, [BF_4]$^-$ and [TfO]$^-$ anions than ILs containing [OH]$^-$, [CH_3SO_3]$^-$ and [Tf_2N]$^-$. The reasonable explanation was that ILs with anion structures containing [CH_3SO_3]$^-$ and [OH]$^-$ have more nucleophilic properties than those containing [PF_6]$^-$ and [BF_4]$^-$. ILs with more nucleophilic properties could easily interact with lipase and consequently there might be conformational change which might be the cause of the activity variances [19,20]. However, Fig. 2 showed that CRL treated with ILs [Emin][Cl] containing [Cl]$^-$ had much higher activity (>40,000 U/g-protein, which was the third best activity among the 17 ionic liquids studied). In fact, if it was focused exclusively on [Emin]$^+$ systems, CRL exhibited the highest activity when it was pretreated with the ILs containing [Cl]$^-$ anions structure (closely followed by [TfO]$^-$). Although [Cl]$^-$ and [TfO]$^-$ have more nucleophilic properties comparison with other anions, both [Cl]$^-$ and [TfO]$^-$ in [Emin]$^+$ systems had higher activity for CRL than the less nucleophilic [PF_6]$^-$. Moreover, in the case of [Bmin]$^+$ systems, ILs [Bmin][TfO] had also higher activity than [Bmin][PF_6]. These phenomena seemed contradictory with those reported by other researchers in literature [19,20]. The reasonable explanation why there was contradict in the case of [Bmin]$^+$ systems was still not known. Interestingly, very low esterification activity of CRL was observed when CRL was treated with ILs with strong water miscible properties, such as in [Hmim][CH_3SO_3] (2436.6 U/g-protein), [Bmim][CH_3SO_3] (446.8 U/g-protein), [Bmim][OH] (3045.8 U/g-protein), [Bmim][Cl] (1015.2 U/g-protein) and [Omim][Cl] (649.6 U/g-protein), probably due to the influence of solvating properties on essential water layer around the CRL surface microenvironment [14].

In the case of the corresponding cation/anion ILs both [Bmim][BF_6], [Emim][BF_6], [Omim][BF_6] and [Bmim][BF_4], [Emim][BF_4], [Omim][BF_4], CRL esterification activity treated with the above-mentioned ILs revealed that cation selections generally showed smaller differences than anion selections, and the treated CRL activity increased gradually with increasing chain lengths of the alkyl substituent of the imidazolium ring. The reason was that increasing chain length of the alkyl substituent enhanced the ILs cations hydrophobicity, which would preserve the essential water layer around the protein molecule, and it would lead to the corresponding reduction in direct protein-ion interactions and subsequent increase of lipase activity [14]. The tendency of CRL activity treated with ILs was similar to that of the lipases from *Candida antarctica* (CALB) [21,22], *B. cepacia* (BCL) [14] and *Aspergillus niger* (ANL) [23]. In this work the highest activity of CRL treated

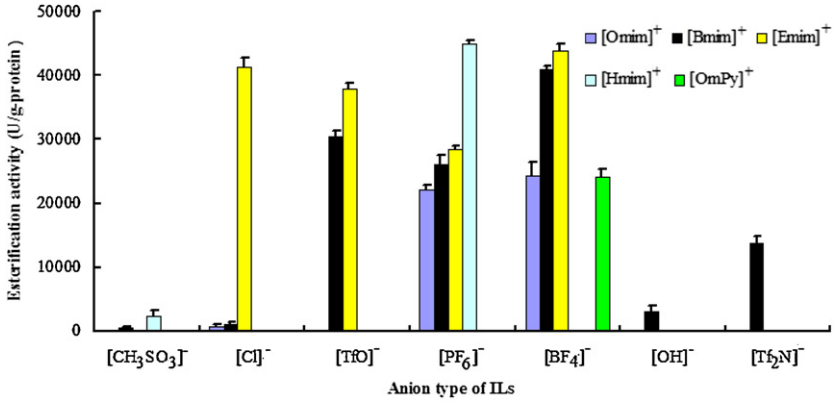

Fig. 2. Effect of ILs pretreatment with different anion and cation structures on esterification activity of CRL (conditions: 40 mg CRL, 0.5 mL ILs, 0.40 g lauric acid (0.2 M), 0.37 g dodecanol (0.2 M), 10 mL isooctane solvent and 0.01 mL PBS, temperature 37 °C, reaction time 4 h).

