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# pH Memory of Immobilized Lipase for (±)-Menthol Resolution in Ionic Liquid

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Magnetic DEAE-GMA-EDMA microspheres were prepared via suspension polymerization and used for the immobilization of *Candida rugosa* lipase by ion exchange. The effect of pH values on the immobilization of lipase was investigated. Resolution of (±)-menthol in the hydrophobic ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate was performed by immobilized lipase-catalyzed enantioselective esterification with propionic anhydride as acyl donor. The effects of pH condition at lipase immobilization on the conversion and enantioselectivity were investigated. As a result, pH memory of the immobilized lipase for catalyzing (±)-menthol resolution in the ionic liquid was observed. Better conversion and the best enantioselectivity were obtained with the immobilized lipase prepared at pH 5.0. Under the condition, (−)-menthyl propionate with enantiomeric excess of >90% was obtained. Moreover, the enantioselectivity of the immobilized lipase decreased gradually with increasing pH value.

**KEYWORDS: pH memory; ionic liquid; immobilized lipase**

## INTRODUCTION

As an alternative to traditional organic solvents, ionic liquids, a new class of green solvents, have attracted many investigations for applications in biocatalysis and biotransformations. Recent research findings have shown that many enzymes keep high activity in ionic liquids, even in an almost anhydrous environment (*1*–*3*). In nonaqueous enzymatic catalysis system, however, the macro-pH of the reaction media cannot be measured, nor can the micro-pH of essential water solution around the enzyme molecules. Therefore, control of the pH value of the reaction media in nonaqueous environments seems to be impossible. Zaks and Klibanov (*4*) dissolved lipase in buffers of different pH values, then lyophilized it, and thus prepared the lipase at different initial pH values. The lipase was then employed to catalyze the hydrolysis of tributyrin in organic solvents. They found that the hydrolysis activity of such lipase with "pH history" had noticeable correlation with the pH of the initial buffers. They introduced the concept for the first time that lipase had "pH memory" in organic solvents. Further research (*5*) on immobilized lipase was made, and the results demonstrated that immobilized lipase also had pH memory; that is, lipase would remember the pH of the final aqueous environment during its immobilization. Therefore, immobilizing lipase at the optimal pH value will benefit its activity in nonaqueous systems. Manohar et al. (*6*) studied porcine pancreas lipase-catalyzed esterification of anthranilic acid with methanol in organic solvents by an artificial neural network analysis. They found that adding a micro amount of phosphate buffer of pH 7.0 could increase the conversion yield of esterification and verified that lipase had pH memory in organic solvents. Up to now, studies of lipase's pH memory are still focused on the reaction system in organic solvents. Whether immobilized lipase also has pH memory in ionic liquids and how it takes effect on the activity and enantioselectivity of lipase have not been reported in the literature.

In this work, magnetic GMA-EDMA microspheres of about 5 $\mu$m were prepared by suspension polymerization and then modified to an anion exchanger with DEAE-Cl. *Candida rugosa* lipase was adsorbed on the magnetic carrier by ion exchange in buffers of different pH values. After lyophilization, the immobilized lipase would retain the pH history of the buffers and was used to esterify and resolve (±)-menthol in the hydrophobic ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim]$PF_6$). Here, the enzyme-catalyzed esterification of (±)-menthol in ionic liquid with propionic anhydride as acyl donor was measured. Especially, it is necessary to investigate the effect of pH on the lipase-catalyzed enantioselective esterification of (±)-menthol, which was not considered by previous researchers (*7*–*9*). Comparison of the activity and enantioselectivity between the free and immobilized lipase in ionic liquid was also performed. The effects of pH value on the activity of the two types of lipases and the capability of pH memory of the immobilized lipase in ionic liquid were studied. Thus, we can control the micro-pH of the essential water solution around the enzyme molecules in ionic liquids and further optimize the enzyme activity for enantioselectively esterifying (±)-menthol.

