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Resolution of *N*-(2-ethyl-6-methylphenyl) alanine via cross-linked aggregates of *Pseudomonas* sp. Lipase

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Abstract

Pseudomonas sp. Lipase (PSL) immobilized with the method of cross-linked enzyme aggregates (CLEAs) using acetone as the optimal precipitant is investigated. The immobilization efficiency (%) and activity retention (%) of the immobilized lipase (CLEA-PSL) are 70.6 and 45.1%, respectively. In the kinetic resolution of *N*-(2-ethyl-6-methylphenyl) alanine, CLEA-PSL not only keeps excellent enantioselectivity (*E*-value > 100) as the free PSL, but also shows higher catalytic activity and thermal stability. CLEA-PSL requires only 12 h to obtain 50% conversion whereas the free PSL needs 48 h; the residual activity of CLEA-PSL and free PSL are respectively 72.2 and 23.3% after incubated 24 h at 60 °C. Furthermore, CLEA-PSL can be re-used through ten cycles and the efficiency loss in activity is found to be only 19.1%.

Keywords: *Pseudomonas* sp. Lipase; Cross-linked enzyme aggregates; *N*-(2-ethyl-6-methylphenyl) alanine; Kinetic resolution

1. Introduction

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) play an important role in the field of biocatalysis, which is mainly based on their extreme versatility with respect to substrate specificity and stereoselectivity. Lipase-catalyzed reactions can be used industrially for a variety of purposes, including hydrolysis of oils and fats [1,2], synthesis of fatty acid esters as cosmetic ingredients or detergent additives [3,4], production of intermediates for organic synthesis [5,6], and so on.

(*S*)-*N*-(2-ethyl-6-methylphenyl) alanine ((*S*)-NEMPA) is an important precursor in the synthesis of most widely used herbicides such as (*S*)-Metolachlor [7]. (*S*)-NEMPA is currently produced by chemical synthesis in large quantities [8]. Nevertheless, the chemical method requires drastic reaction conditions that may cause racemization, decomposition or side reactions. In contrast, enzyme-catalyzed reactions are less hazardous, polluting and energy-intensive than conventional chemistry-based. We had successfully obtained enantiopure (*S*)-NEMPA using a convenient two-step resolution procedure (Scheme 1), in which enantiomerically pure (*R*)-(+)-acid (99% $e.e._p$) was first produced using the excellent enantioselectivity of lipase PSL, and then (*S*)-acid was prepared without any reduction in enantiomeric excess (98% $e.e._p$) by the lipase B from *Candida antarctica* (CAL-B)-catalyzed hydrolysis of the remaining (*S*)-ester [9,10]. In this way, we may obtain higher optically pure (*S*)-NEMPA and (*R*)-NEMPA at the same time.

From the above results, we may conclude it is necessary that the lipase PSL is selected for further investigation. The lipase PSL immobilized on mesoporous molecular sieve (SBA-15) by adsorption had been studied [10], and the stability and reusability of PSL had a little increase during immobilization. However, the immobilized PSL on SBA-15 showed some disadvantages in the reaction of lipase-catalyzed resolution of NEMPA, such as: (1) adsorbed enzyme easily leaching from support in aqueous media; (2) insufficient operational stability (approximate 50% of activity loss after six cycles); (3) the additional cost of the preparation of support. In order to overcome these problems, we attempted to immobilize PSL with the method of cross-linked enzyme aggregates (CLEAs).

The method of CLEAs, which was recently developed in Cao group, is a much simpler technique [11]. In the process of preparing CLEAs [12], the enzyme is first precipitated from an aqueous