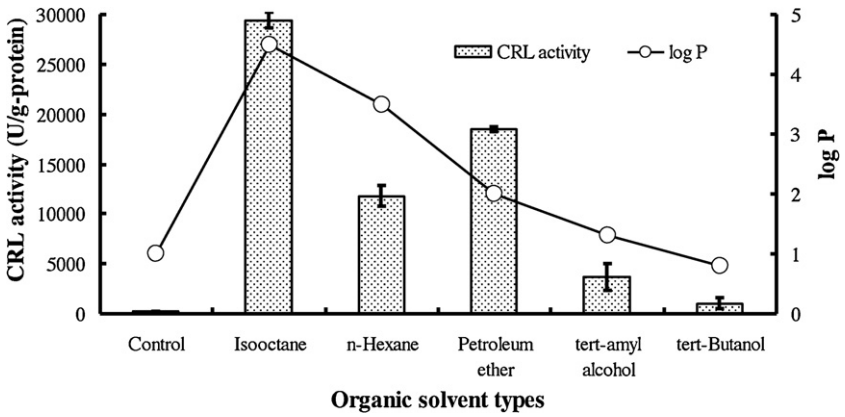

Fig. 3. The influence of organic solvents pretreatment with different log *P* on esterification activity of CRL (conditions: 40 mg CRL, 0.40 g lauric acid (0.2 M), 0.37 g dodecanol (0.2 M), 10 mL organic solvents and 0.01 mL PBS, temperature 37 °C, reaction time 4 h).

with ILs [Hmim][PF_6] was obtained with the value of 45078 U/g-protein. Whilst CALB treated with [Bmim][Tf_2N] showed higher activity than [Emim][Tf_2N], and BCL in ILs [Bmim][Tf_2N] had the highest activity with the value of 100 U/g-protein. In contrast, ANL showed the epoxidation of methyl oleate yield of 89% in hydrophilic ILs [BMI][BF_4]. Therefore, the variance of the lipase activity treated with ILs depended on the enzyme and ILs types, which is very useful for us to choose suitable lipase in biocatalysis and biotransformation reactions in presence of ILs.

3.2. Effect of organic solvents with different log P pretreatment on CRL esterification activity

The influence of five tested organic solvents pretreatment with different log *P* on CRL esterification activity is shown in Fig. 3.

As seen in Fig. 3, the highest esterification activity of treated CRL with the value of 29442.8 U/g-protein was obtained with isooctane, and it decreased by the order of isooctane > petroleum ether > *n*-hexane > *tert*-butanol > *tert*-amyl alcohol. In general, CRL esterification activity was relatively lower treated with hydrophilic solvents (e.g. *tert*-butanol and *tert*-amyl alcohol) with log $P < 2$, because a strong deformation can occur between the essential water and protein interactions [17]. It was reported that hydrophilic solvents would influence the essential water content surrounding the lipase microenvironment of the active site, and subsequently affect the variance of lipase conformation, which caused the lipase activity change [17].

3.3. Effect of sub- and supercritical CO_2 pretreatment on CRL esterification activity

Table 1 depicts the results of the effect of pressure on the CRL esterification activity with sub- and super-critical CO_2 pretreatment. It could be seen that the CRL activity increased 1.2- and 1.3-fold times after pretreatment under the pressures of 6 MPa and 15 MPa at 40 °C for 20 min. The improved activity might be due to the change in conformation structure of CRL treated by high-pressure CO_2. This result fell into a contradiction with that reported by Primo et al. [24], who reported that the specificityon oxidase enzymatic complexes from mate tea leaves treated with high-pressure CO_2 might decrease lipase activity. It was explained that SC-CO_2 might remove the essential water off surrounding the lipase surface responsible for maintaining the lipase conformation, leading to the reduction of the lipase activity [24]. Therefore, it should be very important to evaluate the conformation of lipase treated with high-pressure CO_2, which could be responsible for the variance of the lipase activity.

3.4. The conformation of CRL pretreated with ILs, organic solvents and sub- and super-critical CO_2

FT-IR can be used to examine the lipase secondary structure since proteins absorb infrared wavelengths due to the peptide bond vibrations. The amide I region (mainly due to the C=O stretching vibration) at approximately 1600–1700 cm^{-1} is mostly used in protein secondary structure determination due to its sensitivity in conformational changes and significantly higher signal intensities than those from 1340 to 1220 cm^{-1} amide III bands [17,19,21]. Table 2 shows the secondary structure elements of CRL pretreated with some selected ILs, and organic solvents and under the sub- or super-critical CO_2 conditions.