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## MATERIALS AND METHODS

**Materials.** (±)-Menthol, (−)-menthol, propionic anhydride, diethylaminoehtyl chloride (DEAE-Cl), Coomassie brilliant blue G-250, bovine serum albumin (BSA), and *C. rugosa* lipase (CRL, type VII) were obtained from Sigma (St. Louis, MO). Ethylene glycol dimethacrylate (EDMA) was obtained from Fluka (Buchs, Switzerland). $FeSO_4$, $FeCl_3$, glycidyl methacrylate (GMA), (2,2)-azoisobisbutyronitrile (AIBN), polyvinyl alcohol (PVA), dodecyl alcohol, sodium dodecyl sulfate (SDS), and all other chemicals were of analytical grade and obtained from local sources.

**Preparation of Magnetic DEAE-GMA-EDMA Microspheres.** $Fe_3O_4$ magnetic fluid of nanometer was prepared via chemical coprecipitation process (*10*). Magnetic poly(GMA-EDMA) microspheres were prepared via suspension polymerization (*11*). The organic phase contained magnetic fluid (1.00 g, wet weight), GMA (1.50 g), EDMA (2.10 g), dodecyl alcohol (0.40 g), and 0.30 g of AIBN. The aqueous continuous phase was composed of SDS (0.80 g) in 300 mL of 1% PVA solution. The polymerization reactor, a three-necked flack equipped with a mechanical stirrer, was hatched in a water bath and heated to 80 °C. The stirring speed was set to 100 rpm After 5 h of reaction, the acquired composite magnetic microspheres were collected by a permanent magnet and then were sequentially washed with deionized water, ethanol, and acetone. The product was reacted with $NaBH_4$ to convert the epoxy groups on the microspheres' surface into hydroxyl groups. The microspheres then were modified with DEAE-Cl. The thus obtained magnetic DEAE-GMA-EDMA microspheres had a mean diameter of 5 $\mu$m and could be used as the carrier of lipase immobilization.

**Immobilization of Lipase onto Magnetic DEAE-GMA-EDMA Microspheres.** Magnetic DEAE-GMA-EDMA microspheres of the same dry weight were washed with buffers (50 mmol/L) of different pH values and then suspended in the corresponding buffers, respectively. *C. rugosa* lipase powder was added to the suspension with a dry carrier-to-enzyme weight ratio of 1:1. The reaction was carried out at 25 °C with shaking at 150−170 rpm. At definite time intervals, the microspheres with immobilized lipase were collected by permanent magnet and washed twice by the same buffer solution, respectively. Then they were lyophilized and stored at −20 °C for future use.

The amount of lipase protein immobilized on the carrier was evaluated by determining the soluble protein content in the remaining solution according to the Bradford method (*12*). Activities of the immobilized lipase were determined with the method of olive oil hydrolysis (*13*). One unit of enzyme activity was defined as the amount of lipase that liberates 1 $\mu$mol of fatty acids per minute under the assay condition. The specific activity of immobilized lipase was calculated as the ratio of enzyme activity to the total amount of immobilized enzyme. Experiments were conducted in triplicate. The mean values are presented, and standard deviations are given as error bars in all figures (see below).

**Preparation of [Bmim]$PF_6$.** The hydrophobic ionic liquid [Bmim]$PF_6$ was prepared according to the procedure described by Huddleston (*14*). The ionic liquid was lyophilized for 24 h, and the water activity was 0.07 as assayed by Hygrolab (Rotronic, Switzerland).

**Esterification of Menthol.** A typical experiment was carried out as described in a previously published paper (*15*): 1.0 mmol of (±)-menthol and a certain amount of immobilized CRL (100 units) were added to 3 mL of the ionic liquid in a 10 mL screw-capped vial. The reaction was started after the addition of 1.0 mmol of propionic anhydride and run by shaking at 200 rpm at a temperature of 30 °C for 24 h. At different time intervals, aliquots were taken and analyzed by gas chromatography (GC) after extraction and dilution with *n*-hexane. Immobilized lipase was separated by permanent magnet when the reaction finished and could be reused.

The enantiomeric excess (ee%) and the conversion of menthol (*c*) were based on the GC analyses and calculated by the equations as used previously (*16*).

