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ORIGINAL PAPER

Chemical modification with functionalized ionic liquids: a novel method to improve the enzymatic properties of *Candida rugosa* lipase

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Abstract Chemical modification of lysine residues in *Candida rugosa* lipase (CRL) was carried out using five different functional ionic liquids, and about 15.4–25.0 % of the primary amino groups of lysine were modified. Enzymatic properties of the native and modified CRLs were investigated in olive oil hydrolysis reaction. Improved thermal stability, catalytic activity in organic solvents, and adaptability to temperature and pH changes were achieved compared with the native enzyme. CRL modified by [choline][H_2PO_4] showed the best results, bearing a maximum improvement of 16.7 % in terms of relative activity, 5.2-fold increase in thermostability (after incubation at 45 °C for 5 h), and 2.3-fold increase in activity in strong polar organic solvent (80 % dimethyl sulfoxide) compared with the native enzyme. The results of ultraviolet, circular dichroism and fluorescence spectroscopy suggested that the change of the secondary and tertiary structures of CRL caused by the chemical modification resulted in the enhancement of enzymatic performance. The modification of CRL with functional ionic liquids was proved to be a novel and efficient method for improving the enzymatic properties of CRL.

Keywords Ionic liquid · Chemical modification · *Candida rugosa* Lipase · Enzymatic property

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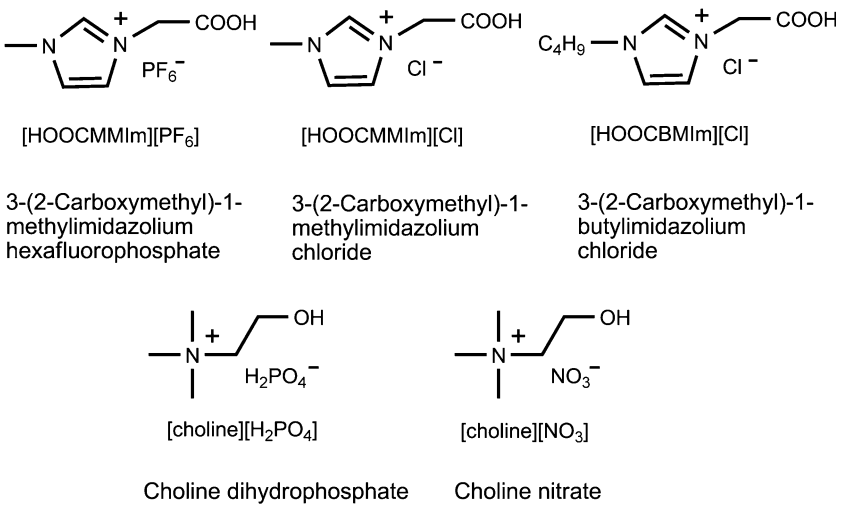
Introduction

Lipases are a series of versatile biocatalyst with excellent selectivity and activity under mild reaction conditions and play an important role in the development of chemical industries, such as pharmaceuticals, food, energy and fine chemicals [1–4]. However, the industrial application of lipase is often hampered by its high cost and easy inactivation in organic solvent, high temperature and other extreme conditions. Thus, most of the strategies for enzyme engineering are focused on microbiology [5], protein engineering [6], medium engineering [7], immobilization on novel supports, etc. [8]. The traditional technique of chemical modification is a very powerful tool for improving the enzymatic properties [9], leading to the introduction of functional and specificity-determining groups that are inaccessible by conventional mutagenesis techniques, and improvements in enzyme activity and/or stability, which can be achieved at a low cost using a relatively straightforward method. The modifiers usually used for chemical modification of enzymes are aldehydes, anhydrides, amines, fatty acids, halohydrocarbons, polyethylene glycol, dextrans, and so on [9–11].

Room temperature ionic liquids have been widely used as solvents or co-solvents in biocatalytic reactions and have been processed over a decade [12, 13]. In our previous work, we synthesized and grafted different functionalized ionic liquids onto the surface of mesoporous silica SBA-15 (IL-SBA). Lipase was successfully incorporated into IL-SBA by various methods, and the enzymatic properties were improved remarkably [14–17]. Recently, we also showed that various functional ILs with different cations and anions could chemically modify porcine pancreatic lipase (PPL) to increase enzyme activity and thermostability in aqueous solution [18].

Scheme 1 Structures of ionic liquids used to chemically modify the CRL



Herein, *Candida rugosa* lipase (CRL), a typical and widely used lipase, was modified with different functionalized ionic liquids (Scheme 1). The structures of native and modified CRLs were studied by ultraviolet, circular dichroism (CD), and fluorescence spectra. The effects of temperature and pH on the enzymatic activity were studied. Moreover, the thermal stability, catalytic activity in organic solvent and kinetic parameters were investigated. This work aims to develop an efficient modification system for improving the enzymatic performance of lipase.

Materials and methods

Materials

CRL (Type VII) and trinitrobenzenesulfonic acid solution (5 % w/v in H_2O) were purchased from Sigma-Aldrich China Inc. Carbonyldiimidazole (97 %) was purchased from Aladdin Chemistry Co., Ltd. Ionic liquids (99 %, HPLC) used in this work were purchased from Shanghai Chengjie Chemical Co. Ltd. Hydrochloric acid, acetone, ethanol, methanol, isooctane, anhydrous dimethyl sulfoxide and other reagents were of analytical grade and purchased from SCRC, China. All the solutions were prepared with distilled water.

