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Crosslinking of enzyme coaggregate with polyethyleneimine: A simple and promising method for preparing stable biocatalyst of *Serratia marcescens* lipase

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ABSTRACT

Crosslinking of enzyme aggregates is a promising method for enzyme immobilization. In this work, crosslinked enzyme coaggregates of *Serratia marcescens* lipase with polyethyleneimine (CLECAs-SML-PEI) were prepared using polyethyleneimine (PEI) as coprecipitant and glutaraldehyde as crosslinking reagent. The crude lipase solution at a low protein concentration (0.1 mg/ml), with PEI at a mass ratio of 3:1 (PEI/protein, w/w), was found to be most adequate for the coprecipitation of SML. After crosslinking of the coaggregate of SML-PEI with 0.2% (w/v) glutaraldehyde under ambient temperature, over 70% of the total lipase activity was recovered. Compared with the free SML, the optimum temperature of the CLECAs-SML-PEI was enhanced from 50 °C to 60 °C and its thermal stability was also significantly improved. CLECAs-SML-PEI showed excellent operational stability in repeated use in aqueous–toluene biphasic system for asymmetric hydrolysis of *trans*-3-(4′-methoxyphenyl)glycidic acid methyl ester (MPGM), without significant inactivation after 10 rounds of repeated use.

1. Introduction

Extracellular lipase excreted by *Serratia marcescens* (SML) showed high enantioselectivity toward many industrially relevant chiral esters, such as flurbiprofen ethyl ester [1], naproxen methyl ester [2], glycidyl butyrate [2], 4-hydroxy-3-methyl-2-(2-propynyl)-cyclopent-2-enone [2], and especially (±)-*trans*-3-(4′-methoxyphenyl)glycidic acid methyl ester (MPGM), an important intermediate for the synthesis of cardiovascular drug diltiazem [3,4]. Due to the importance of SML in chiral resolution, many methods have been employed to realize the reuse of SML and increase its stability, including retaining of the lipase with hollow fiber membrane [5] or immobilization of SML onto appropriate carriers [4,6–8]. Though the stability of SML was markedly enhanced by enzyme immobilization, the volumetric activities of these immobilized enzymes were relatively low due to the high proportion of carriers to the immobilized lipases.

In order to overcome these limitations associated with conventional carrier-immobilized enzymes, novel methods, such as cross-linked enzyme crystals (CLECs) [9] and cross-linked enzyme aggregates (CLEAs) [10], have been developed. In these methods, essentially pure proteins were crosslinked by a bifunctional reagent, such as glutaraldehyde, to form a crosslinked enzyme. Compared with CLECs, the technology of CLEAs has more advantages since tedious purification and crystallization process of the desired enzyme is no longer required, which makes the whole process of enzyme immobilization much simple and universal.

For the preparation of CLEAs, the enzyme protein is firstly aggregated into supermolecular structures, induced by appropriate precipitating agents, including inorganic salts like ammonium sulfate [11] and organic solvents such as acetone [12], 1,2-dimethoxyethane [13], acetonitrile [14] and *tert*-butanol [15,16]. Some nonionic polymers, such as polyethylene glycols (PEGs), can also be used for the precipitation of enzyme proteins [17]. Subsequently, the formed aggregates are crosslinked by a bifunctional reagent, typically glutaraldehyde. In some cases, another protein, such as bovine serum albumin, is added to increase the protein concentration or to improve the enzyme tolerance against high concentration of glutaraldehyde, so as to increase the crosslinking efficiency [18].

Polyethyleneimine (PEI) is a highly aminated hydrophilic polymer. Co-immobilization of mutant penicillin G acylase and PEI on glyoxyl-agarose was performed, attachment of PEI generate a hydrophilic microenvironment covering each enzyme molecule, thus avoiding the attachment of organic solvent molecules to the enzyme and improving the stability of enzyme in the presence of organic solvents [19]. PEI has been used with in coaggregation with several enzymes, including penicillin G acylase [20], lipases QL and CALB [21] and glutaryl acylase [22,23] to prepare CLECAs, with PEG, ethylene glycol dimethyl ether or *tert*-butyl alcohol as precipitant. The highly aminated PEI compensates the low content of surface lysine group of lipase and glutaryl acylase and prevents the leak-

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age of enzyme molecules from CLECAs [21]. Preparation of glutaryl acylase CLECAs with PEI as the sole precipitating agent was also reported [22], but the stability of CLECAs-PEI was lower than that of CLECAs-PEI-PEG.

