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A facile technique to prepare cross-linked enzyme aggregates of bovine pancreatic lipase using bovine serum albumin as an additive

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Abstract−Cross-linked enzyme aggregates from bovine pancreatic lipase were prepared by co-aggregation of lipase and BSA (Lipase-BSA-CLEAs). The main factors in the preparation of lipase-BSA-CLEAs were optimized. The highest activity recovery was around 75% under the condition of using 1% (v/v) glutaraldehyde as cross-linker and 0.05 g/L bovine serum albumin as feeder for 2 h cross linking. The optimum temperature for both lipase-CLEAs and lipase-BSA-CLEAs was measured as 60 °C, which is 10 °C higher than that of free lipase. Moreover, the lipase-BSA-CLEAs evidenced higher thermal stability and excellent reusability in comparison with the lipase-CLEAs. Lipase-BSA-CLEAs retained more than 75% of the initial activity after eight cycles of reuse, while lipase-CLEAs only retained 20% of its initial activity. Additionally, lipase-BSA-CLEAs showed more storage stability than free lipase and lipase-CLEAs. The high stability and recyclability of lipase-BSA-CLEAs make it efficient for different industrial applications.

Keywords: Bovine Pancreatic Lipase, Cross-linked Enzyme Aggregates, Immobilization Enzyme, BSA

INTRODUCTION

Lipases are versatile biocatalysts that can catalyze various types of reactions, such as hydrolysis, esterification, inter-esterification and aminolysis under mild conditions; therefore, lipase is widely used in the food, pharmaceutical, and chemical industries [1]. However, free lipase exhibits poor stability, has difficulty of recovery and recycle during bioconversion, which presents a significant barrier to lipase application [1,2]. Therefore, many attempts have been made to improve lipase activity and stability, such as genetic engineering, protein engineering, fermentation engineering, chemical modification and enzyme immobilization [3-5]. Immobilization technology is a high efficiency method to improve stability of enzymes and easily recycle them [6,7]. Compared with carrier-bound immobilization techniques, cross-linked enzyme aggregates (CLEAs) seem to be a promising method for enzyme immobilization [8,9]. Enzyme can be simply immobilized with high stability and high volume activity by cross-linking the physical enzyme aggregates [10,11]. CLEAs of a number of enzymes have been synthesized in the last decade. For example, CLEAs of seven commercial lipases were prepared and CLEAs with more than ten-fold increase in activity respect to native enzyme were reported [12]. Lipase from *Rhizopus oryzae* (ROL) was immobilized as crosslinked enzyme aggregate (CLEA). The resulting CLEAs retained 91% activity after ten cycles in aqueous medium [13]. Other reports on CLEAs, different than lipases, and stabilization strategies have been reported [14-16]. Generally, glutaraldehyde has been used for CLEAs. However, glutaraldehyde was found to modify some essential ε-amino groups, which resulted in CLEA with significant loss of biological activity [17,18]. Moreover, enzymes having low number of amino groups often undergo inadequate cross-linking and form mechanically fragile CLEA that release enzyme into the reaction medium during a biocatalytic reaction [19,20]. This problem may be partially solved by co-aggregation of the enzyme and some polymers or proteins containing a large number of primary amino groups, such as polyethyleneimine (PEI) [20,21]. The addition of polymers containing amino groups could increase cross-link efficiency. However, the microenvironment that surrounds the enzyme was altered. As a result, the prepared CLEAs did not exhibit more conformationally stable than the standard CLEA. In contrast, addition of BSA is known to facilitate CLEAs preparation in cases in which the protein concentration of the enzyme preparation is low and/or the enzyme activity is vulnerable to high concentrations of cross-linker [22,23]. To date, immobilization of lipases has been accomplished by CLEAs [18,24,25], but to our knowledge, there is no systematic work in the open literature about the immobilization of commercial bovine pancreatic lipase as CLEAs by addition of BSA. Therefore, in this study, CLEAs of bovine pancreatic lipase were prepared for the first time by co-aggregation of lipase and BSA (Lipase-BSA-CLEAs) to avoid the loss of activity of lipase, improve cross-linking efficiency and mechanical strength. The main immobilization parameters which affect the activity of the biocatalyst were investi-

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gated. The morphology of the CLEAs particles was determined by SEM and confocal laser scanning microscopy (CLSM). Furthermore, the catalytic properties of free lipase, lipase-CLEAs, and lipase-BSA-CLEAs were also compared in detail, including optimal temperature, kinetic properties, thermal stability, storage stability, and reusability.