**GC Analysis.** The GC analysis was performed as described previously (*15*) with an Agilent 6890N (Agilent Technologies, Wilmington, DE) equipped with a splitless/split injector, a flame ionization detector, and a CYCLOSIL-B capillary column (0.25 $\mu$m film thickness, 30 m length, 0.25 mm i.d.). The injector and detector were set at 200 and 250 °C, respectively, and nitrogen was used as the carrier gas. The oven temperature was kept at 90 °C for 10 min, programmed to increase from 90 to 150 °C at 2 °C/min, then increased to 165 at 5 °C /min, and finally kept at 165 °C for 5 min. Chromatographic data were acquired and analyzed using the Agilent Chemical Station. The retention times were 30.5 and 31.5 min for (−)- and (+)-menthyl propionate, respectively, and 26.3 min for (±)-menthol.

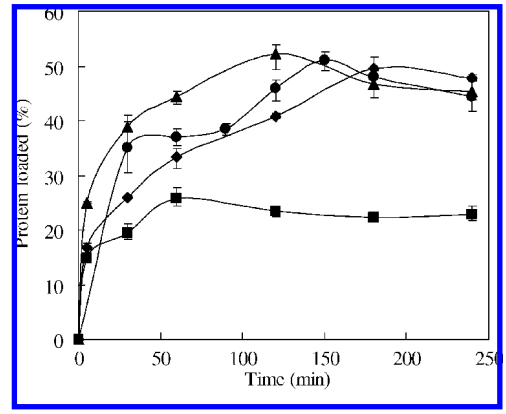


**Figure 1.** Kinetics of the ion exchange immobilization of *Candida rugosa* lipase at different pH values (■, pH 5.0; ◆, pH 6.0; ●, pH 7.0; ▲, pH 8.0).

## RESULTS AND DISCUSSION

**Effect of pH Value on the Adsorption of Immobilized Lipase.** The effect of pH value on the adsorption of free lipase was investigated by immobilizing lipase in buffers of different pH. **Figure 1** shows the adsorption kinetics in buffers of pH 5.0, 6.0, 7.0, and 8.0. It can be seen that pH values affect the adsorption of lipase greatly. The amount of lipase immobilized on the carrier at pH 8.0 was about twice that at pH 5.0. On the other hand, the time point when the highest adsorption amount is reached varies with pH value. For example, at pH 8.0, the loading capacity reaches the maximum at 120 min, whereas at pH 5.0 the time is 60 min.

Some papers (*17–19*) have reported that although commercial *C. rugosa* lipase (type VII, Sigma) had been rid of most impurity proteins, it was still composed of some isoenzymes presenting different catalysis behaviors, such as enantioselectivity. In many resolution reactions (*17–19*), the enantioselectivity of these isoenzymes was distinctive, even opposite. Two components in commercial CRL were separated by ion exchange chromatography, namely, CRLA and CRLB. As analyzed on polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE), CRLA and CRLB are both in the same molecular weight range of 62−64 kDa. On isoelectric focusing, CRLA showed a single band corresponding to an isoelectric point (p*I*) of 5.6 and CRLB was resolved in two bands having p*I* values around 4.2 (*17*). At pH 5.0, only CRLB is negatively charged and can be adsorbed onto DEAE-functionalized anion exchanger. Thus, under this condition, the carrier adsorbed only CRLB and the lipase loaded on the carrier is the lowest. As the pH is increased, more proteins are negatively charged, so the adsorption capacity increases correspondingly. These adsorption curves also give the optimal times of enzyme loading at different pH values. The optimum immobilization time is shortest at pH 5.0 because less protein is immobilized at this condition.