In this study, PEI was shown to be a good sole precipitating agent for SML at a relatively low concentration, and the crosslinked coaggregates of SML with PEI (CLECAs-SML-PEI) were prepared, with excellent thermal and operational stability.

2. Experimental

2.1. Microorganism and materials

The bacterium *S. marcescens* ECU1010 was isolated from soil and is presently deposited at China General Microbiological Culture Collection Center with an accession number of CGMCC No. 1219. Polyethyleneimine (Polymin P′, molecular weight of about 50,000) was obtained from BDH Chemicals (Poole, UK) as product #15047. Glutaraldehyde solution (25%, w/v) was purchased from Lingfeng Chemical Reagents Limited (Shanghai, China). *trans*-3-(4′-Methoxyphenyl)glycidic acid methyl ester [(±)-MPGM] was synthesized ourselves from 4-anisaldehyde and methyl chloroacetate as described elsewhere [24]. *para*-Nitrophenyl acetate (pNPA) was home synthesized as described previously [25] by using *para*-nitrophenol and acetate anhydride as substrates and pyridine as catalyst. All other chemical reagents and solvents were also obtained commercially with the highest grade available and used without further treatment.

2.2. Lipase production

Production of extracellular *S. marcescens* lipase (SML) was performed as described previously [26]. The crude SML solution isolated from the fermentation broth by centrifugation at 10,000 × *g* for 10 min was stored at 4 °C and used directly without further purification.

2.3. Choice of precipitating agents

To 0.2 ml crude SML solution (0.2 mg protein ml^{-1}) precooled at 4 °C, was added 0.4 ml of an organic solvent (acetone, ethanol, *n*-propanol or *iso*-propanol) precooled at −20 °C), 0.8 ml of saturated ammonium sulfate solution or 4 μl PEI solution (1% w/v, pH being adjusted to 7.0 before use). The mixture was placed at 4 °C for 2 h after being vortexed vigorously, and then centrifuged at 8000 × *g* for 1 min. Samples were withdrawn before and after centrifugation, and the SML activity of the precipitate was determined as the difference between the precipitate suspension and centrifuged supernatant.

2.4. Preparation of CLECAs-SML-PEI

The coaggregate of SML with PEI (SML-PEI) was prepared by mixing the SML solution with a PEI solution (pH was adjusted to 7.0 before use) for 2 h. In order to determine the optimal mass ratio of PEI to protein, 50 μl of PEI solution (1%, w/v) was added into 10 ml of crude SML solution (0.1 mg protein ml^{-1}) periodically at an interval of 30 min with gentle agitation, and samples were withdrawn before each addition. Suspension of the coaggregate was centrifuged at 8000 × *g* for 1 min and the precipitate was dissolved in potassium phosphate buffer (KPB, 100 mM, pH 7.0) and used for lipase activity assay.

Crosslinked coaggregates of SML-PEI (CLECAs-SML-PEI) was prepared by the addition of glutaraldehyde (25%, w/v) into 1.0 ml of the coaggregate suspension of SML-PEI and agitated for 1 h. The resultant CLECAs-SML-PEI was collected by centrifuge at 5000 × *g* for 3 min, washed with KPB (100 mM, pH 7.0) to remove the excessive glutaraldehyde and resuspended in KPB for activity assay.

2.5. Lipase activity assay

The activities of soluble SML and CLECAs-SML-PEI were determined by the initial rate of *p*-nitrophenol release in the enzymatic hydrolysis of *p*-nitrophenyl acetate (pNPA).

For assay of soluble SML, 30 μl of lipase solution was added into 2.94 ml KPB (100 mM, pH 7.0). After incubated at 30 °C for 3 min, the reaction was initiated by the addition of 30 μl pNPA solution (100 mM, in dimethyl sulfoxide). The change in the optical absorbance at 405 nm was recorded by a spectrophotometer (UVmini-1240, Shimadzu, Japan).