MATERIALS AND METHODS

1. Microorganism and Chemicals

Lipase (EC 3.1.1.3 Type II, from bovine pancreas) was purchased from International Aladdin Reagent Inc. (Shanghai, China), and stored at 4 °C. The activity is 15-30 U/mg. Glutaraldehyde was obtained from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). 4-Nitrophenyl acetate (98%) was purchased from International Aladdin Reagent Inc. (Shanghai, China). BSA was purchased from Sigma. Other reagents used were of analytical grade.

2. Preparation of Lipase-CLEAs and Lipase-BSA-CLEAs

For the preparation of lipase-CLEAs, 0.1 g of lipase was dissolved in 50 mM sodium phosphate buffer (pH 7.5) and ammonium sulfate was added up to the final concentration of 80% (w/v). The mixture was stirred at 4 °C for 1 h, and then a certain amount of glutaraldehyde (10% v/v in water) was slowly added to the final concentration of 1% (v/v). The mixture was kept at 4 °C for 2 h with constant shaking at 200 rpm. Then the resultant suspension was centrifuged at 10,000 ×g for 10 min at 4 °C. The insoluble CLEAs were washed for three times with 50 mM sodium phosphate buffer (pH 7.5). Finally, the CLEAs were resuspended in 50 mM sodium phosphate buffer (pH 7.5) and stored at 4 °C prior to use. The Lipase-BSA-CLEAs were prepared as the general procedure, but adding a given amount of BSA into the enzyme solution.

3. Activity Assay

The activities of free lipase and CLEAs to catalyze the hydrolysis of 4-nitrophenyl acetate (4NPA) to p-nitrophenol (pNP) were measured following the procedures described by Bustos-Jaimes et al. [26]. The substrate solution contained 50 mM sodium phosphate buffer solution (pH 7.5), 0.5 mM 4NPA and 0.2% Triton X-100. Then a small amount of enzyme sample was added to substrate solution. The resultant reaction medium was incubated at 37 °C for 5 min. The pNP formation was monitored at 410 nm using a 2800H spectrophotometer (Unicoi Instrument Co., Ltd. Shanghai). One unit of lipase activity was defined as the amount of lipase releasing 1 μmol of pNP from pNPP per minute. The activity assay of immobilized lipases was detected by the same procedure as described above. The activity recovery in immobilized lipases was calculated by comparing the activity of enzyme before and after immobilization. As given in Eq:

$$\text{Activity recovery (\%)} = \frac{\text{Total activity of CLEAs (U)}}{\text{Total free lipase activity used for CLEAs production (U)}}$$

4. Structural Characterization of Lipase-CLEAs and Lipase-BSA-CLEAs

SEM (JEOL JSM6700, Japan) was used to examine the particle morphologies of Lipase-CLEAs and Lipase-BSA-CLEAs. Before SEM observation, CLEAs were freeze-dried and coated with platinum under vacuum. CLSM was used to investigate the distribution of CLEAs. Prior to observation, 50 mg/ml FITC solution (FITC in acetone) was added to lipase solution for 3 min to form a highly fluorescent product. Modified FITC labeled lipase was then immobilized.

5. Measurement of Kinetic Parameters

The kinetic parameters, K_m and V_{max}, for free lipase and immobilized lipase were calculated by the Lineweaver-Burk double-reciprocal plot method of Michaelis-Menten Equation between 0.08 and 0.12 mM 4NPA concentrations at a constant enzyme concentration (0.1 mg/mL). The enzymatic reaction was carried at pH 7.5 (50 mM phosphate buffer), 37 °C, and the change in absorbance was measured at 410 nm.

