Journal of Molecular Catalysis B: Enzymatic 57 (2009) 96–103

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Enhancement of the activity and enantioselectivity of lipase in organic systems by immobilization onto low-cost support

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ARTICLE INFO

Article history:
Received 19 April 2008
Received in revised form 11 July 2008
Accepted 12 July 2008
Available online 23 July 2008

Keywords:
Enzyme-aggregate coating
Surface modification
Immobilization
Interfacial adsorption
Lipase

ABSTRACT

Lipase from *Arthrobacter* sp. was immobilized onto low-cost diatomite materials using different protocols for the resolution of 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one (HMPC) by asymmetric acylation. The support surface was grafted various functional groups including methacryloxypropyl, vinyl, octyl, dodecyl and γ-(aminopropyl)-glutaraldehyde. These modifications resulted in various mechanisms during the immobilization and thus introduced different characteristics to the prepared lipases. The interfacially adsorbed lipase onto dodecyl-modified support exhibited both higher activity and stability among these immobilized preparations. The modified enzyme-aggregate coating method was performed based on interfacial adsorption in our work, and the characteristics of this immobilized lipase were investigated and compared with those by cross-linking and interfacial adsorption methods. It was shown that the enzyme-aggregate coated lipase yielded the highest activity with a recovered activity of 8.5-fold of the free enzyme, and the highest operational stability with 85% of initial activity remained after 10 recycles. Excellent enantioselectivity ($E \geq 400$, with e.e. = 99% of *S*-HMPC) was obtained for most lipase preparations in our paper ($E = 85$ for the free enzyme).

1. Introduction

Lipases (glycerol ester hydrolase, E.C. 3.1.1.3) are very likely the most used enzymes in organic chemistry because of their combination of a broad range of substrate specificity with a high regio- and enantio-selectivity in many reactions [1]. They can catalyze esterification, hydrolysis and transesterification reactions to produce many useful chemical products including alcohols [2], esters [3], acids [4], amine [5] and β-hydroxy selenides [6] under mild reaction conditions. In industrial applications, lipases are mostly used in their immobilized states owing to many preferably advantages that facilitate the scaling up of the process, e.g. simple recovery of catalyst and products, ready reutilization of the catalyst, possibility of continuous operation, and easy operation and design of the bioreactor. The characteristics of supports and immobilization strategy have been generally considered the most important factors concerning the properties of the enzyme preparations. Both organic polymers and inorganic materials are often selected as support materials [7–11]. The former ones, such as epoxy [7] and PVA [8] functionalized polymers, are accepted for the rich functional surface groups which can link enzymes onto the support, while the later ones, such as silica gels [9], porous ceramics [10] or diatomite [11], are known to be thermally and mechanically stable, non-toxic, and highly resistant against microbe and organic solvents. In cases of the preparations of relative low valuable products, many applications of immobilized biocatalysts become bottleneck in their cost for further exploitation, which might be mainly associated with the usage of costly materials. On this point the use of inexpensive materials has been proposed as an efficient alternative. Many immobilization techniques for lipases have been developed over the past 30 years. They can be classified into four kinds: adsorption onto polymer-based or inorganic materials [12]; encapsulation [13]; covalent attachment to carriers [14] and cross-linking using for example glutaraldehyde [15]. Among these methods adsorption has been usually received for its simple process and high activity yield. For lipase, adsorption onto hydrophobic supports has been widely reported in these years for its special characteristics, that is, it can be hyperactivated at hydrophobic interfaces, which is called 'interfacial activation' mechanism [16]. However, the adsorbed enzymes usually exhibit poor reutilization owing to the weak interactions between enzymes and the supports, and thus inhibit their industrial applications. In recent years a novel immobilization method of enzyme-aggregate coating [17] has been developed based on the concept of 'cross-linking of enzyme aggregates' (CLEA) [18]. This method involves the following steps: the 'seed' enzyme molecules are covalently attached onto the support,

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doi:10.1016/j.molcatb.2008.07.003

then additional enzyme molecules and their aggregates in solution are cross-linked by glutaraldehyde onto the 'seed' molecules. By this way, higher enzyme loading can be achieved and hence result in increased overall activity and even higher stability of the immobilized biocatalyst.