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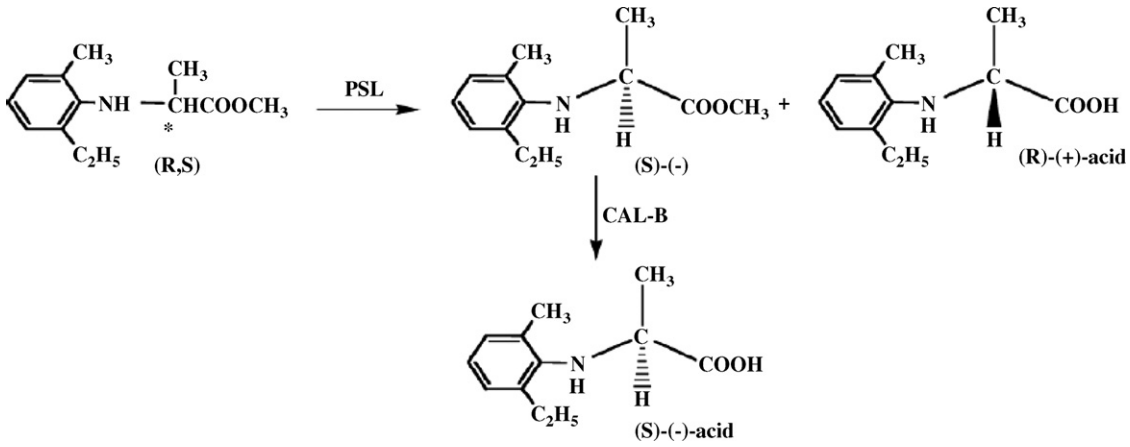

Scheme 1. Preparation of (*S*)-(–)-NEMPA by two-step resolution.

solution by adding a salt or a water-miscible organic solvent or a polymer, and then the physical aggregates of enzyme are cross-linked with a bifunctional reagent. Compared with other kinds of immobilized methods, cross-linked enzyme aggregates exhibit many advantages, for instance, highly concentrated enzyme activity in the catalyst, high stability and the low production cost due to the exclusion of an additional carrier. Another important feature is that one does not have to start with a purified enzyme preparation [13–15].

Herein, the cross-linked enzyme aggregate of *Pseudomonas* sp. Lipase (CLEA-PSL) was prepared. The effects of some factors (such as the selection of precipitants, cross-linked time, concentrations of the cross-linking reagent, etc.) on the activity of CLEA-PSL were examined. In particular, the catalytic activity, thermal stability and reusability of CLEA-PSL were investigated and compared with those of the free lipase in the kinetic resolution of NEMPA.

2. Experimental

2.1. Materials

Pseudomonas sp. Lipase (2500 U/g) was purchased from Amano Pharmaceutical Co. Ltd. (Japan). Tributyrin was purchased from Acros Organics (USA). Glutaraldehyde solution (25% (v/v) in water) was purchased from Tianjin Chemical Reagent (China). (*R*,*S*)-*N*-(2-ethyl-6-methylphenyl) alanine methyl ester ((*R*,*S*)-NEMPA-ME) was prepared in our lab [9]. Other reagents were all analytical grade or better and used without further purification.

2.2. Preparation of CLEAs

Lipase PSL (30 mg enzyme powder) was dissolved in 1 mL Na_2HPO_4/NaH_2PO_4 buffer solution (100 mM, pH 7.0) in a centrifuge tube. Then the different precipitants were added dropwise to the lipase solution under gentle stirring at 4 °C for 2 h. 50 μl glutaraldehyde solution (25% (w/v) in water) was added in the mixture, continuously stirred at 25 °C for 2 h. The mixture was centrifuged at 5000 rpm for 10 min. The supernatant was decanted and the residue was washed with the relevant solvents (5 ml each times) until no more activity was determined in the supernatant. The final enzyme preparations were lyophilized and stored at 4 °C.

2.3. Assay of lipase activity

The activity of the lipase was determined by titrating the butyric acid produced in the hydrolysis of tributyrin [16]. 100 μl tributyrin and 6.0 ml phosphate buffer solution (25 mM, pH 7.5) were mixed and placed in a bath vessel maintained at 37 °C. Then 5 mg free PSL powder or 20 mg immobilized PSL was added and stirred sufficiently for 15 min. The extent of the reaction was monitored by titrating the produced butyric acid with 0.05 M NaOH using a pH-stat. Similarly, a blank experiment without added enzyme was carried by the above assay procedure. Based on the amount of alkali consumed, the produced amount of butyric acid in the samples was calculated and the enzyme activity was determined. One lipase unit (IU) is defined as the amount of enzyme required to produce 1 μmol of free fatty acid per min at 37 °C.