6. Effect of Temperature on Enzyme Activity

To determine the optimum temperature of free lipase, lipase-CLEAs, and lipase-BSA-CLEAs, enzyme activities were measured by incubating the enzyme samples in the substrate solution in the temperature range of 30-70 °C for 5 min. Meanwhile, the time courses of thermal inactivation of Lipase-CLEAs, Lipase-BSA-CLEAs and free lipase were also investigated by incubating them in 50 mM sodium phosphate buffer solution (pH 7.5) without substrate at 60 °C for 1-3 h. At different incubation times, the residual lipase activity was determined by the same procedure as described above.

7. The pH and Storage Stabilities of Free Lipase, Lipase-CLEAs, and Lipase-BSA-CLEAs

To determine the storage stability of the free lipase, lipase-CLEAs, and lipase-BSA-CLEAs, enzyme samples were incubated in 50 mM sodium phosphate buffer solution (pH 7.5) without substrate at 25 °C. At different storage times, the residual activities of free lipase and immobilized lipase were determined. The effect of pH on enzyme stability was determined at various pHs (4-12) incubated at room temperature for 12 h, then measured the residual activity, taking initial activity as 100%.

8. Reusability of CLEAs

Reusability of Lipase-CLEAs, Lipase-BSA-CLEAs for the hydrolytic application was evaluated. 0.5 mM 4NPA was added to 10 ml of 50 mM phosphate buffer (pH 7.5) containing 50 mg of the immobilized lipase. This reaction mixture was incubated at 37 °C for 20 min to hydrolyze 4NPA. CLEAs were reused eight times, with repeated washing after each 20 min reaction. The residual lipase activity of each cycle was calculated by taking the enzyme activity of the first cycle as 100%.

RESULTS AND DISCUSSION

1. Preparation of Lipase-BSA-CLEAs

The general scheme of preparation of Lipase-BSA-CLEAs is illustrated in Fig. 1. The bovine pancreatic lipase was mixed with BSA and then precipitated with ammonium sulfate. The resultant precipitant was subsequently cross-linked with glutaraldehyde. Effect of different amounts of BSA on CLEAs formation was first investigated. As shown in Fig. 2, Lipase-CLEAs, prepared in the absence of BSA, recovered only 58% of the free enzyme activity. However, this recovery was increased significantly with the addition of BSA. It was indicated that the amounts of BSA had remarkable effects on the crosslinking process of CLEAs formation. The maximal

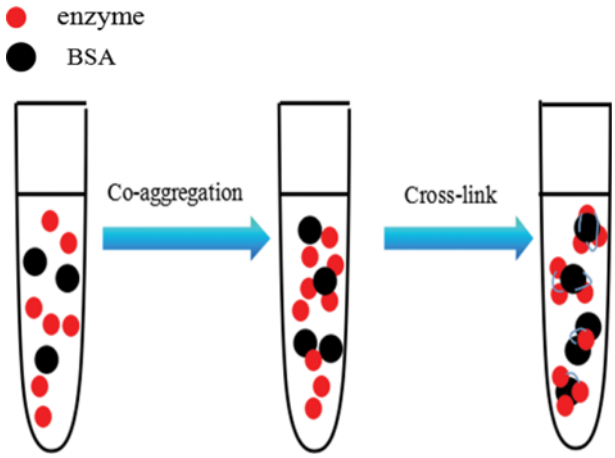

Fig. 1. General scheme of preparation of lipase-BSA-CLEAs.