For assay of the immobilized SML, an appropriate amount of CLECAs-SML-PEI was suspended in 0.5 ml KPB (100 mM, pH 7.0). After incubated at 30 °C for 3 min, 5 μl of pNPA solution (100 mM) was added and shaken with an agitation rate of 1000 rpm. After 3 min of reaction, 0.5 ml acetone was added to quench the reaction. After centrifuge at 10,000 × *g* for 3 min, 0.6 ml reaction supernatant was mixed with 2.4 ml of KPB (100 mM, pH 7.0) and the optical absorbance at 405 nm was recorded by the spectrophotometer. A blank test without the addition of the enzyme was also preformed for a comparison.

One unit (U) of lipase activity is defined as the amount of the enzyme that releases 1.0 μmol of *p*-nitrophenol per minute under above conditions.

2.6. Protein assay

Protein concentration was determined according to the method described by Bradford [27] using bovine serum albumin (BSA) as the standard protein.

2.7. Determination of the enzyme stability

Thermal stabilities of free SML and CLECAs-SML-PEI were measured under 30 °C and 50 °C at pH 7.0 in different time intervals. The pH stabilities of free SML and CLECAs-SML-PEI were determined at varied pH ranging from 5.0 to 11.0 after 24 h of incubation at 30 °C. For the pH ranges of 5.0–8.0, 8.0–9.0, and 9.0–11.0, sodium phosphate buffer (0.1 M), Tris–HCl buffer (0.1 M) and glycine–NaOH buffer (0.1 M) were utilized, respectively. The results were represented as percentages of the residual activities, taking the initial activity as 100%.

2.8. Reusability of CLECAs-SML-PEI

To determine the reusability of the immobilized lipase, the CLECAs-SML-PEI was prepared from 50 ml of SML solution (4 U/ml, 0.1 mg protein ml^{-1}, pH 7.0) with 150 μl of PEI (10%, w/v) and 0.4 ml glutaraldehyde (25%, w/v), and then employed in an organic–aqueous biphasic system for enzymatic hydrolysis of (±)-MPGM. The reactions were performed in a 250-ml three-necked flask. The substrate (±)-MPGM (1.04 g, 5 mmol) dissolved in 50 ml toluene was mixed with 50 ml suspension of CLECAs-SML-PEI in water. The reaction was performed at 30 °C with agitation of 200 rpm. The pH was controlled between 8.0 and 8.5 by automatic titration with 3 M ammonia solution. Samples were taken at time intervals for the determination of reaction conversion and enantiomeric excess of the unreacted substrate (ee_s). When the ee_s reached 99%, the reaction was terminated and the organic phase was removed. The aqueous phase containing CLECAs-SML-PEI was collected and mixed directly with 50 ml of fresh toluene solution of (±)-MPGM (100 mM) to start a new round of reaction. The reaction was repeated for 10 rounds.

Table 1
Comparison of several protein precipitating agents.[a]

Precipitants	Activity recovered in precipitate (%)[b]	Activity remained in supernatant (%)
Acetone	61.3 ± 7.0	15.6 ± 4.2
Ethanol	18.3 ± 2.0	22.8 ± 3.2
Propanol	0	0.3 ± 0
Isopropanol	1.1 ± 0.5	0
Ammonium sulfate	54.9 ± 0	3.2 ± 0.6
PEI	74.3 ± 1.2	8.3 ± 0.5

[a] Reaction conditions: 0.2 ml crude SML solution (13 U/ml, 0.2 mg protein ml^{-1}) was mixed with 0.4 ml of an organic solvent (acetone, ethanol, propanol or isopropanol, precooled at −20 °C), 0.8 ml saturated ammonium sulfate or 4 μl PEI solution (1%, w/v) respectively, and settled down at 4 °C for 2 h.

[b] SML activities recovered in precipitates were determined as the difference between the precipitate suspension and the supernatant after centrifugation at 8000 × *g* for 1 min.

Samples from enzymatic hydrolysis of (±)-MPGM were centrifugated at 10,000 × *g* for 1 min, and 50 μl of organic phase was diluted in 0.95 ml ethyl acetate containing 0.5 mM of *para*-nitroacetophenone as an internal standard. Concentrations of the residual MPGM were determined by HPLC (Agilent 1200) equipped with a chiral column of Chiralcel OJ-H (Ø0.46 cm × 25 cm, Daicel Co. Ltd., Japan). Samples were eluted with *n*-hexane/isopropanol (60:40, v/v) at a flow rate of 0.8 ml/min and detected at 254 nm.