Chemical modification of CRL

The CRL powder was dissolved in distilled water and magnetic stirred for 30 min at 4 °C. The obtained CRL solution was then concentrated with ammonium sulfate precipitation and transferred into a 10 kDa dialysis membrane to remove excess salt. The ionic liquid and carbonyldiimidazole were dissolved in anhydrous dimethyl sulfoxide (DMSO) for a final concentration of 1.36 M, respectively. The mixture was reacted for 4 h at room temperature to activate the ionic liquid. The activated ionic liquid (0.4 ml) was added dropwise into 15 ml of CRL solution (15 mg/ml) under vigorous stirring. The reaction was allowed to proceed for 24 h at 0 °C. The modified CRL was dialyzed exhaustively against distilled water at 4 °C for 48 h to remove the unreacted modifier molecules. The resulting lipases were denoted as [HOOCMMIm]-CRL-[PF_6], [HOOCMMIm]-CRL-[Cl], [HOOCBMIm]-CRL-[Cl], [Choline]-CRL-[H_2PO_4] and [Choline]-CRL-[NO_3], respectively. Protein concentration was determined via the BCA method using bovine serum albumin (BSA) as standard [19].

Determination of the degree of modification

The number of free amino groups presented in CRL before and after chemical modification was estimated using trinitrobenzenesulfonic acid (TNBS) assay procedure [20]. CRL solution (0.5 ml, 100 μg/ml) and 0.01 % TNBS (0.5 ml) were first incubated in 0.25 ml phosphate buffer (0.025 M, pH 8.2) at 37 °C for 2 h. Sodium dodecyl sulfate solution (0.5 ml, 10 % w/v) and hydrochloric acid solution (0.25 ml, 1 M) were added, absorbance was measured at 335 nm in a UV-1200. The degree of modification was calculated by the following equation:

$$\text{DM}\ (\%) = 1 - \text{absorbance}_A/\text{absorbance}_B$$

absorbance_A and absorbance_B mean the absorbance of modified and native enzyme, respectively.

Activity assay

The enzymatic activity of CRL was assayed by the olive oil emulsion method according to the process proposed by Monier et al. [21]. The emulsification solution was

prepared by mixing 50 ml of olive oil with 50 ml of gum arabic solution (7 %, w/v). Olive oil emulsification solution (5 ml) and phosphate buffer (5 ml, 0.025 M, pH 7.0) were mixed and incubated in a water bath at 30 °C for 5 min. CRL solution (1 ml, 100 μg/mL) was then added to initiate the reaction under a moderate stirring speed for 3 min. The reaction was stopped by an addition of 15 ml acetone/ethanol (1/1, v/v). The activity of CRL was determined by titration with 0.05 M sodium hydroxide solution. One unit of CRL activity was defined as the amount of enzyme required to release 1 μmol of acid per minute.

Enzymatic properties

Effect of temperature on activity

The activity of CRL was assayed at a temperature ranging from 20 to 50 °C at pH 7 through the activity assay procedure described above.

Effect of pH on activity

The effect of pH (phosphate buffer) on the activity of CRL was determined by conducting the CRL activity assay within the pH ranging from 6.0 to 8.0 at suitable temperatures.

Thermal stability

Thermal stability of CRL was assayed by incubating it in a water bath at 45 °C for 1, 2, 3, 4 and 5 h, respectively. A certain amount of CRL solution was periodically withdrawn for activity assay.

Catalytic activity in organic solvents

Activity of CRL sample in organic solvent was carried out at suitable temperature and pH. The organic solvents used were DMSO, methanol and isooctane. Reaction mixture was set up with increasing percent volume of organic solvents in phosphate buffer (0.025 M) with 10 or 20 % increment.

Determination of kinetic parameters

Experiments for the determination of kinetic parameters, the maximum rate (V_{max}) and the Michaelis constant (K_M) were performed at suitable temperature and pH using different concentrations of oil emulsification solution from 40 to 240 mg/ml. The values of K_M and V_{max} were calculated from a double reciprocal plot. In all cases, the activity of CRL was determined at 3 min to avoid the possible inhibition that may take place because of the appearance of reaction products.

Characterization of native and modified CRLs

Ultraviolet spectroscopy

Ultraviolet spectrum of CRL was recorded at 25 °C on PerkinElmer-Lambda 25 from 200 to 500 nm. The concentration of enzyme was 25 μg/ml.

Circular dichroism (CD) spectroscopy

The measurement was carried out using a circular cell with 1 mm light path length at 25 °C on JASCO-J810 spectropolarimeter (Jasco Co., Japan) with dilute enzyme solution (14.25 μg/ml). All the CD spectra were averaged by three scans taken under the identical condition and corrected for the solvent background.

Fluorescence spectroscopy

Fluorescence spectrum of CRL (100 μg/ml) was monitored on a spectrofluorometer (PerkinElmer LS55, USA) at 25 °C using a slit width of 5 nm for both excitation and emission. The emission was recorded from 300 to 400 nm, using an excitation wavelength of 270 nm. Three spectra were accumulated and averaged for each sample.