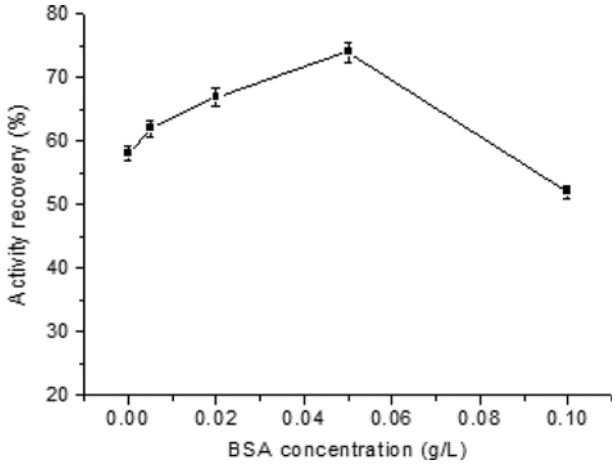

Fig. 2. Effect of BSA concentration on the activity recovery of lipase-BSA-CLEAs.

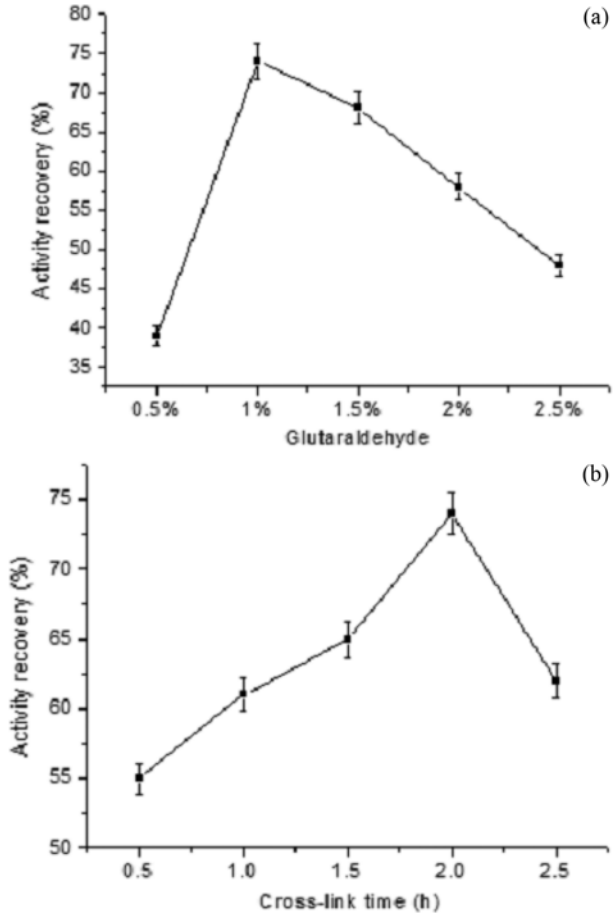

Fig. 3. Effect of glutaraldehyde concentration (a) and cross-link time (b) on the activity recovery of lipase-BSA-CLEAs.

activity recovery (75%) was obtained when 0.05 g/L BSA was used as the additive. However, the activity recovery decreased with addition of excessive amounts of BSA. It could be due to competition between the free amino groups of BSA and those of the lipase. Generally, glutaraldehyde has been used for cross-linking proteins. Cross-linking occurs via reaction of the free amino groups of lysine residues on the surface of neighboring enzyme molecules [27,28]. Therefore, one key variable in the production of CLEAs is cross-linker concentration and cross-link time. Effects of glutaraldehyde concentration and cross-link time on activity recovery of Lipase-BSA-CLEAs are shown in Fig. 3. With an increase in glutaraldehyde concentration, higher amounts of Lipase-BSA-CLEAs could be produced. At 1% (v/v) glutaraldehyde, 75% of the free enzyme activity was recovered in Lipase-BSA-CLEAs. However, the activity recovery decreased with addition of excessive amounts of glutaraldehyde (Fig. 3(a)). The previous reports showed that excessive glutaraldehyde might produce too much extensive cross-linking, which resulted in the enzyme losing flexibility [29]. Moreover, it is clear that cross-link time has a strong effect on the activity recovery of Lipase-BSA-CLEAs (Fig. 3(b)). Activity in the Lipase-BSA-CLEAs increased with the increase of cross-linking time, and the highest activity yield was obtained when the aggregates were cross-linked for 2 h. However, the excessive cross-linking time might influence the active site availability and therefore decreased the activity of the Lipase-BSA-CLEAs (Fig. 3(b)).