2.9. Scanning electron microscopy (SEM) analysis

CLECAs-SML-PEI was spread over a microscope slide and sputtered with gold. Scanning electron microscope images were recorded with JSM-6360lv Electron Microscope (JEOL), equipped with NORAN System 6 X-ray Microanalysis System and Semafore Digitizer.

3. Results and discussion

3.1. Selection of the lipase precipitant

Several types of protein precipitants, including organic solvents such as ethanol, *n*-propanol, *iso*-propanol and acetone, inorganic salts like ammonium sulfate and aminated polymer (e.g., PEI) were investigated for evaluating their abilities of precipitating SML. The results are shown in Table 1. Short chain alcohols seemed not good precipitants, since SML was denatured with low activities recovered both in the precipitate and in the supernatant when *n*-propanol and *iso*-propanol were used as precipitants. Ethanol was better than *n*- and *iso*-propanol, resulting in a recovery of more than 40% initial activity. Ammonium sulfate and acetone were better precipitating agents, with 54.9% and 61.3% of the initial SML activity recovered in the precipitates. PEI gave the best result, giving 74.3% and 8.3% recovery of activity in the precipitate and the supernatant, respectively. Therefore, PEI was selected as the best precipitating agent for preparing the immobilized SML in subsequent investigation.

3.2. Optimization of CLECAs-SML-PEI preparation

3.2.1. Effect of protein concentration

Protein concentration significantly affects the activity recovery in precipitation of enzymes. Generally speaking, precipitation of an enzyme will be easier at a relatively higher protein concentration when organic solvents and ammonium sulfate were chosen as the precipitants. While in the case of SML-PEI preparation, a low concentration of protein was found favorable both for the formation of SML-PEI and for the recovery of lipase activity. When the protein concentration was increased from 0.1 to 0.3 mg/ml while keeping the mass ratio of PEI to protein at 1:1, the activity recovery of the coaggregate decreased and the activity recovery from the supernatant increased with the increase of protein concentration, as shown in Table 2. The highest activity recovery from the precipitate was observed at a protein concentration of about 0.1 mg/ml, affording about 85.7% activity recovery in SML-PEI coaggregates.

Table 2
Effect of protein concentration on activity recovery of SML-PEI coaagregates.

Protein concentration (mg/ml)	Activity recovered in precipitate (%)	Activity remained in supernatant (%)
0.06	82.6	7.1
0.12	85.7	9.4
0.18	80.7	12.1
0.24	67.5	18.8
0.30	67.1	19.1

The mass ratio of PEI to protein was kept at 1:1 and the precipitation process was performed at 4 °C with gentle stirring for 30 min. Experiments were performed in triplicate.

3.2.2. Influence of mass ratio of PEI to protein

As shown in Fig. 1, a high activity recovery of SML-PEI was obtained when the mass ratio of PEI to protein was in a range of 1:1–3:1 (w/w). When the mass ratio was lower than 1:1, only a little precipitate was obtained, and when the mass ratio was higher than 3:1, the SML-PEI formed would be redissolved again, making the activity recovery in precipitate decrease sharply.

It was reported that PEI has a pK_a of about 8.7 [28], while the p*I* of SML from *S. marcescens* ECU1010 was about 4.2 [2]. Therefore, under a neutral condition, the negatively charged SML would combine tightly with the positively charged PEI through ionic adsorption, forming stable and insoluble coaggregates, especially when the mass ratio of PEI to protein was within such a proper range that the amount of PEI's positive charge could just balance the protein's negative charge. Whereas when the proportion of PEI was too high or too low, the formed aggregates could not be well deposited due to the repellence between the same charges.