Racemic compound of 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one (HMPC) [19] has been widely used as the alcoholic moiety of allethrin, a member of synthetic pyrethroids which are a group of esters with splendid insecticidal activities and low toxicity to mammals [20]. However, tested with structure–activity relationship, allethrin of *S*-HMPC moiety presents higher activity than that of *R*-form by fourfold. Therefore, efficient methods for the preparation of optically pure *S*-HMPC is essential for the improvement of the insecticidal activity and the decrease of environmental contaminations. Many efforts have been developed to prepare *S*-HMPC, e.g. Danda et al. [21] prepared the *S*-HMPC product with an e.e. value of 78% and yield of 80% by combination with the enzymatic hydrolysis and stereochemical inversion; Wu et al. [22] reported a kind of surfactant-modified lipase (*Pseudomonas* sp. lipase) for the asymmetric acylation of HMPC and realized a high e.e. value of the *S*-form nearly 100%. This modified lipase possessed a high V_{max} value as much as 160 times and a similar K_m value to that of native lipase. However, no study engaging in investigating the stabilities of the enzyme was reported therein. Asymmetric acylation of *rac*-HMPC was adopted with lipase as the biocatalyst and vinyl acetate as both the acyl donor and organic solvent in our experiment (Scheme 1). The reaction turned out to be irreversible because enols was released from vinyl acetate and automatically tautomerized to carbonyl compounds. This methodology has been established as a standard method on the enzymatic preparation of enantiomerically pure alcohols and their acetates from chiral secondary or prochiral primary alcohols [23].

In this paper, we applied the inorganic matrix of cheap diatomite as the support to immobilize lipase from *Arthrobacter* sp. Various immobilization methods including enzyme-aggregate coating, interfacial adsorption, and cross-linking were employed to enhance the activity, enantioselectivity and stability of the enzyme. The catalytic characteristics of immobilized lipases by different methods were assessed in the asymmetric acylation of HMPC.

2. Materials and methods

2.1. Materials

Lipase from *Arthrobacter* sp. called as KM in this paper was a gift from Institute of Microbiology, Chinese Academy of China (IMCAC) and has been deposited in China Center of Industrial Culture Collection (CICC No. 10193). The lipase activity was 5000 U/mg powder using tributyrin as substrate, and one unit of enzyme corresponds to hydrolysis of 1 μmol of tributyrin per minute. Bovine serum albumin (BSA) and all the silane reagent including (methacryloxypropyl)trimethoxy-, octyltrimethoxy-, dodecyltrimethoxy-, vinyltrimethoxy- and γ-(aminopropyl)triethoxy-silane were purchased from Sigma. Diatomite with an average diameter of 1.0 mm came from Shanghai Fengcheng Reagent Factory. *rac*-HMPC was gifted by Changzhou Kangmei Chemical Industry Co., Ltd. (purity > 98% by gas chromatograph). All other chemicals were of analytical grade and commercial available.

2.2. Hydroxylation of diatomite

The hydroxylation process of the support surfaces was carried out as follows: the supports were first washed with distilled water twice and then treated by a mixture of $HCl/H_2O_2/H_2O$ with a volumetric ratio of 35/35/175 at 80 °C for 10 min. Thereafter the supports were washed with distilled water several times and successively rinsed in ethanol. Finally, the supports were dried in an air oven at 110 °C for 8 h before used. After the process the hydrophilic surface of the support was obtained.

2.3. Functionalization of diatomite granules

2.3.1. Silanization of supports

Diatomite derivatives with various functional groups were obtained by silanization treatment with a procedure described as follows: the hydroxylized supports were rinsed twice in ethanol, three times in toluene and then treated with silane coupling agents (0.14 mmol/g support) in toluene at room temperature for 60 min. The silane coupling agents used in our study included (methacryloxypropyl)trimethoxy-, octyltrimethoxy-, dodecyltrimethoxy-, vinyltrimethoxy- and γ-(aminopropyl)triethoxy-silane. After silanization the supports were rinsed three times in toluene, twice in ethanol and successively dried in air oven at 110 °C for 8 h before used.

2.3.2. Glutaraldehyde-activated support

Glutaraldehyde-activated support was prepared as follows: 1.0 g of support grafted aminopropyl group was soaked in 10 ml of 5% aqueous glutaraldehyde solution for 2 h. Then the support was rinsed three times in distilled water, once in ethanol and finally dried in air oven at 110 °C for 8 h before used.

2.4. Immobilization of lipase

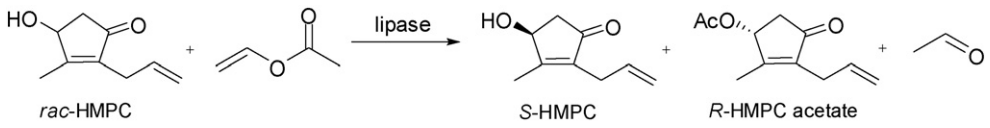
The enzyme-aggregate coating derivatives were prepared as follows: a little amount of 0.02 g of lipase was dissolved in 4 ml of sodium phosphate buffer (pH 7.0, 0.03 M) and centrifuged at 1000 rpm for 6 min to remove the insolubles. Then the supernatant was added to amount of functionalized diatomites, which were prewetted with ethanol if the surface was hydrophobic and rinsed with the same buffer twice. The mixture was incubated at room temperature and 150 rpm for 4 h, and then kept at 4 °C overnight. Afterwards 0.4 ml of 1% glutaraldehyde solution was added and the mixture was treated for 2 h. Finally, the solids were filtered out, washed several times with Tris–HCl buffer (pH 9.0, 0.03 M) till no traces of enzyme protein were found in the rinse solutions. After that, the solids were dried overnight over sillica gel under vacuum.