2.4. Lipase-catalyzed hydrolysis of (R,S)-NEMPA-ME in aqueous media

The same units (50U) of free PSL or CLEA-PSL were added to a suspension of (*R*,*S*)-NEMPA-ME (0.5 mM) in phosphate buffer solution (100 mM, pH 8.0, 5.0 ml). The resulting mixture was shaken at 37 °C, 160 rpm. Aliquots were periodically drawn and analyzed by capillary zone electrophoresis (Beckman Coulter P/ACE^{TM} MDQ, USA) [17]. The conversions and enantiomeric excess (e.e.$_p$) of (*R*)-NEMPA were determined according to the literature [9].

2.5. Determination of kinetic parameters

The kinetic parameters (K_m and V_{max} in Michaelis–Menten Equation) for both free and immobilized enzymes were calculated by the Lineweaver–Burk double-reciprocal method, under

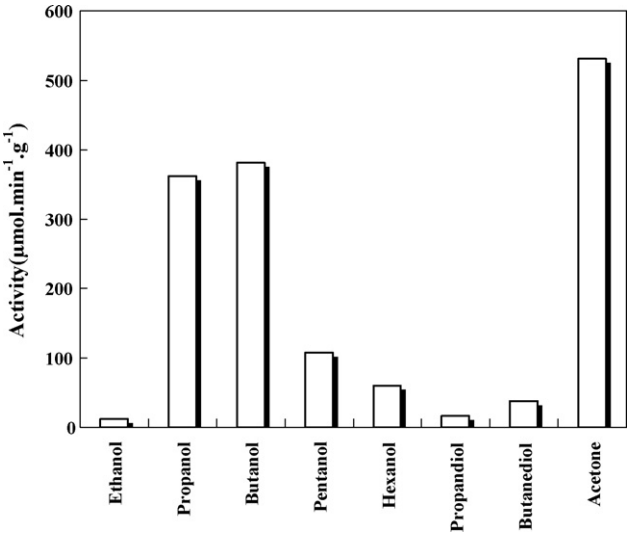

Fig. 1. The activity of CLEAs prepared with different precipitants. Prepared conditions: PSL 30 mg/ml, 1 ml; varying solvents 5 ml; glutaraldehyde 20 mM.

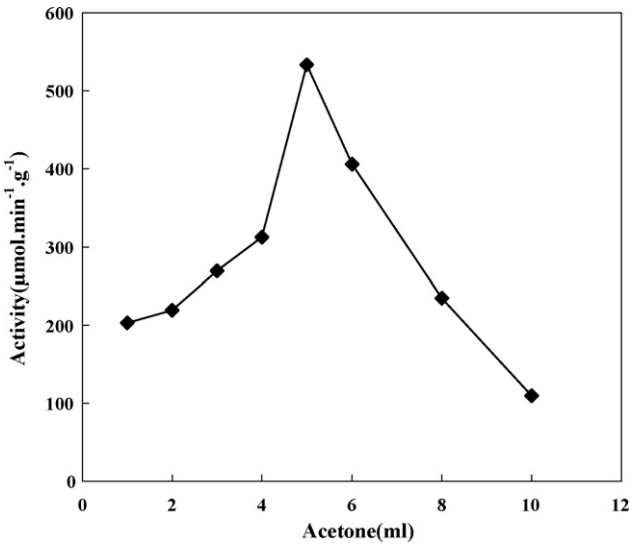

Fig. 2. Effect of adding amount of acetone on CLEAs activity. Prepared conditions: PSL 30 mg/ml, 1 ml; acetone 1–10 ml; glutaraldehyde 20 mM.

the conditions of coequal temperature and pH at different substrate concentrations between 0.1 and 1 mM.

2.6. Fourier transform infrared spectra (FT-IR)

FT/IR spectra of free PSL and CLEA-PSL were recorded at 25 °C in the spectral range 4000–400 cm^{-1} using Nicolet™ 5700 spectrometer (Thermo Electron Corporation, USA) equipped with a DTGS (deuterated triglycine sulphate) detector at 4 cm^{-1} resolution, 32 scans coaddition and triangular apodization. Powdered samples were grounded with KBr and pressed into pellets. The spectra were corrected by KBr (blank) as a background spectrum.