3.2.3. Effect of glutaraldehyde concentration on the activity recovery in CLECAs-SML-PEI

Glutaraldehyde is a commonly used crosslinking reagent. The concentration of glutaraldehyde significantly affects the activity recovery of CLECAs. A low concentration of glutaraldehyde is favorable for a high activity recovery, while a high concentration of glutaraldehyde tends to inactivate the enzyme. When the concentration of glutaraldehyde was increased from 0.2 to 1.0% (w/v), the activity recovery of CLECAs-SML-PEI decreased from 54% to 33%,

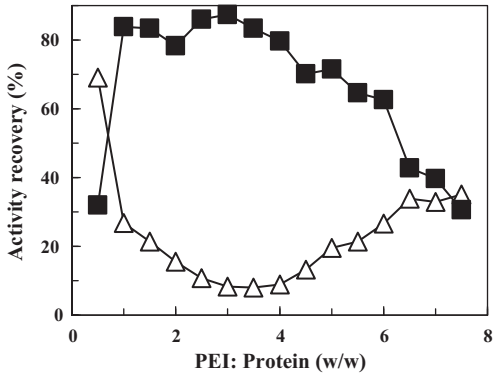

Fig. 1. Effect of PEI concentration on activity recovery in SML-PEI. (■) Aggregate; (△) supernatant. To 10 ml crude SML solution (0.1 mg protein ml^{-1}), 50 μl of PEI solution (1%, w/v, pH was adjusted to 7.0 before use) was added periodically with an interval of 30 min.

Table 3
Effect of glutaraldehyde concentration on the activity recovery of CLECAs-SML-PEI.

Glutaraldehyde concentration (%, w/v)	Activity recovered in CLECAs (%)	Activity remained in supernatant (%)
0.2	54	8.7
0.4	42	7.5
0.6	40	7
0.8	35	5.9
1	33	4.7

SML solution (0.1 mg/ml) was mixed with PEI (final concentration of 0.3 mg/ml) and gently stirred at 4 °C for 15 min, then further maintained at 4 °C with gentle stirring for 1 h. Experiments were performed in triplicate.

as shown in Table 3. Therefore, 0.2% (w/v) of glutaraldehyde was employed for the crosslinking of SML-PEI.

3.2.4. Fast formation of CLECAs-SML-PEI

Times needed for the formation and crosslinking of SML-PEI were determined. Results showed that both the two processes proceeded fast. For the coaggregation of SML with PEI, as high as 75% of initial lipase activity could be recovered in the aggregates after only 15 min mixing of PEI with the crude SML solution. And for the crosslinking of SML-PEI, visible particles of crosslinked coaggregates were formed in 5–10 min after the addition of glutaraldehyde into the suspension of coaggregates and nearly 60% of the initial activity was recovered from the CLECAs-SML-PEI in only 10 min.

3.2.5. Effect of temperature on preparation of CLECAs-SML-PEI

Effect of operation temperature on the activity recovery of CLECAs-SML-PEI was investigated. The same operation was performed at 4 °C and 25 °C, respectively. Negligible difference in the activity recovery of CLECAs-SML-PEI was observed (data not shown). This is very favorable for practical preparation of CLECAs since the operation at 4 °C is tedious and energy consuming.

3.2.6. Increase of CLECAs-SML-PEI activity by ultrasonic treatment

Using PEI as precipitant and glutaraldehyde as crosslinking reagent, the formed particles of CLECAs-SML-PEI were larger than the CLEAs formed using other precipitant reagents, so mass transfer limitation appeared. Ultrasonic treatment promoted the dispersion of CLECA particles and resulted in higher activity. Activity recovery was increased from 53.9% to 72.6% by ultrasonic treatment of the CLECAs-SML-PEI for 2 min.

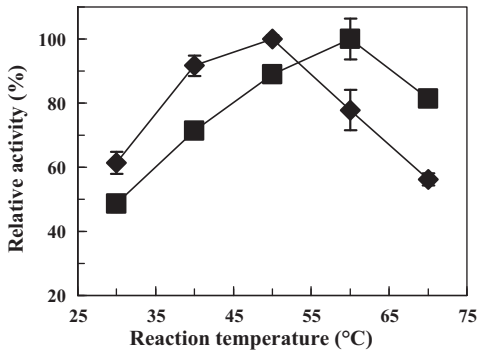

Fig. 3. Effect of temperature on the activities of free and CLEA immobilized SML. Experiments were performed in triplicate. (♦) Free SML; (■) CLECAs-SML-PEI.