Results and discussion

Determination of modification degree and catalytic activity in aqueous environment

Table 1 summarized the results of the modification degree of CRL. In general, 15.4–25.0 % of the primary amino groups of lysine reacted with the functionalized ionic liquid, and the modification degrees at the same conditions followed the decreasing order [HOOCBMIm]–CRL–[Cl] > [Choline]–CRL–[H_2PO_4] > [Choline]–CRL–[NO_3] > [HOOCMMIm]–CRL–[Cl] > [HOOCMMIm]–CRL–[PF_6]. For the same cation (anion), the more kosmotropic the anion (cation), the higher the modification degree was obtained. The kosmotrope was reported to have stronger interactions with water molecules, thus breaking the hydrated shell of the enzyme and allowing the activated cation to graft easily onto the enzyme. The kosmotropicity for the cations was following the order: choline$^+$ < MMIm$^+$ < BMIm$^+$, and for the anion was PF_6^- < NO_3^- < Cl^- < $H_2PO_4^-$ [22–25].

Generally, chemical modification of CRL will cause a decreased hydrolytic activity. Sánchez-Montero reported that

Table 1 Modification degrees and activities of native and modified CRLs

Sample	Specific activity (U/mg protein)[a]	Relative activity (%)[b]	Degree of amino groups modified (%)[c]
CRL	214.6 ± 7.2	100	–
[HOOCMMIm]-CRL-[PF_6]	179.3 ± 5.8	83.6	15.4 % ± 1.6
[HOOCMMIm]-CRL-[Cl]	183.4 ± 6.6	85.5	16.2 % ± 2.8
[HOOCBMIm]-CRL-[Cl]	89.8 ± 7.1	41.8	25.0 % ± 3.9
[Choline]-CRL-[H_2PO_4]	250. 4 ± 6.3	116.7	22.7 % ± 0.8
[Choline]-CRL-[NO_3]	191.7 ± 7.9	89.3	21.5 % ± 2.0

All data in the table were the averages of the triplicate of experiments. Error bars represent deviation from the mean for three separate experiments

[a] Reaction conditions: activity was determined at suitable temperature and pH in phosphate buffer (0.025 M) for 3 min, respectively

[b] Relative activity of unmodified CRL (214.6 U/mg protein) was defined as 100 %

[c] CRL (0.5 ml, 100 μg/mL) and 0.01 % TNBS (0.5 ml) were incubated in 0.25 ml phosphate buffer (0.025 M, pH 8.2) at 37 °C for 2 h, then sodium dodecyl sulfate solution (0.5 ml, 10 % w/v) and hydrochloric acid solution (0.25 ml, 1 M) were added, absorbance was measured at 335 nm in a UV-1200

CRL lost more than 80 % hydrolysis activity after modification with polyethylene glycol [26], and over 20 % decrease of activity using dextrans modification [27]. Also, Park showed that copolymer of polyethylene and maleic acid anhydride modification onto CRL caused nearly 20 % decrease of activity in the suitable conditions [28]. By contrast, the modified CRLs obtained in our study maintained a relative higher level of enzyme activity (Table 1). It was found that the activity of the modified CRLs followed the nature of ionic liquids: higher activity was achieved using ionic liquid with chaotrope (cation)–kosmotrope (anion) combination. Compared with the native CRL, the relative activity of CRL was improved by modifying it with a chaotrope–kosmotrope combination of [choline][H_2PO_4]. Previous study has reported similar activation of the PPL by chaotropic cations and kosmotropic anions in an aqueous environment [18]. It was worth to note that the modification degrees in this study have no association with hydrolysis activities, which was different from the previous research result [18].

Enzymatic properties

Effect of temperature on enzyme activity

Temperature has a profound influence on the enzyme activity. The variations of the relative activities of native and modified CRLs at different temperatures were shown in Fig. 1. As expected, the activities of all the lipases increased gradually with increasing temperature, and the maximum activity was obtained at 30 °C for CRL and 35 °C for modified CRLs. At temperature beyond 35 °C, the relative activity of the modified CRLs exhibited a slow decrease, while it still maintained almost 80 % of its activity from 35 to 50 °C. By contrast, the activity of native CRL declined rapidly, indicating that the modified CRLs had good heat resistance, and the modification possibly altered the conformation of the enzyme.