3. Results and discussion

3.1. Preparation of CLEAs

The simplified procedure for the synthesis of CLEAs included the formation and cross-linking of protein aggregates. The CLEAs prepared with three types of precipitants were investigated, including salts (NaCl and $(NH_4)_2SO_4$), nonionic polymer (polyethylene glycol (PEG)) and organic solvents (alcohols and acetone) [18]. It was discovered that the physical aggregate of lipase was hardly induced using PEG and NaCl as precipitants and no CLEAs were produced. CLEA prepared with $(NH_4)_2SO_4$ could be facilely redissolved in aqueous solution although exhibiting high activity, which was disadvantageous to the successive reuse of lipase. Better results were obtained with three organic solvents (propanol, butanol and acetone) as precipitants, especially, CLEAs gave the highest activity when acetone was utilized (Fig. 1). So acetone was selected as the optimized precipitating solvent for further investigation.

The adding amount of precipitant also affected the activity of CLEAs (Fig. 2). The free lipases in solution were gradually precipitated by increasing the adding amount of acetone; however, the excessive adding of acetone possibly resulted in the partial deactivation of the lipase and the reduced activity of CLEA was observed. An optimum adding amount of acetone was 5 ml for 1 ml lipase solution (30 mg/ml).

In addition, the activity of CLEAs was also influenced by the concentrations of glutaraldehyde solution (Fig. 3). At lower concentrations very little insoluble aggregates could be obtained; whereas at higher concentrations the amount of insoluble aggregates increased but no significant activity was detected. The highest activity could be obtained when 20 mM glutaraldehyde solution and 2 h cross-linked time were adopted. The excessive cross-linking might influence the active site availability and therefore decrease the activity of the CLEA [19].

Herein, a cross-linked aggregate of PSL (CLEA-PSL) was prepared in the above-mentioned optimal conditions. The activ-

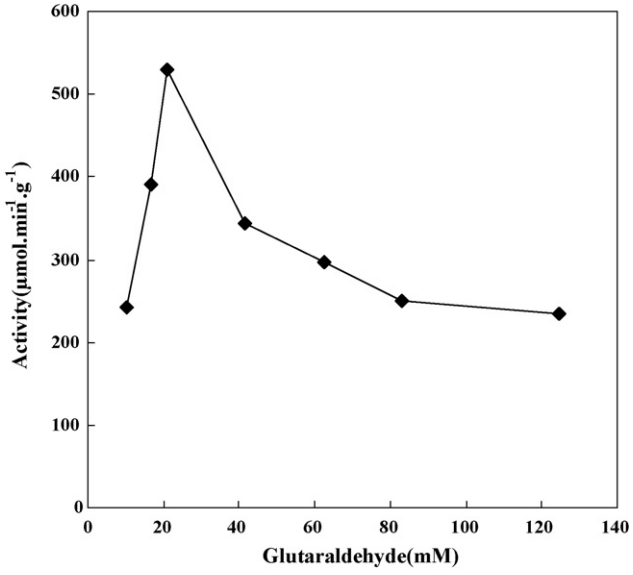

Fig. 3. Effect of the concentrations of glutaraldehyde on CLEAs activity. Prepared conditions: PSL 30 mg/ml, 1 ml; acetone 5 ml; glutaraldehyde 10–125 mM.

ity, immobilization efficiency (%) and activity recovery (%) of CLEA-PSL are 533 U/g, 70.6 and 45.1%, respectively. The residual enzyme activity (%) in the supernatant and the washing of CLEA-PSL are respectively 20.1 and 9.3%, which are calculated by taking the total enzyme activity offered for immobilization as 100%. So, it is approximately considered that the extent (%) of deactivation versus leaching is 25.5% versus 29.4% in the immobilization procedures.