3.3. Characterization of CLECAs-SML-PEI

3.3.1. Morphology of CLEAs-SML

According to the scanning electron microscopy (SEM) images (Fig. 2B and C), the CLECAs-SML-PEI showed relatively uniform structures after crosslinking. The average diameter of the CLEA-SML-PEI particle was around 0.3 μm, close to that of the type 2 aggregates obtained by Schoevaart et al. [29]. Considering the protein size of SML to be about 8.0 nm × 4.5 nm × 3.0 nm [30], one can estimate that a single CLECAs-SML-PEI particle contains a maximum of about 1.3×10^5 SML molecules. Single particle of CLECAs-SML-PEI can form larger clusters with sizes of 5–100 μm, as displayed in Fig. 2A and B.

3.3.2. Effect of temperature on the activities of free and immobilized SML

Activities of free and immobilized SML at different temperatures ranging from 30 to 70 °C were measured, as shown in Fig. 3. After enzyme immobilization, the optimum reaction temperature

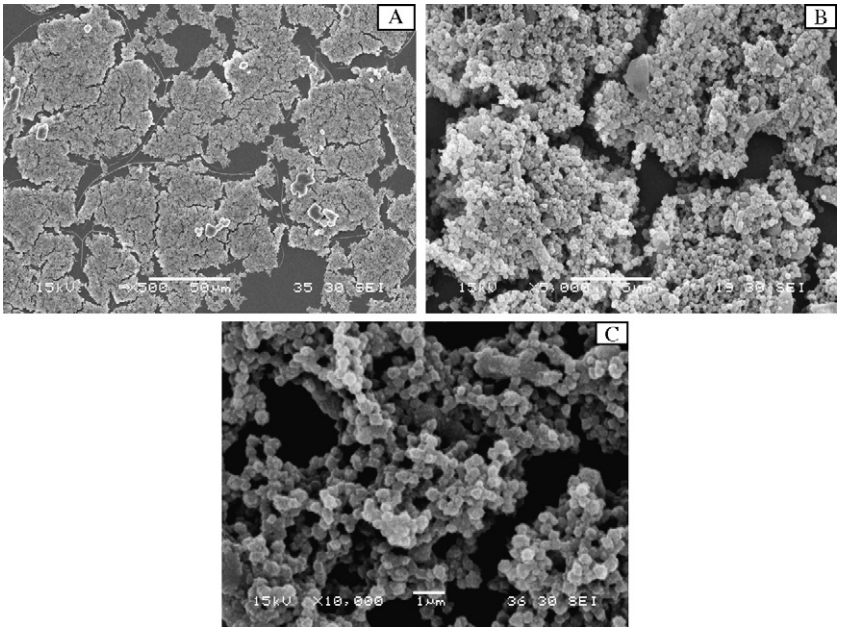

Fig. 2. Scanning electron microscope images of CLECAs-SML-PEI with different magnifications. (A) 500×; (B) 5000×; and (C) 10000×.

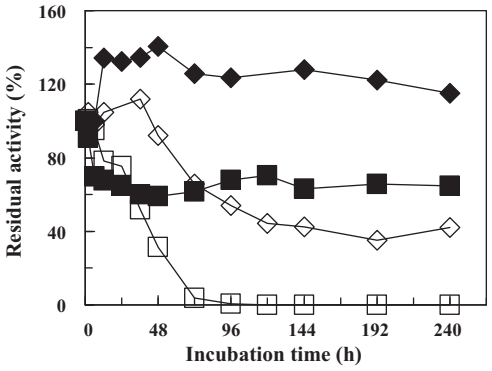

Fig. 4. Thermal stability of free SML at 30 °C (◊) and 50 °C (□) and CLECAs-SML-PEI at 30 °C (♦) and 50 °C (■). Inactivation was performed in 100 mmol/L KPB (pH 7.0) at 30 °C and 50 °C, respectively.

increased from 50 °C for the free SML to 60 °C for the CLECAs-SML-PEI.

3.3.3. Thermal and pH stability of free and immobilized SML

Thermal stability of both free SML and CLECAs-SML-PEI was evaluated by incubating enzyme samples at 30 and 50 °C, respectively (Fig. 4). The free SML was not very stable at high temperature. Nearly 50% of its initial activity was lost after only 36 h incubation at 50 °C. While at 30 °C, the stability of the free SML was much better: after 10 days of incubation, nearly 40% of the activity was retained. The stability of SML was significantly enhanced in the form of CLECA. Notably, an obvious hyperactivation phenomenon was observed for the CLECAs when incubated at 30 °C. Nearly 35% enhancement of its initial activity was observed when CLECAs-SML-PEI was incubated at 30 °C for 12 h. The SML activity in the CLECAs remained at high level, and only a slight loss was detected after 10 days of incubation. Even so, the residual activity at 10 days was still higher than the initial activity. A short time of hyperactivation was also observed by others [11,31], and was explained as the increase in the diffusion rates of substrate and product [11]. When CLECAs-SML-PEI was incubated at 50 °C, the SML activity decreased sharply to 69.7% within the initial period of 6 h, but thereafter kept stable with only slight changes. After 10 days of incubation at 50 °C, still 64% of the initial activity was retained, giving a half life of 520 h.

