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A facile technique to prepare cross-linked enzyme aggregates using *p*-benzoquinone as cross-linking agent

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Abstract−To obtain robust and thermo-stable enzyme aggregates, *p*-benzoquinone was used as cross-linker and bovine serum albumin (BSA) as crowding macromolecules to prepare cross-linked enzyme aggregates (CLEAs) of lipase. Effects of cross-linking time and cross-linker content on the activity, thermal stability and characteristics of enzyme aggregates were examined carefully. It was observed that when the content of *p*-benzoquinone was 5 mM and amount of BSA was 125% of that of lipase (w/w), the specific activity of cross-linked co-aggregates of lipase and BSA was 79.8 U mg^{-1}, 2.44-fold of that of cross-linked enzyme aggregates of lipase without BSA. Moreover, after heat treatment for 96 h at 50 °C, the CLEAs prepared with this facile routine kept 75.18% of their initial activity, 5.01-fold more than that of the just CLEAs using glutaraldehyde. Furthermore, BSA macromolecules in lipase CLEAs enhanced the catalytic efficiency of free and just lipase CLEAs without BSA by 1.45 and 2.83 times, respectively. The proposed cross-linking technique would rank among the potential strategies for efficiently preparing robust and thermo-stable enzyme aggregates.

Key words: Cross-linked Enzyme Aggregates, *p*-Benzoquinine, Macromolecule Crowding, Lipase, Bovine Serum Albumin

INTRODUCTION

Enzyme-catalyzed reactions are of increasing interest for the industrial synthesis of fine chemicals and pharmaceutical, due to the increase in environmental awareness. The skill to stabilize and reuse enzyme catalyst through immobilization has proven one of the key steps to render an enzymatic process economically viable [1,2]. By now, enzyme immobilization has been revealed as a very powerful tool to enhance most of enzyme properties, if properly designed: e.g., stability, activity, specificity and selectivity, reduction of inhibition [3,4]. Among the immobilization technologies, a promising strategy is cross-linked enzyme aggregates (CLEAs), a kind of carrier-free immobilization [5], using a cross-linking reaction between enzyme molecules to yield immobilized enzyme [6-9]. This immobilization technology is attractive for its low cost, simplicity and efficiency.

The general approach for CLEAs preparation consists of protein aggregation followed by cross-linking with glutaraldehyde or aldehyde-dextran as cross-linking agent [10-14]. Glutaraldehyde cannot be used to crosslink the hydroxyl and sulfhydryl group of enzyme with amino and hydroxyl of polymer. In the case of an enzyme with a low number of reactive ε-amino groups, the aggregation has to be performed in the presence of a polymer and macromolecules with many amino groups [15]. However, Schiff bases are unstable under acidic conditions and tend to break down to regenerate the aldehyde and amine [16], which might impair binding and lead to gradual release of the enzyme into buffer solutions.

In the present work, lipases (triacylglycerol acylhydrolases, EC 3.1.1.3), versatile biocatalysts, were used as model and *p*-benzoquinone was used as cross-linker and BSA was used as macromolecule reagent to form the CLEAs, enhancing the activity and thermal stability based on our previous covalent enzyme immobilization work [17]. This novel technique was expected to obtain more stable CLEAs than that using glutaraldehyde because C-O and C-N bonds are tolerant to variation in pH and stable even in acidic condition.

EXPERIMENTAL SECTION

1. Preparation of Lipase Cross-linked Enzyme Aggregates

Lipase, a gift from Dr. Xiaolin Pei, was from the recombinant strain *E. coli* BL21 (pET28a-lip2). This strain contains the gene from *Geobacillus* sp. Substrate, p-nitrophenyl acetate (p-NPA), was obtained from TCI (Shanghai) Development Co., Ltd. bovine serum albumin (BSA) commercially available was obtained from Sigma-Aldrich. *p*-Benzoquinone was provided by Sinopharm Chemical Reagent Co., Ltd. Deionized water with a resistance greater than 18 MQ was obtained from a Millipore-Q Plus water purifier. *p*-Benzoquinone cross-linked co-aggregates of lipase and BSA was prepared by adding BSA solution of high content in phosphate buffer under agitation to 300 μL of lipase solution (3.2 mg mL^{-1}, PBS, pH 6.5) to precipitate the enzyme. After 10 min, *p*-benzoquinone solution was added to cross-link the enzyme precipitate, and the mixture was kept under stirring for certain period of time at 25 °C. The CLEAs produced were repeatedly washed with 100 mM sodium phosphate buffer at pH 6.5 for three times and centrifuged at 10,000

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rpm for 8 min each time. All operations were conducted at room temperature, and the pH was controlled at a value of 6.5. Just lipase CLEAs were prepared using the above method without adding BSA into the mixture.