The adsorbed derivatives were prepared in the same procedure as the enzyme-aggregate coating method, but no glutaraldehyde was added for the treatment.

The covalently attached derivatives were prepared with the same mixture in enzyme-aggregate coating method, and the mixture was incubated at room temperature and 150 rpm for 4 h, and then kept in 4 °C overnight. After that, the solids were filtered and treated with 0.4 ml of 1% glutaraldehyde solution for 2 h. Finally, the solids were filtered out, and treated in the same procedure as the enzyme-aggregate coating method.

2.5. Protein assay

Protein was determined according to Bradford's method [24] using bovine serum albumin (BSA) as a standard. The amount of bound protein was determined indirectly by comparing the difference between the amount of protein introduced into the supports and the amount of protein both in the filtrate and in the washing solutions after immobilization.



Scheme 1. Kinetic resolution of HMPC with lipase by asymmetric acylation.

2.6. Assay of lipase activity and enantioselectivity

The asymmetric acylation of HMPC was used to determine the activity and enantioselectivity of the lipase derivatives. All the commercial organic solvents and *rac*-HMPC were dehydrated with anhydrous $CaCl_2$ at room temperature for 2–3 days before used. Both the free and immobilized lipases were dried in silica gel for 2 days and then stored in closed vessels over freshly reactivated 4 Å molecular sieves at 4 °C for 2 days before used. In a general experiment, a mixture of 3 ml vinyl acetate containing 1.8 mmol HMPC was placed into a 5 ml screw-capped vial. Then the reaction was started by adding 60 mg of free lipase or 80 mg of immobilized lipase and carried out at 30 °C and 220 rpm. Aliquots were withdrawn from the reaction mixture at different reacting intervals, and analyzed by gas chromatography (carrier gas: N_2 at a flow rate 50 ml/min; column: 120 °C, FID detector: 230 °C, injector: 230 °C) equipped with a chiral column (WCOT fused silica column with CP Chirasil Dex CB, 25 m × 0.32 mm). The retention times were 10.6 min (*S*-HMPC), 11.1 min (*R*-HMPC), 7.4 min (*S*-HMPC acetate) and 7.0 min (*R*-HMPC acetate), respectively. The conversion (*C*) of substrate was calculated as follows: $C = \text{e.e.}_s/(\text{e.e.}_s + \text{e.e.}_p)$, where e.e._s and e.e._p denoted enantiomeric excess of substrate and enantiomeric excess of product, respectively. All the activity data of free or immobilized lipases were determined at <5% conversion and one unit (U, $\mu\text{mol}\,g^{-1}\,min^{-1}$) of activity was defined as 1 μmol of HMPC acetate produced by the catalysis of 1 g of free or immobilized lipase in 1 min under the assay conditions. Enantioselectivity was expressed as *E* value and calculated as follows: $E = \ln[1 - C(1 + \text{e.e.}_p)]/\ln[1 - C(1 - \text{e.e.}_p)]$ [25].

2.7. Operational stability

A batch-wise fashion was adopted to investigate the operational stability of the immobilized lipase. The activity was determined according to Section 2.6. Each reaction was terminated at approx. 50% conversation. Then the immobilized lipase was recovered by filtration, washed by 3 × 5 ml of vinyl acetate and subsequently used in the next reaction.

2.8. Thermal stability

The thermal stability of the free or immobilized lipase was determined as follows: lipases were incubated in 3 ml of pure vinyl acetate at different temperatures from 30 to 50 °C for 10 h. After incubation, the solvent was removed carefully, and the fresh reaction solution which was incubated at 30 °C for 10 min was added. The residual activity was determined under the same reaction conditions in Section 2.6. The activity of the enzyme without incubation was considered as the control (100%).

2.9. Storage stability

The immobilized lipase was stored at 4 °C in refrigerator and the storage stability of enzymes was determined by measurement of the activity of samples taken at regular time intervals.

3. Results and discussion

3.1. Effect of surface modification on immobilization

The nature of the support surface is crucial to the properties of the lipase derivatives [26]. In this experiment different functional groups, including methacryloxypropyl, octyl, dodecyl, vinyl and aminopropyl groups, were grafted onto the surface of the diatomite. These support derivatives were utilized to immobilize lipase KM, and the activity and enantioselectivity of lipases were investigated with the results displayed in Table 1.