3.2. Comparing the catalytic activity of immobilized lipase with that of free lipase

The time-course of the free and immobilized lipase were tested in the optimized conditions of hydrolysis of (*R*,*S*)-NEMPA-ME (Fig. 4). It shows that CLEA-PSL exhibits higher catalytic activity compared with the free lipase. CLEA-PSL needed only 12 h when the conversion of (*R*)-NEMPA achieved 50%, whereas the free lipase needed 48 h. The initiative velocities (V_0) of the free and immobilized PSL are 0.22 and 0.42 μmol min^{-1}, respectively. It should be stressed that CLEA-PSL displays the same enantio-preference towards (*R*)-isomer and very high enantioselectivity ($E > 100$) as the free PSL [10], and the enantiomeric excesses (e.e.$_p$) of (*R*)-NEMPA are maintained 99% before 50% of hydrolysis of the racemic (*R*,*S*)-NEMPA-ME. However, the drop of e.e.$_p$ is observed when the conversions greater than 50% are obtained because the reaction rate of (*S*)-isomer become faster after the (*R*)-ester was entirely hydrolyzed.

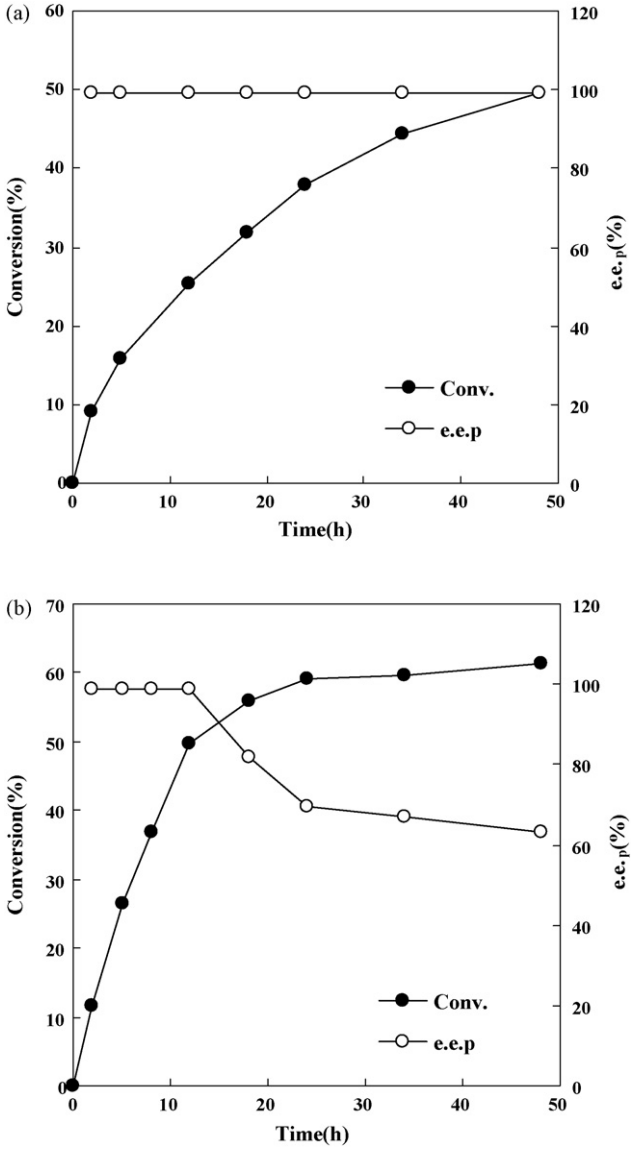

Fig. 4. The time-course for enantioselective hydrolysis of (*R*,*S*)-NEMPA-ME catalyzed by the free PSL (a) and CLEA-PSL (b). Reaction conditions: substrate 0.5 mM, phosphate buffer (pH 8.0, 100 mM, 5.0 ml), free or immobilized enzyme 50U, temperature 37 °C.