**Effect of pH on the Activity of Immobilized Lipase.** The activity and specific activity of the immobilized lipases prepared at different pH values were investigated to examine the effect

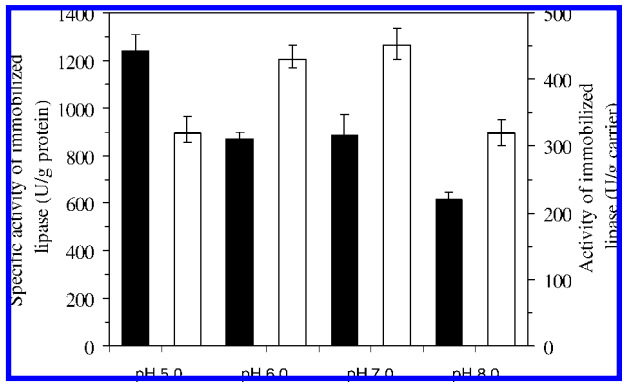


**Figure 2.** Effect of pH on the specific activity (solid bars) and activity (open bars) of the immobilized lipase.

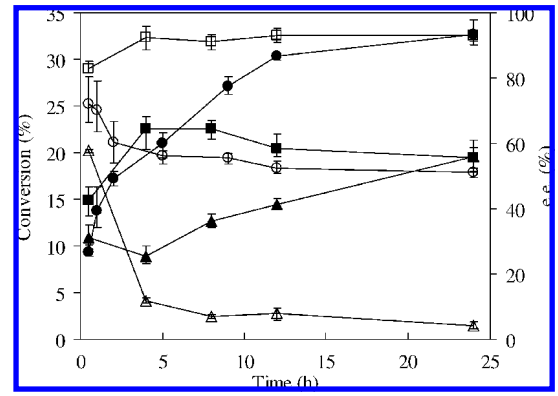


**Figure 3.** Effect of immobilization pH on the conversion of menthol and enantiomeric excess. Menthol conversion: ■, pH 5.0; ●, pH 7.0; ▲, pH 8.0. Enantiomeric excess (ee%): □, pH 5.0; ○, pH 7.0; △, pH 8.0.

of pH on the biocatalysis activity. The results are shown in **Figure 2**. It can be seen from the figure that the pH value shows dramatic impacts on activity and specific activity of the immobilized lipase, especially on the latter. The highest activity, 452 units/g of carrier, appears at pH 7.0. At pH 5.0 and 8.0, the activity was relatively lower (about 320 units/g of carrier). As for the specific activity, the highest value (1240.31 units/g of protein) appears at pH 5.0, whereas the lowest one, 614.20 units/g of protein, appears at pH 8.0. The results demonstrate that at different pH environments, higher enzyme adsorption does not mean higher catalytic activity (see also **Figure 1**). Such results might be attributed to the fact that pH 8.0 is much higher than the p*I* of the lipase, resulting in the change of the secondary structure and tertiary structure as well as the conformation of active center in such a basic environment. Thus, the activity and specific activity of the lipase decrease at pH 8.0. On the contrary, in an acidic environment of pH 5.0, although the enzyme loading capacity is the lowest, selective adsorption of CRLB and the intact natural structure of lipase result in the highest specific activity. These results are in agreement with the finding that CRL has a higher activity in neutral or weak acid environment (*20*). On the other hand, most impurity proteins in the commercial CRL have p*I* > 5.0, so the isoenzymes and impurities are most adsorbed on the carrier at pH 6.0−8.0, which decreases the specific activity of the immobilized lipase.

**pH Memory of the Immobilized Lipase in Ionic Liquid.** Resolution of (±)-menthol was carried out in the hydrophobic ionic liquid [Bmim]$PF_6$ by employing the immobilized lipase with different pH histories (pH 5.0, 7.0, and 8.0). The results are shown in **Figure 3**. The results indicate that the conversion of menthol is highest at the neutral condition (pH 7.0), whereas it is lowest at the basic condition (pH 8.0). This is consistent with the results discussed above. Moreover, pH also presents a significant effect on the enantiomeric excess. Lipase immobilized at pH 5.0 gives the best result of resolving the enantiomers. The enantiomeric excess decreased drastically with increasing pH at the reaction time of 24 h. These results demonstrate that the lipase has a pH memory effect in ionic liquid.

Previous studies (*20, 21*) have suggested the optimal pH values at which CRL displayed the best activity of esterification and transesterification in nonaqueous media. Although the optimum pH value herein is not identical with that of previous work (*17, 18*) because of the different reaction system and method of assay, the results in this research agree with the widely recognized phenomenon that CRL shows higher activity in neutral and weak acid environments. Furthermore, the enantioselectivity of lipase was reduced with increasing pH value, as illustrated in this work, which is coincident with that reported previously (*22*).