All the immobilized enzymes still preferred the *R*-form of HMPC in the asymmetric acylation as the free lipase. It was exciting to see that all the prepared lipases clearly improved the enantioselectivity towards HMPC compared with the free enzyme. The reasons for improvement of enantioselectivity by immobilization was not clear until now, however, some literature [27,28] reported that this phenomenon might be attributed to some distortion on the protein conformation, reducing the overall flexibility of the enzyme molecules generated from the interactions between enzyme and the supports during the immobilization. The lipases immobilized on the supports suffering organic functional group

Table 1
Immobilization of lipase on various support derivatives

Entry	Lipase derivatives[a]	Bound protein (mg/g immobilized lipase)	Bound protein (%)	Total activity[b] (U)	Specific activity (×10^5 U/g protein)	Activity yield (fold)	*E*
1	Diatomite-KM	0.21	38	2.9	0.17	1.7	255
2	Methacryl-KM	0.32	57	29.2	1.14	11.4	≥400
3	Vinyl-KM	0.24	43	9.8	0.51	5.1	≥400
4	Octyl-KM	0.36	65	17.0	0.58	5.8	≥400
5	Dodecyl-KM	0.43	76	24.0	0.70	7.0	≥400
6	Aminopropyl-KM	0.49	65	7.4	0.25	2.5	≥400
7	Free KM	–	–	3.7	0.10	–	85

The lipase loading was 0.02 g lipase/0.6 g aminopropyl glutaraldehyde-activated support and 0.02 g lipase/0.8 g support for all the other support derivatives. Other immobilization conditions were described in Section 2.4.

[a] The amount of silanizing agent was 0.14 mmol/g support for every kind of support derivatives.

[b] The reaction conditions were described in Section 2.6 and the activity of the free KM enzyme was 187 U. For the immobilized derivatives total activity meant the sum of the activity of all the prepared lipases obtained at the end of the immobilized process, while for the free KM enzyme, total activity meant the sum of the activity of soluble lipases offered to the supports before the immobilized process.

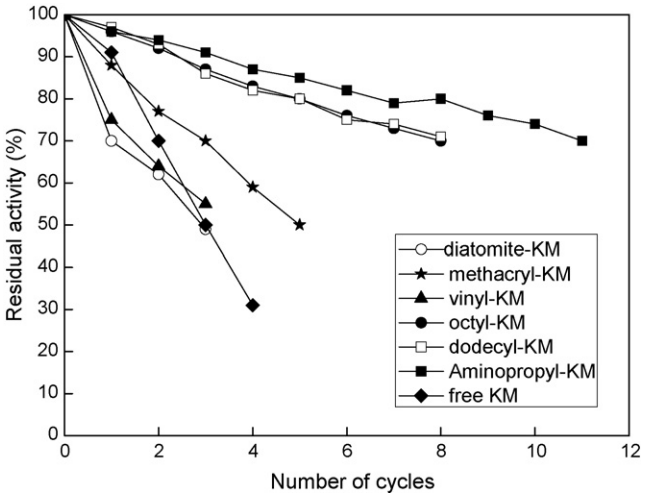


Fig. 1. The operational stability of immobilized lipases on different support derivatives. All the conditions were the same with those in Table 1.

modification (entry 2–6) exhibited much higher values of specific activity than the free enzyme by 2.5–11.2-fold, while on the crude diatomite (entry 1) the specific activity of enzyme was only improved by 1.7-fold. Therefore it was evidenced that the improved activity of enzymes should be ascribed to not only the dispersion of lipase on the support but also the interactions between the enzyme and the functional groups. Lipases have a unique characteristic called 'interfacial activation', that lipases can be hyperactivated at hydrophobic interfaces like oil drops by removing the 'lid' covering their active sites and making them more accessible to substrates [16]. The same action might also occur when the lipases were absorbed onto the methacryl-, vinyl-, octyl- and dodecyl-modified support derivatives [29]. From the data in Table 1, the activities of lipases on the support derivatives followed the order: methacryl-KM > dodecyl-KM > octyl-KM, which seemed that the high activity of lipase was not only preferred by long spacing arm of support derivatives but also surface characteristics of the alkoxycarbonyl group. It should be mentioned that the lipase immobilized on aminopropyl glutaraldehyde-activated support, i.e. aminopropyl-KM (entry 6), was covalently attached onto the support by forming schiff base between amine groups in the enzyme and aldehyde groups of the support, and the specific activity of aminopropyl-KM exhibited 2.5-fold of that of the free lipase, but lower than that of the immobilized preparation by interfacial adsorption.

3.2. Operational stability of immobilized lipases on different support derivatives

Apart from the enantioselectivity and activity, the operational stability was also a significant criterion for the immobilized lipase, especially in industrial applications. Hence, we investigated the operational stabilities of the lipase preparations immobilized on different support derivatives, and the results were presented in Fig. 1. Among all the prepared lipases in our experiments, aminopropyl-KM which was immobilized by covalent attachment exhibited the most stable operational stability with 70% of initial activity remained after 11 recycles. Both dodecyl- and octyl-KM kept 70% of initial activity after 8 recycles. Unfortunately, methacryl-KM lost 50% of initial activity after 5 recycles. The reasons why methacryl-KM presented higher activity but lower operational stability are attractive to us and the attempts