The stability of free and immobilized SML at different pH values was compared at 30 °C after incubation of these biocatalysts in buffers within the pH rang of 5–11 for 24 h. As can be seen from Fig. 5, free SML was relatively stable under near neutral conditions and the activity of free SML decreased sharply in basic environments. In contrast to the free SML, CLECAs-SML-PEI showed relatively high stability in basic environments (pH 8–10). This behavior was very favorable for bioresolution of (±)-MPGM, since

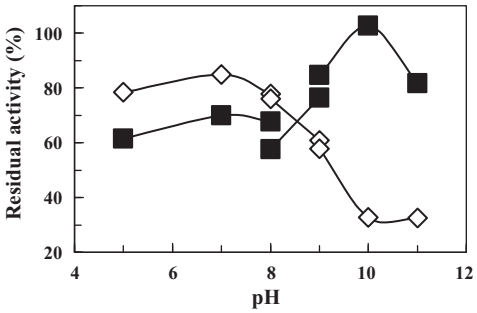

Fig. 5. Stability of free and CLEA immobilized SML. Activity was measured after incubation in buffers with different pH at 30 °C for 24 h. (◊) Free-SML; (■) CLECAs-SML-PEI.

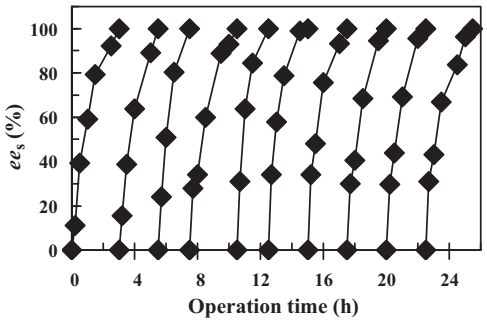

Fig. 6. Repeated batch reaction for the bioresolution of (±)-MPGM by CLECAs-SML-PEI. Reaction was performed at 30 °C in aqueous–toluene biphasic system with 0.1 M (±)-MPGM in toluene. After each batch of reaction, the aqueous phase containing CLECAs-SML-PEI was withdrawn and mixed with fresh organic phase containing the substrate (±)-MPGM for the next batch of reaction.

the substrate (±)-MPGM is relatively stable under basic condition, especially at pH 8–9 [3].

3.3.4. Operational stability of CLECAs-SML-PEI

Operational stability is one of the most important characteristics for immobilized enzymes. Since the lipase of *S. marcescens* ECU1010 has high activity and enantioselectivity toward (±)-MPGM, the operational stability of CLECAs-SML-PEI was investigated in repeated reactions of (±)-MPGM hydrolysis in toluene–aqueous biphasic system. The reaction was repeated for 10 batches. After termination of each round of reaction, the toluene phase was removed and new solvent containing the substrate was added for the next round of reaction. As shown in Fig. 6, the reaction time of each round was kept within 2–3 h, and no significant activity loss was observed. In fact, the activity of CLECAs-SML-PEI was increased in the first 5 rounds of reaction and the activity maintained thereafter, indicating the very high operational stability of the immobilized lipase.

4. Conclusions

Stable CLECAs were prepared from a crude *S. marcescens* lipase solution by using PEI as coprecipitant and glutaraldehyde as crosslinking reagent with several outstanding advantages as follows. (1) PEI was used as sole precipitating agent. Compared with other precipitating agents, low amount of precipitating agent required and less waste is exposed. (2) High activity recovery at a relatively low protein concentration. This is very favorable since the SML is an extracellular enzyme and the protein concentration is relatively low, so no further protein concentration step is necessary. (3) Significantly enhanced stability. All these advantages make PEI an excellent coprecipitation agent for preparing stable CLECAs.

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