2. The Morphology of CLEAs and Kinetic Properties

The morphologies of lipase-CLEAs and lipase-BSA-CLEAs were characterized by scanning electron microscope (SEM), which are presented in Fig. 4. Lipase-CLEAs displayed the amorphous morphology with many small enzyme aggregates packed together (Fig. 4(a), 4(c)), while Lipase-BSA-CLEAs showed larger aggregates with the ball-like shape than Lipase-CLEAs (Fig. 4(b), 4(d)). The results were further confirmed by fluorescence micrograph using fluorescein isothiocyanate (FITC) labeled CLEAs since the labeled CLEAs showed a typical green fluorescence image. Compared with Lipase-CLEAs, particles of Lipase-BSA-CLEA exhibited larger fluorescent size (Fig. 4(e), 4(f)). This result can be explained by the fact that the degree of the cross-linking of lipase protein was greatly increased in number due to the help of the lysine-rich BSA protein. Moreover, addition of BSA to the enzyme solution (co-precipitation) also increased the total protein content. Therefore, the size of Lipase-BSA-CLEAs was larger than lipase-CLEAs.

Kinetic properties of free lipase, lipase-CLEAs, and lipase-BSA-

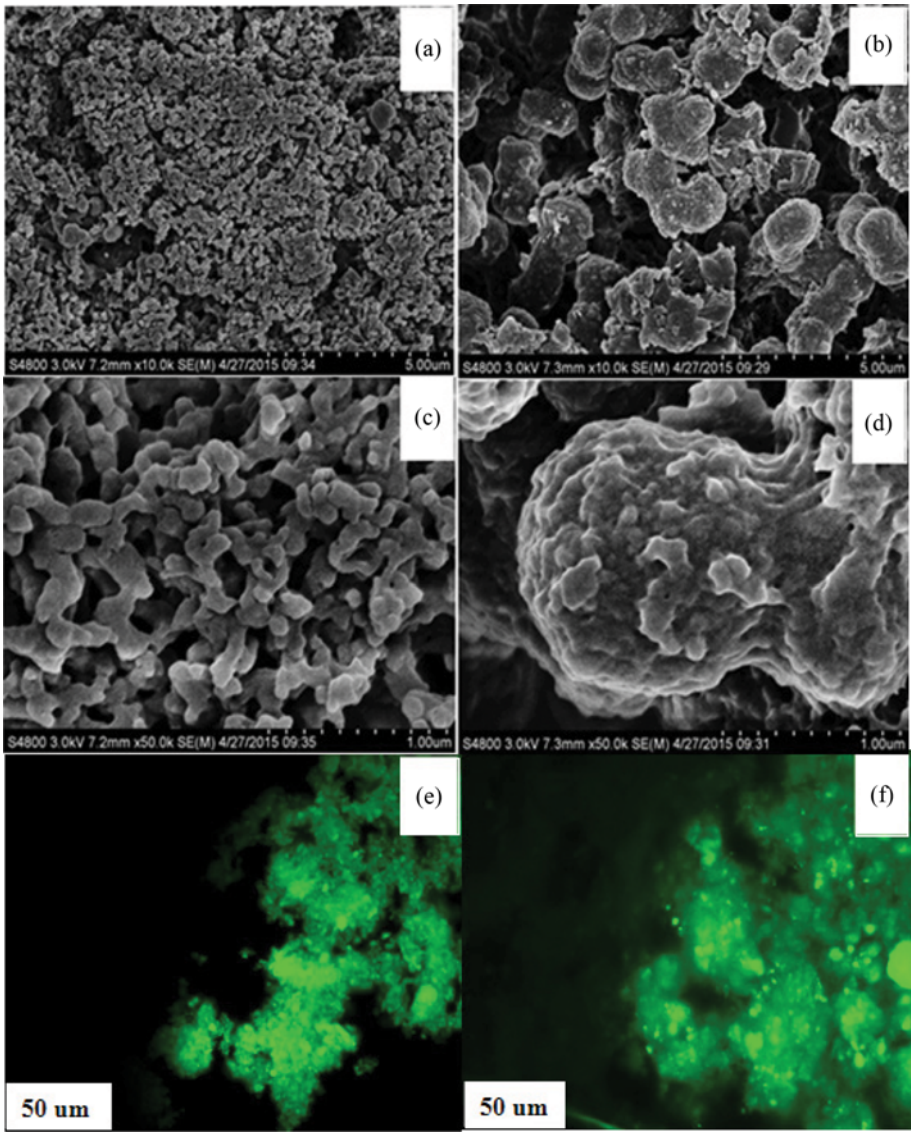

Fig. 4. SEM images of CLEAs with different magnifications: Lipase-CLEAs (a), (c), lipase-BSA-CLEAs (b), (d); LCSM images of lipase-CLEAs (e) and lipase-BSA--CLEAs (f).

Table 1. Comparison of kinetic parameters of free lipase, lipase-CLEAs, and lipase-BSA-CLEAs

Enzyme	K_m (mM)	V_{max} (μM/mL·min)	V_{max}/K_m
Free lipase	123.84	11.09	0.089
Lipase-CLEAs	224.86	4.85	0.021
Lipase-BSA-CLEAs	149.62	4.62	0.031