In many resolution reactions, the enantioselectivity of isoenzymes of lipase is greatly different, or even opposite (*18, 19*). In our research, the immobilized lipase with pH 5.0 history is mostly CRLB, which conformation may be more suitable for the stereoselective resolution of certain menthol enantiomer in ionic liquid. Given that the mechanism and regularity of the medium's impact on lipase enantioselectivity are unclear, further research is necessary.

The effect of pH on the ionization of lipase determines its conformation (*13*). At a certain pH condition, the ionic groups around the active center of the lipase molecule achieve the optimum ionic state for lipase-catalyzed reactions, which is essential to its activity and enantioselectivity (*23*). In traditional aqueous system, the pH value of lipase-catalyzed hydrolysis reaction is adjusted with bulk buffers. In a nonaqueous reaction system, however, water activity has a significant influence on the activity, and water content must be under rigorous control. Hence, it is impossible to control the pH of the reaction by adding buffers. The hydrophobic ionic liquid [Bmim]$PF_6$, which is a liquid salt at vicinal room temperature (from −30 to 50 °C), is also sensitive to the water activity as a medium of lipase-catalyzed reaction. The *C. rugosa* lipase has activity and enantioselectivity only at very low water content (*24*). Therefore, we made immobilized lipase with pH history during the preparation procedure. Such an immobilized lipase with pH history was used to resolve (±)-menthol in the ionic liquid [Bmim]$PF_6$ (water activity $a_w$ = 0.07). Experimental results verified that essential water around the lipase molecules remained at the immobilization pH when it was used in the ionic liquid reaction system.

Zaks and Klibanov (*4, 5*) had reported that free and immobilized lipases both had pH memory effects in organic solvents after lyophilization. Here, we found that in a novel green nonaqueous solvent–ionic liquid, immobilized CRL also had pH memory after lyophilization, and a new method was supplied to prepare immobilized lipase with pH memory. Via a simple and easily performed selective adsorption, we are able to combine the immobilization with the purification. This method is suitable to separate isoenzymes of slight structure difference and improves the catalytic activity of enzymes. Especially, the control and adjustment of pH were realized in biocatalysis with ionic liquid as a reaction medium, which is advantageous in the production of some food additives via enzyme technology.

## LITERATURE CITED


(1) Yang, Z.; Pan, W. Ionic liquids: green solvents for nonaqueous biocatalysis. *Enzyme Microb. Technol.* **2005**, *37*, 19–28.

(2) Zhang, D.-H.; Bai, S.; Sun, Y. Lipase-catalyzed regioselective synthesis of monoester of pyridoxine (vitamin $B_6$) in acetonitrile. *Food Chem.* **2007**, *102*, 1012–1019.

(3) Zhang, D.-H.; Bai, S.; Dong, X.-Y; Sun, Y. Optimization of lipase-catalyzed regioselective acylation of pyridoxine (vitamin $B_6$). *J. Agric. Food Chem.* **2007**, *55*, 4526–4531.

(4) Zaks, A.; Klibanov, A. M. Enzymatic-catalysis processes in organic solvents. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3192–3196.

(5) Zaks, A.; Klibanov, A. M. Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.* **1988**, *263*, 3194–3201.

(6) Manohar, B.; Divakar, S. An artificial neural network analysis of porcine pancreas lipase catalysed esterification of anthranilic acid with methanol. *Process Biochem.* **2005**, *40*, 3372–3376.

(7) Othman, S. S.; Basri, M.; Hussein, M. Z.; Rahman, M. B. A.; Rahman, R. N. Z. A.; Salleh, A. B.; Jasmani, H. Production of highly enantio selective (−)-menthyl butyrate using *Candida rugosa* lipase immobilized on epoxy-activated supports. *Food Chem.* **2008**, *106*, 437–443.

(8) Shih, I. L.; Hung, S. H.; Chen, F. Y.; Ju, H.-Y.; Shieh, C.-J. Optimized synthesis of lipase-catalyzed L-menthyl butyrate by *Candida rugosa* lipase. *Food Chem.* **2007**, *100*, 1223–1228.