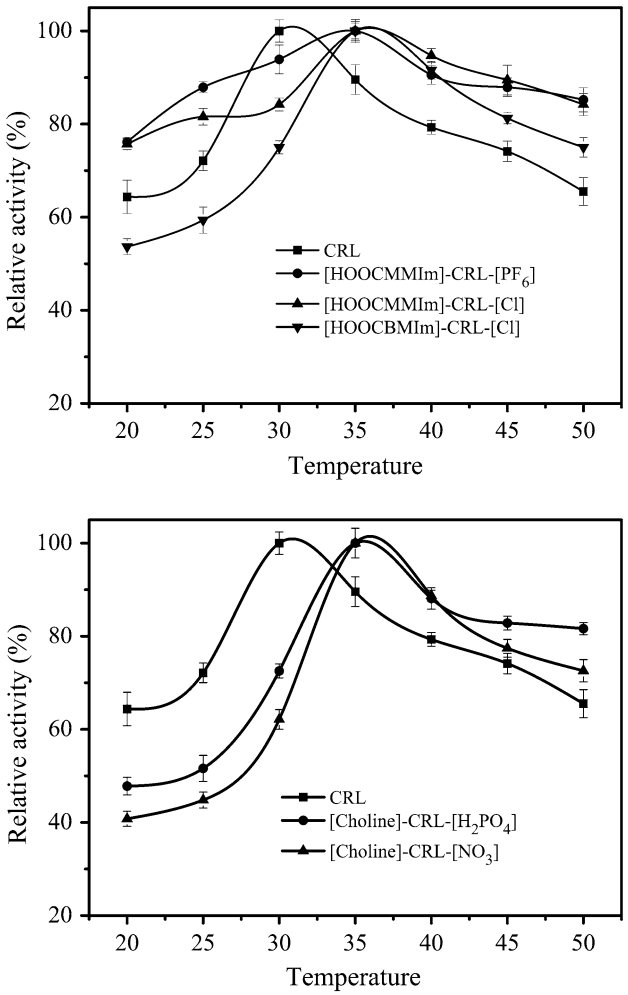


Fig. 1 Effect of temperature on the activity of CRLs. Enzyme activity was determined in phosphate buffer (0.025 M, pH 7.0) at different temperatures for 3 min. The maximum activity was defined as 100 % and all data in the figure were the averages of the triplicate of experiments

Effect of pH on enzyme activity

The pH dependence of the CRL hydrolysis reaction was studied within the range of 6.0–8.0, and the maximum activities of the native and modified CRLs were defined as

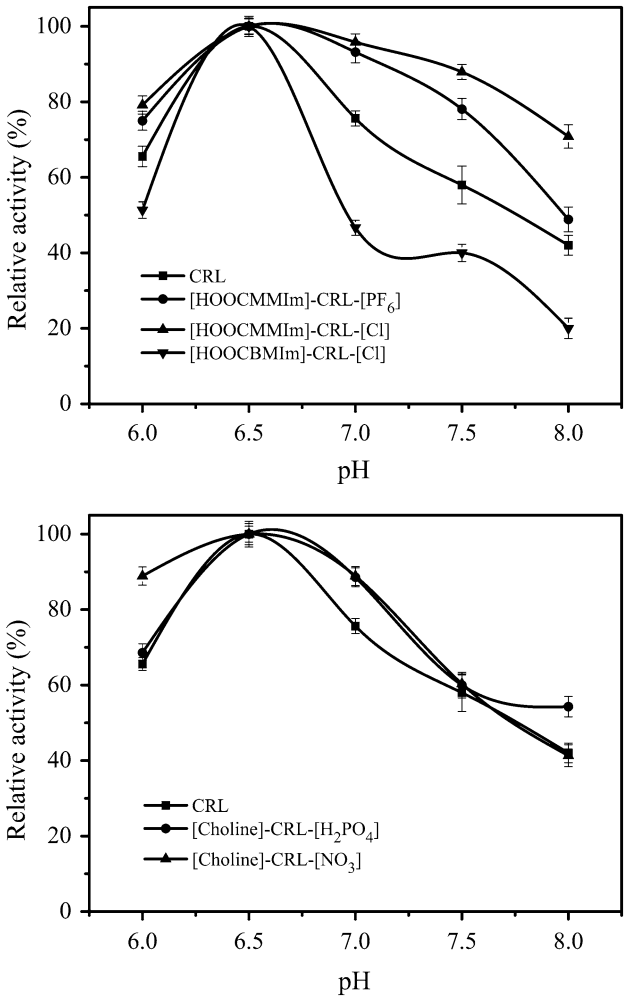


Fig. 2 Effect of pH on the activity of CRLs. Enzyme activity was determined at suitable temperature within the pH ranging from 6.0 to 8.0 for 3 min. The maximum activity was defined as 100 % and all data in the figure were the averages of the triplicate of experiments

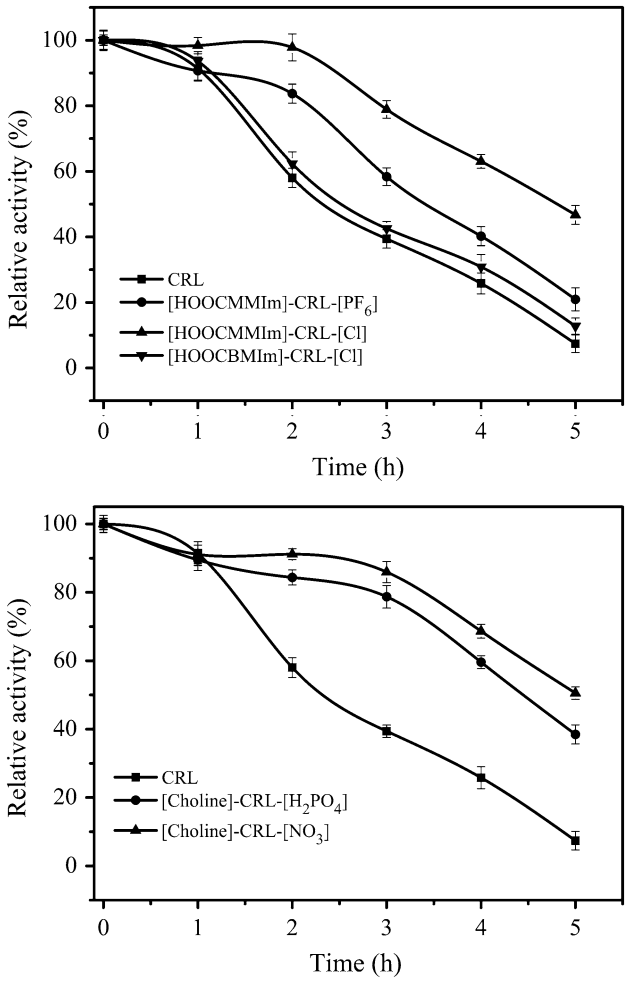


Fig. 3 Thermal stability of CRLs. Enzyme activity was determined under suitable temperature and pH in a water bath at 45 °C for 1, 2, 3, 4 and 5 h, respectively. The initial activity was defined as 100 % and all data in the figure were the averages of the triplicate of experiments