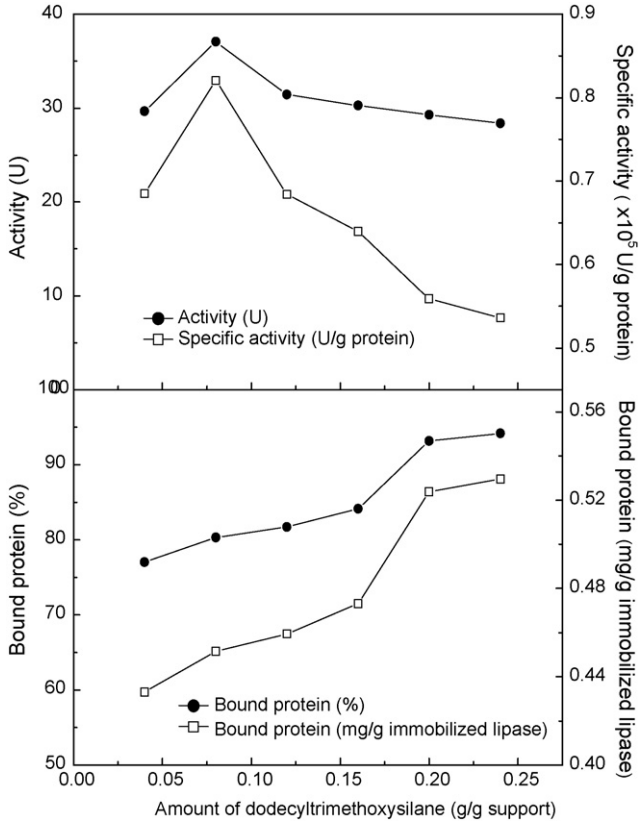


Fig. 2. Effect of the amount of dodecyltrimethoxylsilane on the bound protein (bottom) and activity (top). The lipase loading was 0.02 g lipase/0.8 g support and other immobilization conditions were the same as Section 2.4. The reaction conditions were the same as Section 2.6.

to find out the answers are underway. Based on the overall investigations of activity and operational stability, dodecyl-KM was a kind of prospective immobilized lipase and worth further studying.

3.3. Effect of activation degree of support on lipase preparations

Further efforts were made to improve the characteristics of dodecyl-KM. The effect of activation degree on the lipase immobilization was investigated, which was controlled by the addition amount of dodecyltrimethoxylsilane during the silanization treatment. The results were illustrated in Fig. 2. The bound protein increased with the rising amount of dodecyltrimethoxylsilane in the range of 0.04–0.24 g/g support and reached a maximum value of 0.53 mg/g immobilized lipase. The data gave a direct proof that dodecyl groups did exist on the surface of the support and they were favorable to adsorb enzyme proteins. However, the specific activity of the immobilized lipase ascended with increasing amount of dodecyltrimethoxylsilane up to 0.08 g/g support, and then came to a fall with further increase in the amount of dodecyltrimethoxylsilane. It showed that the specific activity of the prepared lipase was directly controlled by the addition amount of hydrophobic dodecyltrimethoxylsilane, and that appropriate hydrophobicity of the surface was probably necessary for lipase to maintain a suitable protein conformation [30]. All the lipase preparations immobilized on the different activation degrees of the supports kept nearly the same level of enantioselectivity in the resolution of HMPC with an E value more than 400.