2. Study of the Enzyme Release from the Aggregates and Sufficient Crosslinker Content

To determine the covalent crosslink of lipase with each other and sufficient crosslinker content, different aggregates, prepared using different content of *p*-benzoquinone, were boiled for 5 min in 500 μL of 5% sodium dodecyl sulfate (SDS). In this way, any noncovalently attached molecule in the aggregate was released to the medium [15]. Then, the boiled mixture was centrifuged and the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was stained with Coomassie blue and analyzed by densitometry.

3. Enzyme Activity Assay

The activity of free lipase and CLEAs was determined by a previously reported assay method using *p*-nitrophenyl acetate as a substrate [18]. The reaction mixture (3.0 mL) was composed of 2.85 ml phosphate buffer (pH 6.5, 0.01 M), enzyme solution of appropriate dilution and appropriate CLEAs in which the buffer was used for the blank. The mixture was incubated for 2 min at 37 °C. Reactions were initiated by addition of 0.15 mL of a *p*-nitrophenyl acetate solution and stopped by the addition of acetone (3 mL) after 3 min. The mixtures were clarified by filtration, and the absorbance of solution due to the release of *p*-nitrophenol was measured at 405 nm. One unit of lipase activity was defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol per min. Coupled yield and relative activity of immobilized enzyme were calculated as our previous work [19]. Experiments were carried out in triplicate, and standard error was never over 5%.

4. Determination of the Km and Vmax for Lipase Preparations to Hydrolyze the *p*-Nitrophenyl Acetate

The kinetic constants, Km and Vmax values of free and lipase CLEAs, were determined by measuring initial rates of the reaction with *p*-NPA (0.1-3.0 mM) as a substrate in phosphate buffer solution (0.01 M, pH 6.5) at 37 °C. Km and Vmax were calculated from the Lineweaver-Burk plots using the initial rate of the reaction data.

$$-\frac{1}{Vs}=\frac{Km}{Vmax\bullet[S]}+\frac{1}{Vmax} \quad (1)$$

Where, [S] is the concentration of the substrate, Vs and Vmax represent the initial and maximal rate of the reaction, respectively, and Km is the Michaelis constant.

5. Thermal and Storage Stability of Different Lipase Preparations

For thermo-stability, free enzyme and immobilized enzyme were transferred into the reaction medium and incubated at 50 °C for different time period. Periodically, samples of the suspension were withdrawn and their remaining activities were assayed as described above. Thermal stability is given as residual activity of the immobilized derivatives or soluble enzyme. The initial activity of enzyme preparations was set as 100%.

For storage stability experiments, enzyme preparation and free enzyme in solution were stored in sealed bottles and placed at 4 °C in the refrigerator without further protection. Aliquots were taken periodically and enzyme activity was measured as described above.

RESULTS AND DISCUSSION

1. Preparation of Lipase CLEAs Using *p*-Benzoquinone as Cross-linker and BSA as Crowding Macromolecules

Lipase CLEAs were prepared by cross-linking enzyme molecules with BSA macromolecules using *p*-benzoquinone as cross-linker. Fig. 1 shows the procedures for cross-linking the nucleophilic groups such as amino and hydroxyl groups of enzyme and BSA using *p*-benzoquinone. BSA was used as crowding molecules to stabilize the enzyme structure based on macromolecular crowding theory [20]. As is known, glutaraldehyde can easily cross-link the amino groups of enzyme by forming the unsaturated bond and Schiff base reactions