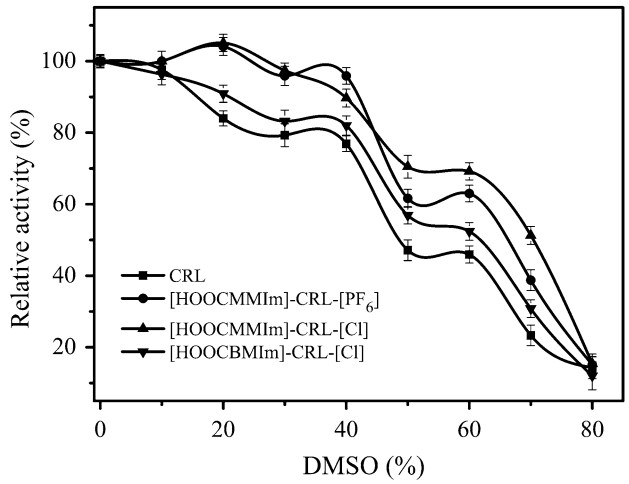
100 %. As shown in Fig. 2, the suitable pH was 6.5 for all CRLs, the relative activity increased from pH 6.0 to pH 6.5, and then decreased at higher pH levels, especially for native CRL and [HOOCBMIm]-CRL-[Cl]. The broad pH adaptability of the modified CRLs may be attributed to chemical modification. As shown in Fig. 2, the lipases modified by imidazolium-based cations showed better pH adaptability, which may be attributed to the ionic liquid possessing an imidazole ring and a carboxyl functional group. Both have the ability to release H^+, resulting in better interaction of the charged group with the lipase molecule, thus reduced the sensitivity of the enzyme to high pH levels [29].

Thermal stability of CRL

The thermal stability of native and modified CRLs was evaluated by incubating them in a water bath at 45 °C. As presented in Fig. 3, the native lipase lost its initial activity within approximately 2 h (58.0 %), whereas the modified forms retained their initial activity by about 97.8 % for [HOOCMMIm]-CRL-[Cl] and 84.4 % for [choline]-CRL-$[H_2PO_4]$ under the same conditions. These results indicated that the thermal stability of the modified lipases was much better than that of the native CRL due to the chemical modification with ionic liquids. Our modification method showed better thermal stability than that the modification using citraconic anhydride and maleic anhydride as modifier [30]. In comparison to our previous study for PPL modification [18], the kosmotropic cation modification did not cause a higher stability of CRL, as showed by ionic liquid modification of different lipases which yielded different results.

Catalytic activity in organic solvents

The catalytic activities of the native and modified CRLs in organic solvents were investigated in DMSO, methanol and



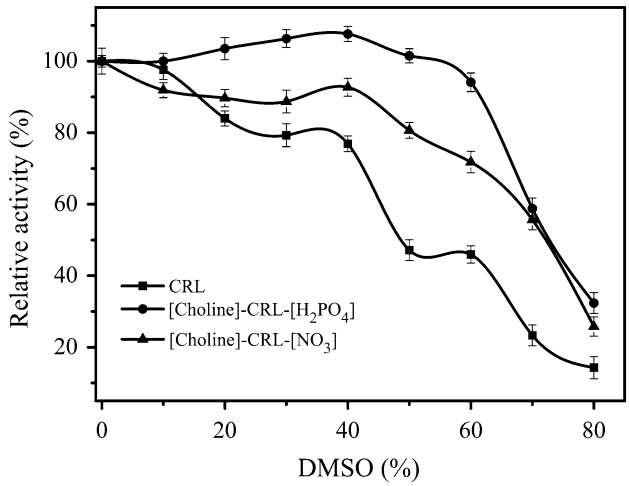
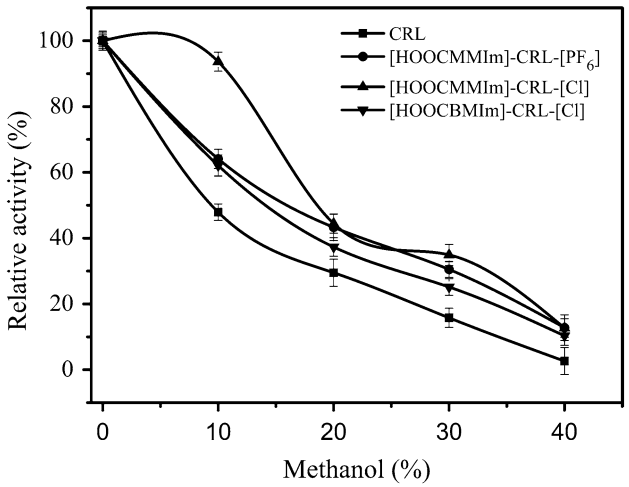