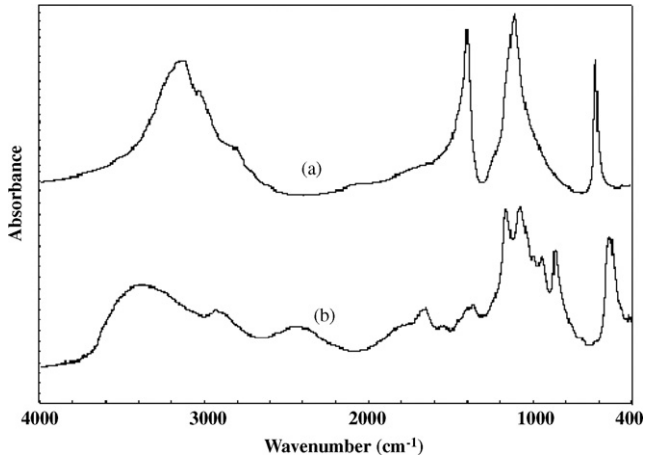

Fig. 5. FT-IR spectra of free PSL (a) and CLEA-PSL (b).

Kinetic studies were also performed to determine the Michaelis constant (K_m) and the maximum velocity (V_{max}) of the free and immobilized lipase. The K_m values of the free PSL and CLEA-PSL are 2.39 and 1.65 mM, while the values of V_{max} are 6.5 and 8.8 μmol min^{-1}, respectively. It is suggested that the activity and the affinity toward the substrate of PSL are enhanced after immobilization.

This may be accounted for that the free PSL is dispersed during immobilization, which decreases the chance of free enzyme convergence in the pure buffer, thus improving accessibility for the substrate to the active site of enzyme and enhancing the activity of immobilized enzyme.

A second possibility is that the conformation of lipase changed in the course of the CLEA preparation. The changes of the second structure of PSL after immobilized were observed by analyzing the infrared spectra of free PSL and CLEA-PSL (Fig. 5) [20]. The FT-IR spectrum of lyophilized PSL powder contains two broad bands near 3100–3300 cm^{-1} due to N–H stretching frequencies. Two sharp bands at 1400 and 1110 cm^{-1} correspond to C–N stretching frequencies. A sharp band at 617 cm^{-1} corresponds to the C–H out of plane bending. The band near 3134 cm^{-1} in PSL has been shifted to 3373 cm^{-1} in CLEA-PSL, and the decrease in the broadness of the peak in CLEA is probably due to the conglomeration of the lipase. The band near 1650 cm^{-1} in CLEA-PSL is obviously observed rather than PSL owing to the intensification of C=O stretching vibrations, which might be caused by the covalent interaction of

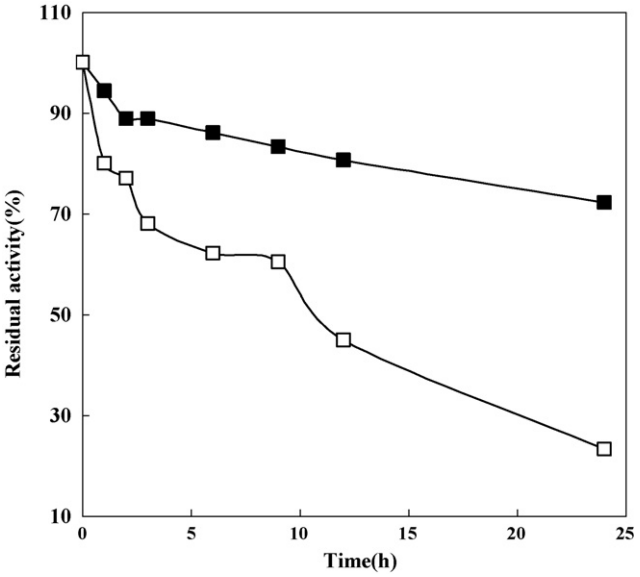

Fig. 6. Effect of thermal (60 °C) incubation of free PSL (□) and CLEA-PSL (■) on activity.