As seen in Table 2, comparing with the crude CRL conformation, the CRL pretreated with the selective ILs, organic solvents and SC-CO_2 underwent alterations in secondary structure to some content. For example, in the cases of α-helix content, it was found that the lower content in α-helix when CRL was treated with isooctane, [Emim][TfO], [Hmim][PF_6], [Bmim][BF_4] and sub-/super-critical CO_2. It was hypothesized that the lower the α-helix content, the higher the "open" conformation of the active site which allows easier access of the substrate, leading to enhancement of the CRL esterification activity [17]. From the relationship between CRL activity and its conformation, it was maybe speculated that the decrease in α-helix content of CRL probably affected the lipase active site, which was responsible for its activity alterations. This hypothesis was also demonstrated for other enzymes in non-aqueous media in literature. For instance, Pan et al. [14] verified that activity of lipase from *B. cepacia* increased in ILs and/or organic

Table 1
Effect of sub-critical and super-critical CO_2 pretreatment on the CRL catalytic esterification activity.

Pressure (MPa)	Temperature (°C)	Time (min)	Final activity (U/g-protein)	Residue activity (%)
6	40	20	218.30 ± 10.15	119 ± 5.6
15	40	20	243.65 ± 15.20	133 ± 8.3

*Control: final activity 182.75 ± 15.25 U/g-protein. Residue activity is defined as the ratio of the final activity after and before pretreated with high pressure CO_2.

Table 2
Secondary structure elements of CRL pretreated with some ILs and organic solvents and under sub-critical or super-critical CO_2.

	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coil (%)
Control[a]	19.5	25.1	39.1	16.3
Isooctane	17.6	20.5	49.2	12.7
Petroleum ether	17.3	21	48.7	13.1
n-Hexane	17.4	21.2	48.3	13.1
tert-Amyl alcohol	17.6	19.6	50.8	12
tert-Butanol	16.4	17.8	53.4	12.4
[Bmim][BF_4]	15.4	36.9	20.4	27.3
[Bmim][PF_6]	19.6	34.7	27.2	18.5
[Bmim][Tf_2N]	18.3	35.5	32.7	13.5
[Emim][TfO]	15.9	15.1	44.9	24.1
[Hmim][PF_6]	14.9	34.7	28	22.4
[Omim][BF_4]	20.3	34.6	28.3	16.8
[Omim][PF_6]	30.3	30.2	21.4	18.1
[Emim][Cl]	19.9	35.4	26.7	18.0
Subcritical CO_2[b]	16.8	14.8	54.8	13.6
Supercritical CO_2[c]	17.5	13.7	53.3	15.6

[a] The structure of untreated CRL measured by FT-IR was used as the control.
[b] Conditions: 6 MPa, 40 °C, 20 min.
[c] Conditions: 15 MPa, 40 °C, 20 min.

solvents was related to the α-helix decrease in conformation structure. However, Pavlidis et al. [25] demonstrated that the higher catalytic behaviors of lipases from *C. rugosa*, *Chromobacterium viscosum* and *Thermomyces lanuginose* in water-in-ionic liquids microemulsions were related to the increase in β-sheet content of secondary structure, which is just reverse to the results in our present work. Therefore, the relationship of these enzyme activity enhancements correlated with the structural changes is worthwhile to further study in future.

4. Conclusions

Cation and anion structures of ILs could significantly affect CRL activity. Generally, Anion selection had much greater influence on CRL esterification activity than cation choice. ILs with strong water-miscible properties would lower CRL esterification activity. Organic solvents with different log *P* also obviously affected CRL activity, and showed increasing tendency with log *P* decrease of organic solvents when it was lower than 2.0. It was observed that the CRL activity pretreated in sub- and super-critical CO_2 was 1.2- and 1.3-fold higher compared with the untreated CRL under the pressures of 6 MPa and 15 MPa at 40 °C for 20 min. Overall, the esterification activity of CRL treated with ILs was higher than with organic solvents and SC-CO_2. FT-IR analyses revealed that change in secondary structure of treated CRL was probably responsible for the variance in the esterification activity, but these enzyme activity enhancements cannot be correlated with the structural changes generated from the experimental data.

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