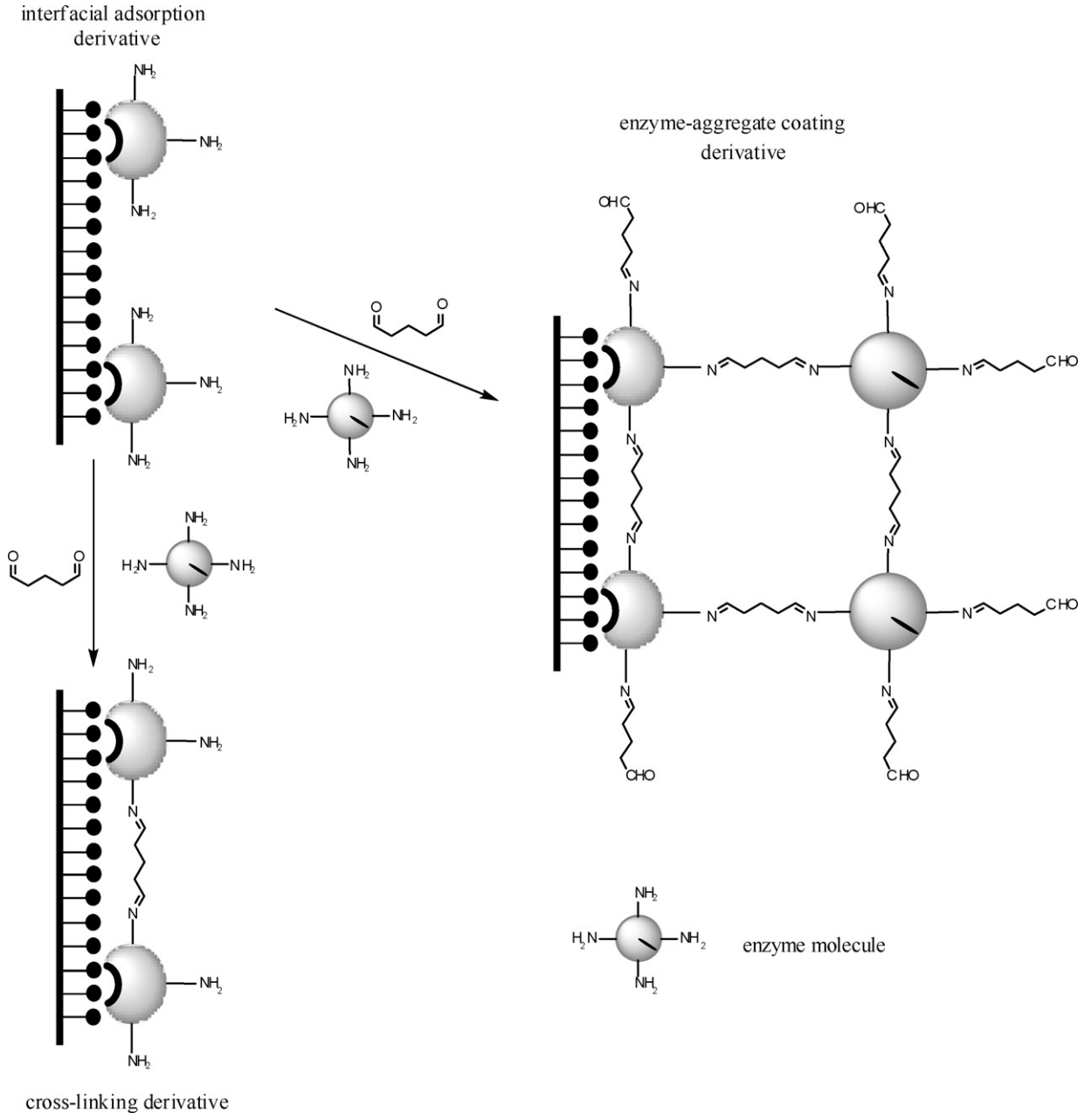
Table 2
Effect of lipase loading on the immobilized lipase

Lipase loading (mg/g support)	Bound protein (%)	Bound protein (mg/g immobilized lipase)	Specific activity ($\times 10^5$ U/g protein)	Activity yield (fold)	Activity (U)	Total activity[a] (U)	Recovered activity[b] (fold)
20	96	0.38	0.69	5.3	26.1	13.1	5.4
25	86	0.43	1.04	8.0	44.0	17.6	7.3
33	73	0.49	1.10	8.5	53.6	16.1	6.7
50	68	0.68	0.85	6.5	57.3	11.5	4.8
Free lipase	–	–	0.13	–	238.8	2.4	–

The adding amount of dodecyltrimethoxylsilane during the silanization was 0.08 g/g support. All the immobilization conditions expect lipase loading were the same as Section 2.4. The reactions were carried out at 40 °C and other conditions were the same as Section 2.6.

[a] For the immobilized derivatives total activity meant the sum of the activity of all the lipase preparations obtained at the end of the immobilized process, while for free KM, total activity meant the sum of the activity of soluble lipase offered to the supports before the immobilized process.

[b] Recovered activity (%) = (total activity of immobilized lipase/total activity of free lipase) × 100%.



Scheme 2. Diagram for the preparation of enzyme-aggregate coating derivative and cross-linking derivative based on interfacial adsorption.

Table 3
Comparison of characteristics of different immobilized lipase preparations

Entry	Lipase derivatives	Bound protein (%)	Bound protein (mg/g immobilized lipase)	Specific activity ($\times 10^5$ U/g protein)	Activity yield (fold)	Activity (U)	Recovered activity (fold)	E
1	Dodecyl-KM	72	0.44	1.08	10.8	48.0	7.7	≥400
2	CL-KM	72	0.44	0.75	7.5	33.2	5.3	≥400
3	EAC-KM	82	0.51	1.05	10.5	53.2	8.5	≥400
4	Free lipase	–	–	0.10	–	187	–	85

The lipase loading of the dodecyl-KM enzyme was 33 mg/g support and other immobilization conditions were the same as those in Table 2. The reactions were carried out as Section 2.6.

3.4. Effect of immobilization conditions on lipase preparations

The immobilization conditions such as the lipase loading and pH value of the buffer were critical to the characteristics of the immobilized lipase. The influences of the lipase loading (from 20 to 50 mg/g support) on the bound protein and activity were investigated. The data were presented in Table 2.