(9) Kobayashi, T.; Nagao, T.; Watanabe, Y.; Yamauchi-Sato, Y.; Negishi, S.; Shimada, Y. Enrichment of CLA isomers by selective esterification with L-menthol using *Candida rugosa* lipase. *J. Am. Oil Chem. Soc.* **2006**, *83*, 93–99.

(10) Xue, B.; Tong, X. D.; Sun, Y. Characterization of PVA-based magnetic affinity support for protein adsorption. *Sep. Sci. Technol.* **2001**, *36*, 2449–2461.

(11) Lu, M.; Bai, S.; Yang, K.; Sun, Y. Synthesis and characterization of magnetic polymer microspheres with a core-shell structure. *China Articuol.* **2007**, *5*, 180–185.

(12) Bradford, M. M. A rapid and sensitive method for the quantition of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(13) Zhang, D.-H.; Guo, Z.; Dong, X. Y.; Sun, Y. Characterization of lipase in reversed micelles formulated with cibacron bule F-3GA modified Span 85. *Biotechnol. Prog.* **2007**, *23*, 108–115.

(14) Huddleston, J. G.; Willauer, H. D.; Swatloki, R. P. Room temperature ionic liquids as novel media for 'clean' liquid−liquid extraction. *J. Chem. Soc., Chem. Comm.* **1998**, 1765–1766.

(15) Yuan, Y.; Bai, S.; Sun, Y. Comparison of lipase-catalyzed enantioselective esterification of (±)-menthol in ionic liquids and organic solvents. *Food Chem.* **2006**, *97*, 324–330.

(16) Bai, S.; Guo, Z.; Liu, W.; Sun, Y. Resolution of (±)-menthol by immobilized *Candida rugosa* lipase on superparamagnetic nanoparticles. *Food Chem.* **2006**, *96*, 1–7.

(17) Xin, J.-Y.; Xu, Y.; Hu, X.-X.; Wang, L.-L.; Li, S.-B.; Xia, C. Selective immobilization of *Candida rugosa* lipase isoenzyme. *Ion Exchange Adsorption (Chinese Ed.)* **2002**, *18* (1), 17–22.

(18) Lundell, K.; Raijola, T.; Kanerva, L. T. Enantioselectivity of *Pseudomonas cepacia* and *Candida rugosa* lipases for the resolution of secondary alcohols: the effect of *Candida rugosa* isoenzymes. *Enzyme Microb. Technol.* **1998**, *22*, 86–93.

(19) Colton, I. J.; Ahmed, S. N.; Kazlauskas, R. J. Isopropanol Treatment increases enantioselectivity of *Candida rugosa* lipase toward carboxylic acid esters. *J. Org. Chem.* **1995**, *60*, 212–217.

(20) Pronk, W.; Boswinkel, G.; Riet, K. Parameters influencing hydrolysis kinetics of lipase in a hydrophilic membrane bioreactor. *Enzyme Microb. Technol.* **1992**, *14*, 214–220.

(21) Wang, Y.-J.; Sheu, J.-Y.; Wang, F.-F.; Shaw, J.-F. Lipase catalyse oil hydrolysis in the absence of added emulsifier. *Biotechnol. Bioeng.* **1988**, *31*, 628–633.

(22) Chang, R.-C.; Chou, S.-J.; Shaw, J.-F. Multiple forms and functions of *Candida rugosa* lipase. *Biotechnol. Appl. Biochem.* **1994**, *19*, 93–97.

(23) Saiz-Díaz, C. I.; Wohlfahrt, G. ; Nogoceke, E.; Hernández-Laguna, A.; Smeyers, Y. G.; Menge, U. Molecular structure and conformational analysis of chiral alcohols application to the enantioselectivity study of lipases. *Mol. Struct. (THEOCHEM)* **1997**, *390*, 225–237.

(24) Kitaoka, S.; Nobuoka, K.; Ishikawa, Y. Ionic liquids for tetraarylporphyrin preparation. *Tetrahedron* **2005**, *61*, 7678–7685.


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