CLEAs showed that K_m was increased when lipase was immobilized. The increase on K_m may be due to diffusion limitations, which indicated the apparent affinity of immobilized lipase towards the substrates was lowered (Table 1). Although K_m of lipase-BSA-CLEAs was larger than that of lipase-CLEAs, lipase-BSA-CLEAs was an overall better catalyst as reflected in its higher V_{max}/K_m of 33 min^{-1} as compared to V_{max}/K_m of 22.05 min^{-1} for lipase-CLEAs obtained (Table 1). The possible reason is that addition of BSA in the presence of adequate free amino groups facilitates the production of active CLEA particles through efficient cross-linking, which is helpful for prevention of extensive cross-linking of the enzyme molecules and preservation of structural integrity of the enzyme. Furthermore, addition of BSA also enhanced monodispersity of CLEAs to a certain extent.

3. Effect of pH on Stability of Lipase-BSA-CLEAs

After free lipase was immobilized into lipase-CLEAs and lipase-BSA-CLEAs, the pH tolerance was enhanced. As shown in Fig. 5, both lipase-CLEAs and lipase-BSA-CLEAs were much more stable than the free lipase between pH 4.0 and 12.0. Moreover, both CLEAs forms were similarly stable at a broader pH range of 4.0-12.0 (Fig. 5). The resistance against pHs can presumably be related to the fact that most of the available amino groups on the surface of the enzyme would have been engaged in the cross-linking with glutaraldehyde, which caused the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site [30].

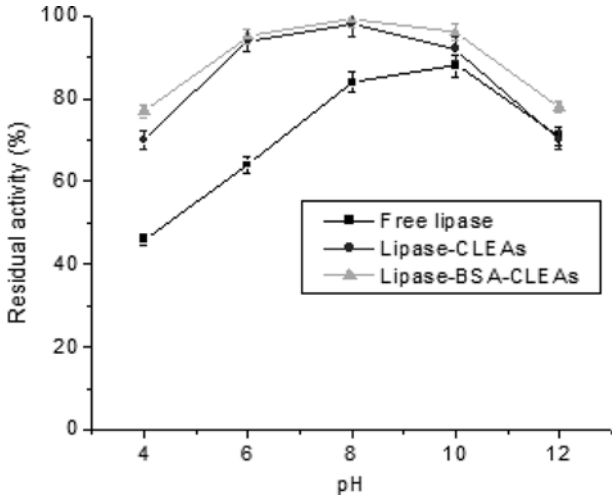

Fig. 5. pH Stability of free lipase, lipase-CLEAs, and lipase-BSA-CLEAs.