From the data we could see that higher activity yield and recovered activity were achieved when the lipase loading was located in the range of 25–33 mg/g support. Higher lipase loading could induce higher tendency of lipases to form inactive enzyme aggregates or even multilayer enzyme molecules and hence diminished catalytic efficiency. When the lipase loading was lower, the adsorbed enzyme molecules might suffer conformation distortion due to strong interaction with the support.

In addition, the effect of pH value of the buffer on the immobilization was also investigated in the range of 6.0–10.0 (data not shown). No apparent differences were found on the characteristics of prepared lipases when using different types of buffers with the same pH value. The results turned out that the bound protein under the varying pH values kept almost the same level while both the activity and specific activity exhibited the highest values at pH 9.0. Under the optimum immobilization conditions discussed above, the bound protein absorbed on the support was 0.46 mg/g immobilized lipase, meanwhile the specific activity and activity reached up to 10.4- and 7.8-fold, respectively, of that of free lipase (the reactions were carried out at 40 °C and other conditions were the same as Section 2.6). No apparent differences were observed for the enantioselectivity of all the prepared lipases ($E \geq 400$).

3.5. Investigation of enzyme-aggregate coating derivatives

In recent years the enzyme-aggregate coating method was put forward to attach more molecules and aggregates in the solution onto 'seed' enzyme molecules which had already been covalently attached onto the support. In our work, in order to further improve the activity and stability of dodecyl-KM, we attempted to prepare the enzyme-aggregate coating derivative based on interfacial adsorption and investigated the characteristics of the immobilized preparation. Moreover, we also prepared the cross-linking immobilized lipase based on interfacial adsorption which was likely to enhance the stability. The comparisons of activity and specific activity between interfacial adsorption derivative (dodecyl-KM, entry 1), cross-linking derivative (CL-KM, entry 2) and the enzyme-aggregate coating derivative (EAC-KM, entry 3) were exhibited in Table 3 and the immobilization mechanisms of the three preparations were illustrated in Scheme 2. Within data in Table 3, EAC-KM achieved the highest activity with a recovered activity of 8.5-fold of the free form among all the three enzymes. Comparable to dodecyl-KM, EAC-KM possessed higher bound protein and activity, but lower specific activity. The results should partially attribute to the facts that the additional enzymes which were attached by cross-linking with glutaraldehyde exhibited lower activity than those interfacially adsorbed onto the hydrophobic surface. In addition, we noticed that CL-KM got the same bound protein with that of dodecyl-KM, however, the activity and specific activity were much lower than those of dodecyl-KM. This indicated that the nonselective intermolecular cross-linkage by glutaraldehyde possibly was harmful to the active conformation of enzyme molecular, which might happen in the functional domain of the protein molecular.

We investigated the reaction courses of the resolution of HMPC with the three immobilized and free lipases (the same protein content of the immobilized lipases with free one), and the results were shown in Fig. 3. A sharp decrease in reaction time was observed by the three immobilized preparations when the conversion reached 50% and only about 1/10 of reaction time was needed compared to the free lipase. At the end of the reaction high optically pure *S*-HMPC was obtained with an e.e. value about 99% for each of the three immobilized preparations. The operational stabilities of the three immobilized lipases were investigated and the results were shown in Fig. 4. It was obviously showed that EAC-KM and CL-KM remained approx. 85% of initial activity after 10 and 8 recycles, respectively, while dodecyl-KM only remained about 70% after 8 recycles. No decrease of enantioselectivity was observed in recycling usage of the three prepared lipases. The operational stabilities reported herein of these immobilized lipases, especially EAC-KM, were much better than those of some reported ones using commercial materials as supports. For example, *Candida rugosa* lipases immobilized onto Eupergit C and Eupergit C 250 L supports remained only about 40% of initial activity after 12 cycles in the reaction of enantioselective esterification of *rac*-menthol in organic solvents [31]. *Pseudomonas* sp. lipase immobilized onto SBA-15 by physical adsorption maintained 90% of its initial activity after reused five times, however, the *E* value decreased from 114 to 92 [32]. The operational stability of EAC-KM was poorer

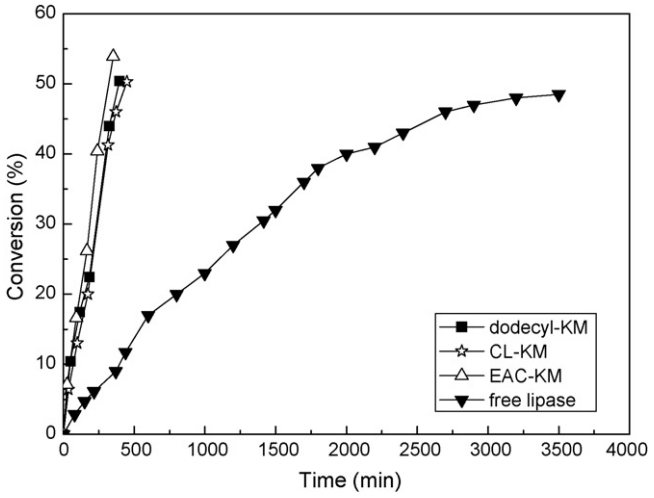


Fig. 3. Comparison of reaction courses of kinetic resolution of HMPC with free lipase, dodecyl-KM, CL-KM and EAC-KM preparations.