Fig. 4 Effect of DMSO on the activity of CRLs. Enzyme activity was determined in phosphate buffer (0.025 M) containing different amount of DMSO at suitable temperature and pH. The initial activity was defined as 100 % and all data in the figure were the averages of the triplicate of experiments



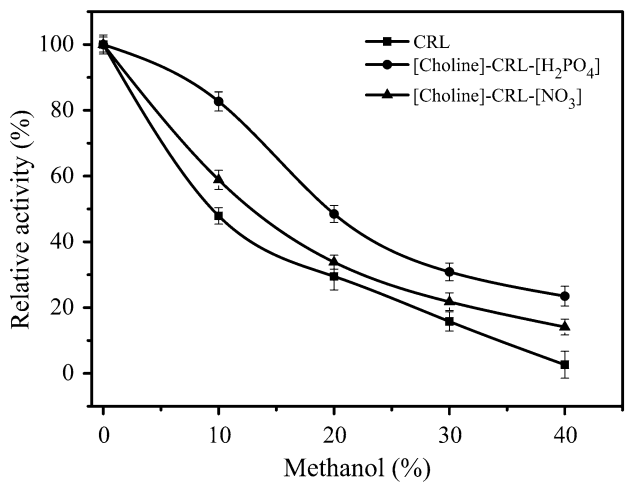


Fig. 5 Effect of methanol on the activity of CRLs. Enzyme activity was determined in phosphate buffer (0.025 M) containing different amount of methanol at suitable temperature and pH. The initial activity was defined as 100 % and all data in the figure were the averages of the triplicate of experiments

isooctane at their own suitable temperature and pH. As shown in Figs. 4, 5, 6, the modified CRLs showed better catalytic activity in organic solvents compared with the native one. In strong polar organic solvent (80 % dimethyl sulfoxide), enzymes with choline ionic liquids modification owned more than twofold activity compared with the native enzyme (Fig. 4). And in aqueous methanol, the activities of the lipases decreased, especially the native CRL, whereas the various modified forms showed better catalytic activity (Fig. 5). In aqueous isooctane (Fig. 6), the activity of the lipases all increased to some degree. In 80 % isooctane, the activities of the modified forms ranged from 98.8 to 122.7 %, whereas that of the native CRL retained 96.6 % of the initial activity under the same conditions. The difference in activity may have been a consequence of the altered conformation of the modified enzyme [30, 31].

Kinetic parameters

The kinetic constants (K_M and V_{max}) of the native and modified CRLs were determined. The values for all the lipases were calculated by using Lineweaver–Burk plots. The K_M value of the native CRL was 150.2 mg/ml, whereas the apparent K_M values of the modified CRLs ranged from 142.8 to 208.3 mg/ml, as was shown in Table 2. The V_{max} value of [choline]-CRL-[H_2PO_4] was 295.0 (μmol/min mg protein), which is noticeably higher than that of the native CRL. These results may be interpreted as follows: the modification changed the confor-

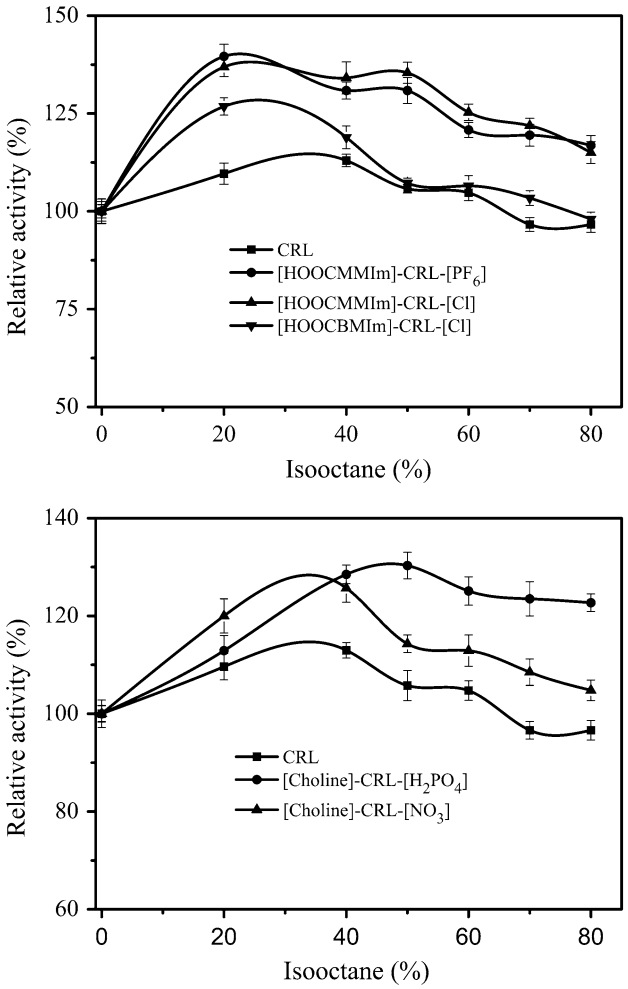


Fig. 6 Effect of isooctane on the activity of CRLs. Enzyme activity was determined in phosphate buffer (0.025 M) containing different amount of isooctane at suitable temperature and pH. The initial activity was defined as 100 % and all data in the figure were the averages of the triplicate of experiments