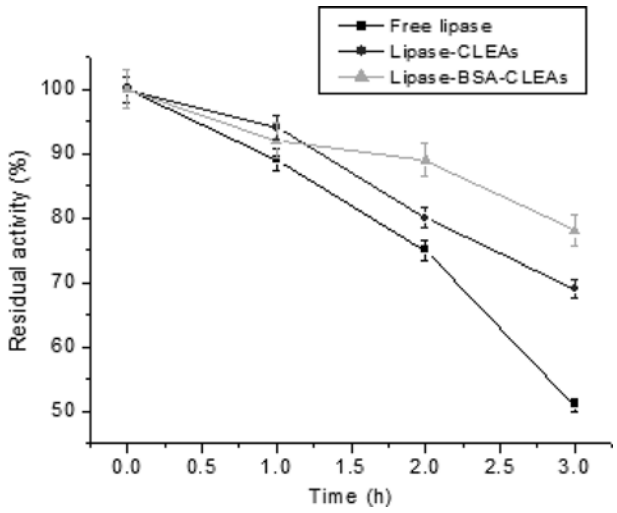

Fig. 7. Thermal stability of free lipase, lipase-CLEAs, and lipase-BSA-CLEAs at 60 °C.

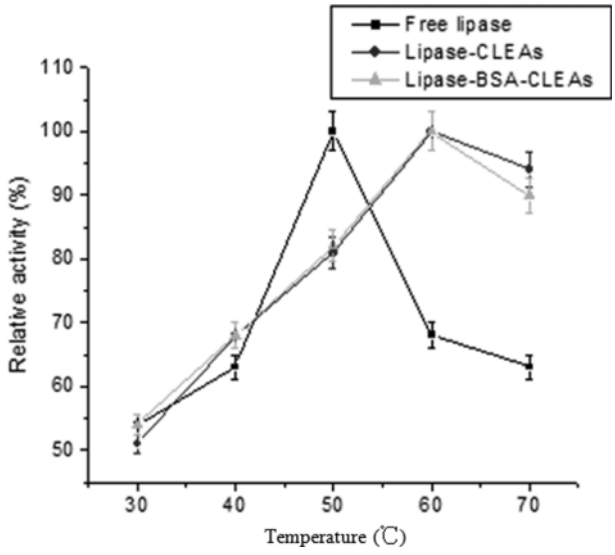

Fig. 6. Optimal temperature for lipase-BSA-CLEAs.

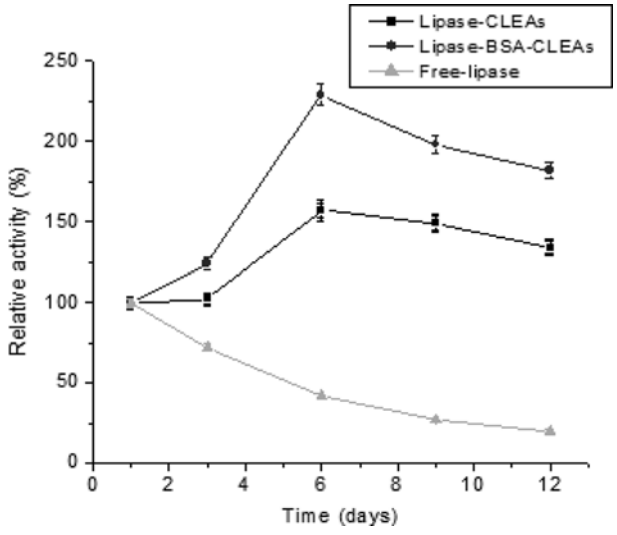

Fig. 8. The storage stability of free lipase, lipase-CLEAs, and lipase-BSA-CLEAs at 25 °C.