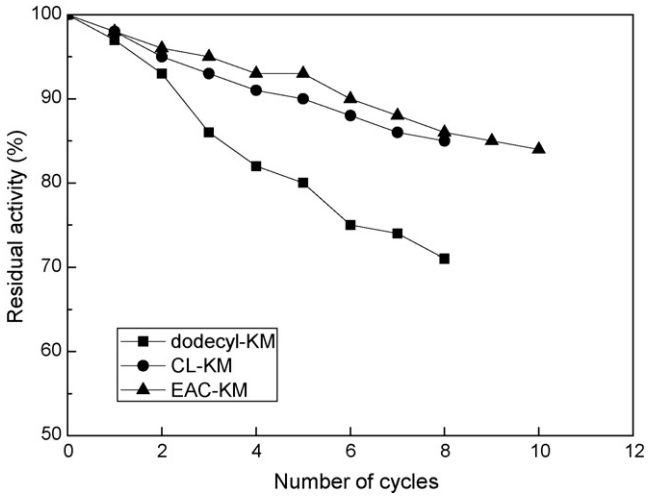


Fig. 4. The operational stability of dodecyl-KM, CL-KM and EAC-KM preparations.

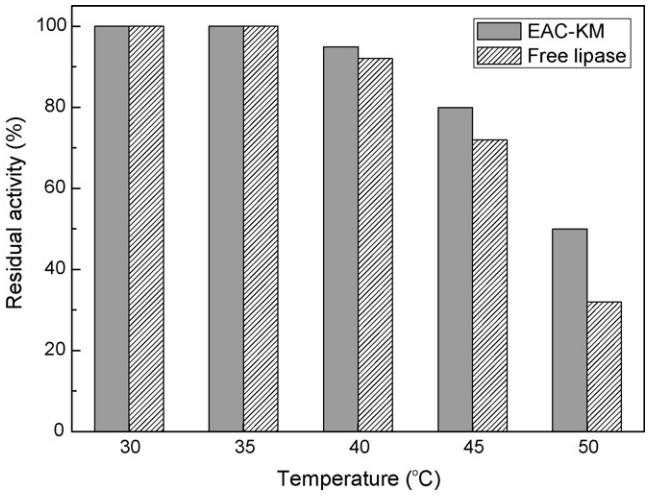


Fig. 5. Investigations of thermal stability of EAC-KM and free lipase. The reaction conditions were described in Section 2.6.

than that of enzyme-aggregate coating derivatives in some other reports [17] which used covalently attached enzymes as 'seed' molecules, and we thought it should mostly attributed to the relative poor interaction during interfacial adsorption in our work. Based on these results, it was concluded that EAC-KM got the highest activity and operational stability among the three enzyme preparations.

Additionally, we investigated the thermal stability of EAC-KM and compared it with that of the free one, and the results were shown in Fig. 5. Comparable to free lipase, a slight enhancement in the thermal stability was observed with EAC-KM. Nearly no loss of activity for both EAC-KM and free lipase could be found at the incubation temperatures of less than 35 °C, but obvious decreases in the activity occurred with the temperature above 40 °C. This meant that EAC-KM was suitable to be used at relative low temperatures. Investigation on the storage stability for EAC-KM revealed that the residual activity reduced to about 50% after 200 days.

4. Conclusion

In this work, lipase from *Arthrobacter* sp. was immobilized onto low-cost diatomite materials for asymmetric acylation of 4-hydroxy-3-methyl-2-(2-propenyl)-2-cyclopenten-1-one. By surface modification of the support surface and further via different immobilization protocols including interfacial adsorption, covalent attachment, enzyme-aggregate coating and cross-linking based on interfacial adsorption, various lipase preparations were obtained. Evaluations on the characteristics of these preparations have been performed and the results showed that EAC-KM was a better one concerning the activity and operational stability. The recovered activity of EAC-KM reached up to 8.5-fold of the free lipase and the residual activity remained 85% of initial activity after 10 recycles. Excellent enantioselectivity ($E \geq 400$) was obtained for most lipase preparations in our paper ($E = 85$ for the free enzyme) with an e.e. value of *S*-HMPC about 99%. On the basis of these results, we recommended EAC-KM as a prospective preparation for continuous industrial applications. And the enzyme-aggregate coating method was worthy of further investigations to achieve higher activity and stability of enzymes compared to conventional immobilization methods.

Acknowledgements

The authors would like to thank the Chinese National Natural Science Foundation (Nos. 20606030 and 20336010), Key Project of Chinese National Programs for Fundamental Research and Development (No. 2003CB716008) and Hi-Tech Research and Development Program of China (No. 2006AA02Z238) for financial support.

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