Table 2 Kinetic constants of native and modified CRLs

Samples	V_{max} (μmol/min mg protein)	K_M (mg/ml)
CRL	248.1 ± 8.5	150.2 ± 13.2
[HOOCMMIm]-CRL-[PF_6]	172.4 ± 9.1	166.8 ± 9.2
[HOOCMMIm]-CRL-[Cl]	178.9 ± 4.6	153.8 ± 7.3
[HOOCBMIm]-CRL-[Cl]	124.8 ± 7.3	208.3 ± 10.5
[Choline]-CRL-[H_2PO_4]	295.0 ± 5.4	142.8 ± 8.4
[Choline]-CRL-[NO_3]	211.4 ± 6.9	168.2 ± 6.8

Experiments were performed at different concentrations of oil emulsification solution from 40 to 240 mg/ml for 3 min. All data in the table were the averages of the triplicate of experiments. Error bars represent deviation from the mean for three separate experiments

mation of CRL, resulting in a greater probability for substrate-enzyme complex formation or higher accessibility for the substrate to the CRL active site.

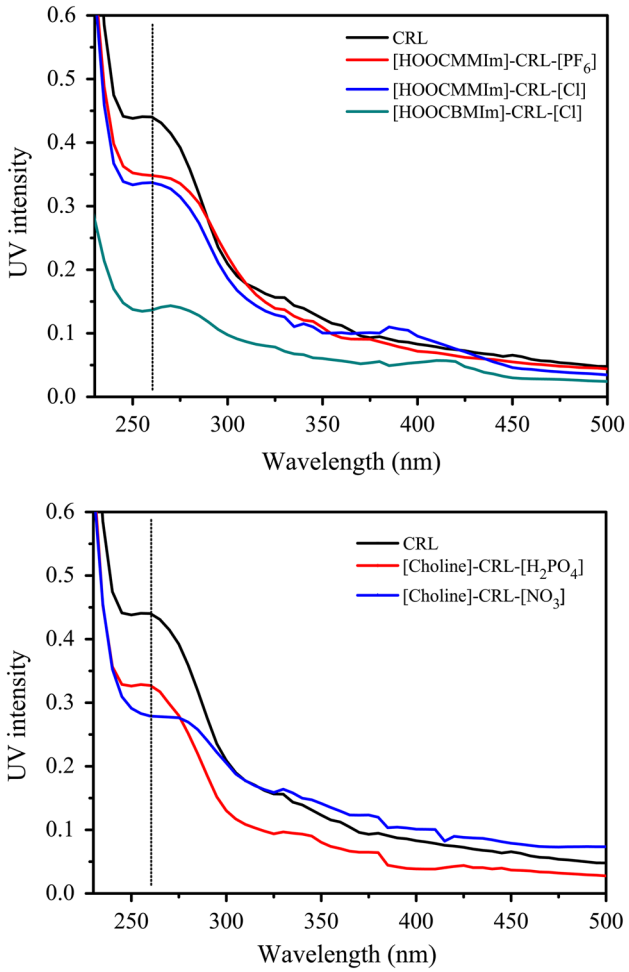


Fig. 7 The ultraviolet absorption spectra of native and modified CRLs

Characterization of native and modified CRLs

Ultraviolet spectroscopy

Protein ultraviolet absorption is mainly due to the electronic excitation of aromatic amino acids such as tryptophan and tyrosine. The absorption spectra of these chromophores could be changed with varying conditions. To explore the mechanism of the improvement of the enzymatic properties of CRL upon modification, the ultraviolet spectra of the native and modified CRLs were determined. As shown in Fig. 7, the absorption peak of CRL was approximately 260 nm, with an absorption intensity of 0.45. Compared with the native CRL, the modified forms showed a slight red shift in their ultraviolet peaks and their absorbance values decreased. Changes in the ultraviolet spectra of proteins in the 230 to 270 nm regions are related to transformations in conformation and a decrease in aromatic amino acid exposure [32]. The

present results indicated that the ionic liquid modification caused microenvironmental changes in the enzymes.

CD spectroscopy

CD measurements were performed to elucidate the secondary and tertiary structures of the native and modified CRLs in phosphate buffer (pH 7.0). As shown in Fig. 8, the native CRL had negative bands at 208 to 220 nm, which agreed with previous reports [30, 33]. However, a change was observed in the CD spectra of the modified lipases with respect to that of the native CRL, as could be attributed to transformations in both secondary and tertiary structures [34]. The percentages of the secondary structure elements were analyzed using Jwsse32 software (Table 3), the ratios of α-helical, β-sheet, and β-turn structures were altered after modification. The differences in secondary structures of the modified lipases in aqueous buffer may be a result from the changes in the enzymatic properties of the modified CRLs [35].