4. Effect of Temperature on the Activity and Stability of Lipase-BSA-CLEAs

Effects of temperature on the activity of different forms of lipase were presented in Fig. 6. The optimum temperature of CLEAs forms was measured as 60 °C, which is 10 °C higher than that of free lipase. However, the optimum temperature of lipase-CLEAs and lipase-BSA-CLEAs is similar. Fig. 7 shows that the lipase-BSA-CLEAs were more thermally stable than the free lipase and lipase-CLEAs. This indicated that efficient cross-linking provided more molecular conformation stability of Lipase. For lipase-CLEAs, the number of amine groups of lipase was not enough to achieve efficient cross-linking of all the enzyme molecules. Therefore, the enzyme could be released from the aggregates under high temperature, leading to the lower stability of lipase-CLEAs than lipase-BSA-CLEAs.

5. Storage Stability and Reusability of Lipase-BSA-CLEAs

Storage stability of lipase-BSA-CLEAs is presented in Fig. 8. Storage time played a key role in the activity exhibited by CLEAs. Free lipase decayed rapidly, the residual activities of free lipase after 12 d retained only 20%. Interestingly, activity of both CLEAs forms enhanced with the increase of storage time. The highest retained activity of lipase-CLEAs and lipase-BSA-CLEAs raised to 158% and 229% with respect to free lipase after 6 d, respectively. Furthermore, the retained activity of both CLEAs forms still higher than that of free lipase during 12 d storage time. The phenomenon was also observed by Ferreira group [31]. These findings might be due to the fact that the cross-linking with glutaraldehyde induced conformational changes in lipase. During storage time, the glutaraldehyde molecules on the CLEAs surface might react with amine residues of the lipase surface positioned close to the active site, and thus induce changes in its conformation that could modify lipase activity. In addition, the reusability of immobilized lipase was also evaluated. As shown in Fig. 9, although the activities of the immobilized lipase decayed with increasing number of recycles, the lipase-BSA-CLEAs retained

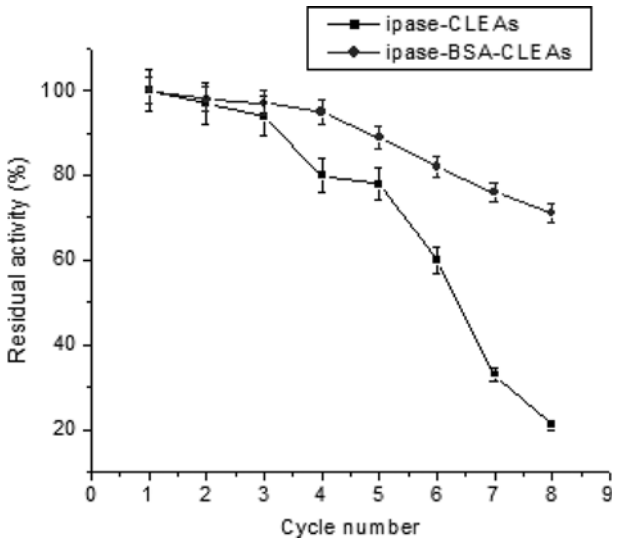

Fig. 9. Reusability of lipase-CLEAs and lipase-BSA-CLEAs.

more than 75% of its initial activity until eight cycles. However, the lipase-CLEAs only retained 20% of its initial activity. The excellent reusability of lipase-BSA-CLEAs could be due to the improvement of cross-linking efficiency of lipase molecules by addition of BSA, which prevented their escape.

CONCLUSIONS

Lipase-BSA-CLEAs were successfully prepared using 0.05 g/L BSA and 1% (v/v) glutaraldehyde for 2 h. Co-aggregation of lipase with BSA dramatically increases cross-linking efficiency. A higher activity recovery of 75% was obtained. Compared to lipase-CLEAs, CLEAs of lipase prepared in the presence of BSA exhibited more stability against various deactivating conditions, including high temperature and extreme pH. Moreover, the lipase-BSA-CLEAs displayed excellent reusability and storage stability, which would allow its exploitation in industrial. These results illustrated that co-aggregation with BSA is an attractive, effective and economic way to produce high active and stable CLEAs.

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