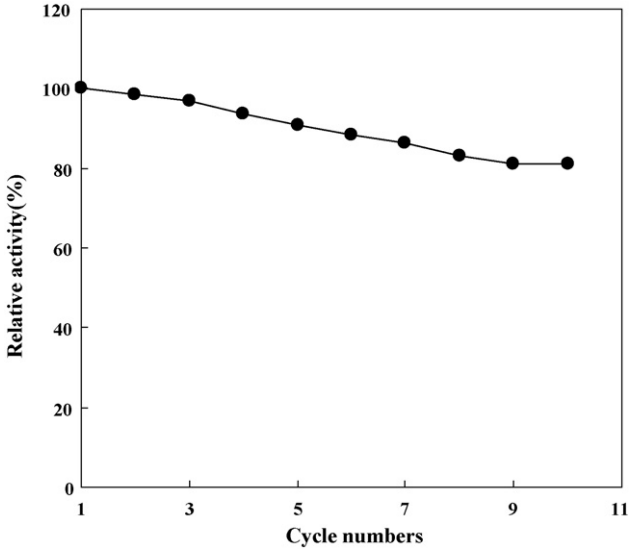

Fig. 7. Recycle of the CLEA-PSL.

amino group of lipase with aldehyde group of glutaraldehyde. The bands at 1400 and 1110 cm^{-1} in PSL, absent in CLEA-PSL, are divided to several sharp bands. These transformations suggest the second structure of lipase PSL has been changed during immobilization, which possibly make the lipase adopt a more active conformation.

3.3. Comparing the thermal stability of free PSL and CLEA-PSL

The CLEA-PSL catalyzed hydrolysis of (*R*,*S*)-NEMPA-ME at pH 8.0 in a temperature range from 30 to 70 °C was investigated, and the highest activity of CLEA was obtained at 60 °C. Therefore, the thermal stability of the free and immobilized lipase was studied under the temperature. The immobilized and free PSL were incubated at 60 °C and pH 8.0 phosphate buffer for 24 h, and the activity was measured periodically during the time. Herein, the relative activity was calculated by assuming the initial activity of PSL was 100%. As shown in Fig. 6, the thermal stability of the both enzymes were diminished to a certain extent, and the free lipase lost 76.7% activity after 24 h, while only 27.8% activity was lost for CLEA-PSL. It may be concluded that the stability of PSL could be improved through the immobilization with the method of cross-linked enzyme aggregates.

3.4. Recycle of CLEA-PSL

As Tischer and Kasche perceptively noted, the major purpose of immobilization is in the design of reusable biocatalysts [21]. So, in this section, we investigated the durability of CLEA-PSL in the repeated batch hydrolysis of (*R*,*S*)-NEMPA-ME. The reaction using 100 mg CLEA-PSL and 0.5 mM (*R*,*S*)-NEMPA-ME in phosphate buffer solution (100 mM, pH 8.0) was carried out at 37 °C for 12 h. The CLEA in each case was filtered and washed after each use and then suspended again in a fresh reaction mixture to measure the conversion and enantiomeric excesses of (*R*)-NEMPA. The residual activity was calculated by taking the enzyme-catalyzed activity of the first cycle as 100%. The relative activity of immobilized lipase during recycling 10 times was given in Fig. 7. After 10 cycles, the immobilized lipase still shows 80.9% of its initial activity. It should also be stressed that the enantiomeric excesses of (*R*)-NEMPA were maintained 99% throughout the 10 repeated reactions.

The results indicated that CLEA-PSL had doughty operational stability in aqueous media, but it was still not ideal because the activity of immobilized lipase was decreased with increasing the recycle number, which may have a close relationship with the leakage and deactivation of partial lipase aroused by long-playing mechanical stirring under the continuous reactions. In addition, the man-caused loss might take place during the recoveries.

4. Conclusions

The cross-linked enzyme aggregates of *Pseudomonas* sp. Lipase (CLEA-PSL) were investigated, and the optimal CLEA was obtained using acetone as the precipitant under the optimized operating conditions including acetone (83.3%, v/v), lipase solution (30 mg/ml), glutaraldehyde (20 mM) and cross-linked time (2 h). It is remarkable that CLEA-PSL exhibits considerable promise as an immobilized biocatalyst for the kinetic resolution of NEMPA. The present results show that the catalytic activity, thermal stability and reusability of CLEA-PSL are superior to those of free PSL while giving the excellent enantioselectivity (*E*-value > 100) as the free PSL. It indicates the method of CLEAs has a higher potential in the immobilization and application of PSL, but the low immobilization efficiency and activity retention are still a major problem. This might be improved by the search of cross-linking strategy or using the protein feeders. Further work will be reported in due course.

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