Fluorescence spectroscopy

A fluorescence emission from a 270 nm excitation was attributed to tryptophan residues. Thus, tryptophan fluorescence was used to probe structural changes of the modified enzyme compared with the native one. As shown in Fig. 9, the emission maximum at 310 nm of the native CRL did not change upon modification, but an increase was observed in the relative fluorescence intensity as the

Table 3 The percentage of secondary structure elements of native and modified CRLs

Sample	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
CRL	20.4	37.4	11.0	31.2
[HOOCMMIm]-CRL-[PF_6]	18.3	38.9	7.1	35.7
[HOOCMMIm]-CRL-[Cl]	28.5	5.4	17.3	48.8
[HOOCBMIm]-CRL-[Cl]	17.2	26.5	6.6	49.7
[Choline]-CRL-[H_2PO_4]	20.2	32.1	3.9	43.8
[Choline]-CRL-[NO_3]	15.4	36.3	5.0	43.3

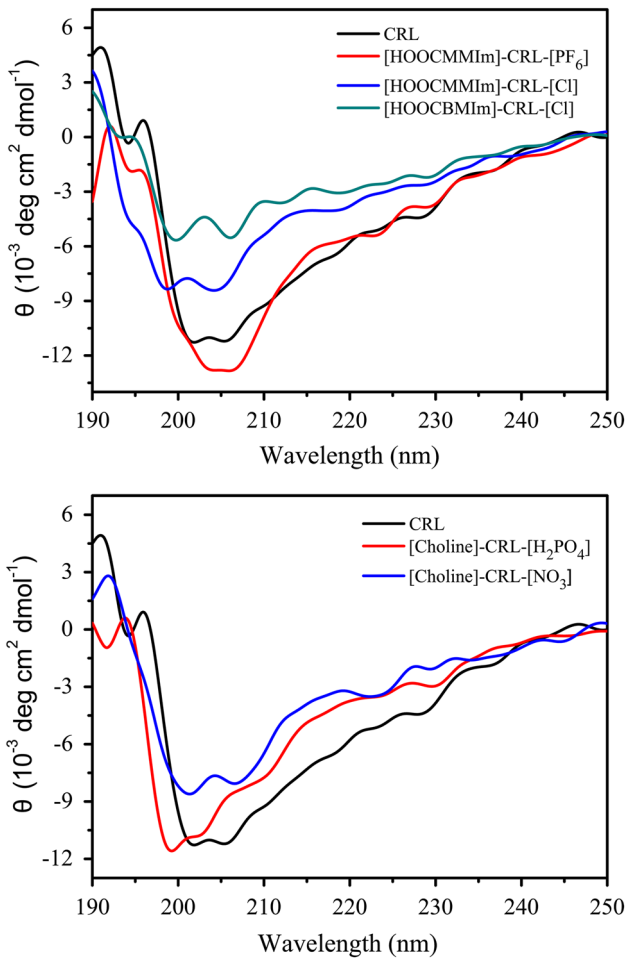


Fig. 8 CD spectra of native and modified CRLs

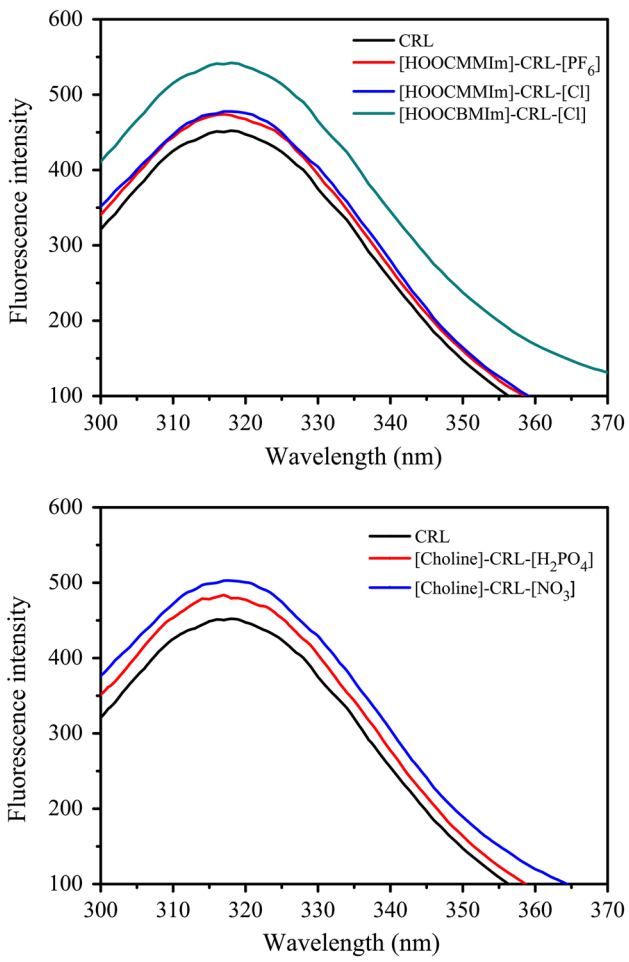


Fig. 9 Fluorescence spectra of native and modified CRLs

modification progressed. A previous study reported that the modified CRLs showed a more compact conformation [34]. Combining results from ultraviolet and CD spectroscopy studies, we believe that ionic liquid modification of CRL occurred, and the conformation of CRL was altered to some degree.

Conclusions

In this study, various functional ionic liquids with different cations and anions were grafted onto CRL through lysine coupling, resulting in different degrees of modification. The chemically modified CRLs exhibited improved thermal stability, catalytic activity in organic solvents and adaptability to temperature and pH changes. Modification with ionic liquid [choline][H_2PO_4] resulted in maximum improvement of CRL in terms of activity, as well as better thermal stability. Furthermore, the ultraviolet, CD, and fluorescence measurements demonstrated that the chemical modification caused change of enzyme conformation to different extent. In addition, in comparison to the previous modification of PPL, the results showed that different kinds of enzymes require different modifiers. Now we are trying to evaluate the mechanism of various catalytic performance improvements caused by different ionic liquids modification using the molecular simulation and new spectroscopy characterization technology.

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