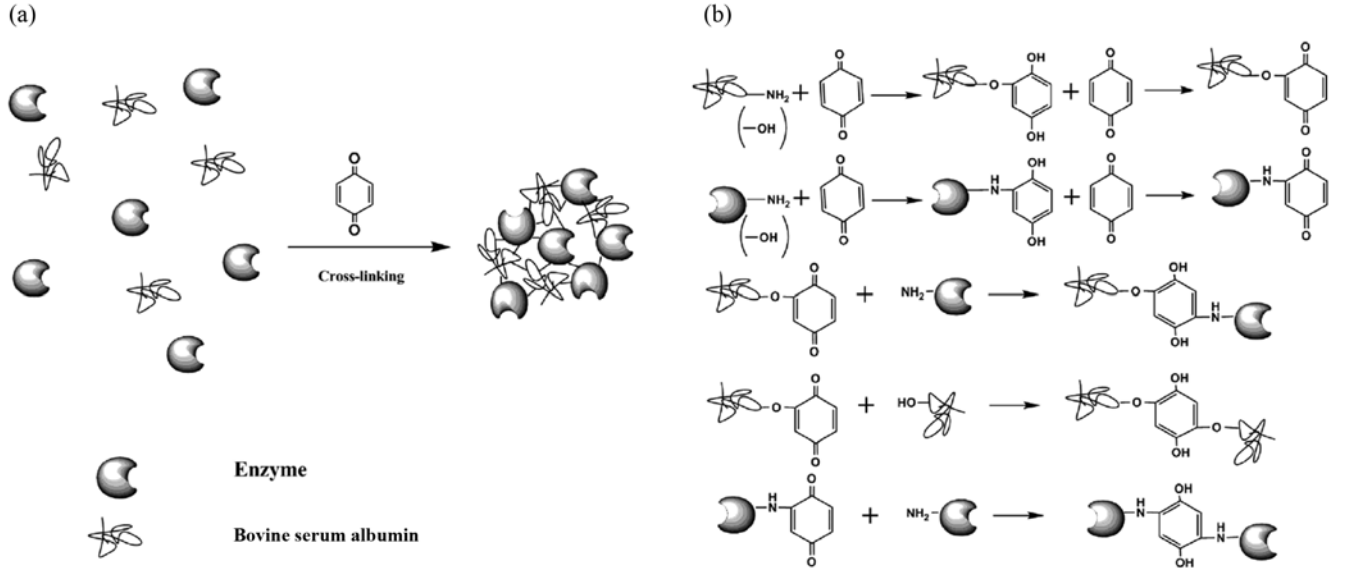

Fig. 1. Facile route to prepare CLEAs and possible mechanism for the cross-linking reaction using *p*-benzoquinone as cross-linker. (a) route to prepare CLEAs; (b) possible mechanism for the cross-linking reaction using *p*-benzoquinone as cross-linker.

with proteins [15,21]. However, Schiff bases are unstable under acidic conditions and tend to break down to regenerate the aldehyde and amine [16], which might impair binding and lead to gradual release of enzyme into buffer solutions. In the present work, lipase was cross-linked using *p*-benzoquinone to produce stable CLEAs based on our previous work [17,22], which possibly resulted from that C-O and C-N bonds were tolerant to variations in pH and is stable even in acidic conditions.

2. Effect of Cross-linking Time on the Coupled Yields, Activity and Thermal Stability of Lipase CLEAs

Table 1 presents the influence of cross-linking time on the coupled yield and activity of lipase CLEAs. When Lipase was immobilized with CLEAs method using *p*-benzoquinone as cross-linker, the coupled yield of lipase seemed stable as cross-linking time grew. It seems that 60 min is enough for the cross-linking between the enzymes. Unlike the couple yield, the specific activity of lipase CLEAs decreases, and it decreased to 22.86 U mg^{-1} when cross-linking time was 240 min.

Fig. 2 shows the effect of cross-linking time on the thermal stability of lipase CLEAs. 150 minutes may be suitable for the lipase cross-linking to form thermally stable CLEAs. After heating for 48 hours at 50 °C, the residual activity of CLEAs with cross-linking time of 60 and 240 min was 46.4% and 27.6% of the initial activity, respectively. However, the residual activity of CLEAs with cross-linking time of 150 min was 60.0% of its initial activity after even 96 hours at 50 °C. This indicates that 150 min is more suitable than 60 min while preparing the thermally stable lipase CLEAs using using *p*-benzoquinone as cross-linking agent.

Table 1. Effect of cross-linking time on the coupled yield and activity of lipase CLEAs

Cross-linking time (min)	Coupled yield (%)	Specific activity (U mg^{-1})
60	73.07	27.66
150	72.93	26.29
240	72.93	22.86

The contents of lipase and *p*-benzoquinone were 3.2 mg mL^{-1} and 10 mM in the immobilization mixture, respectively. The mixture was stirred at 150 rpm for different period of time. The temperature for cross-linking reaction was 25 °C and the pH was controlled at a value of 6.5.

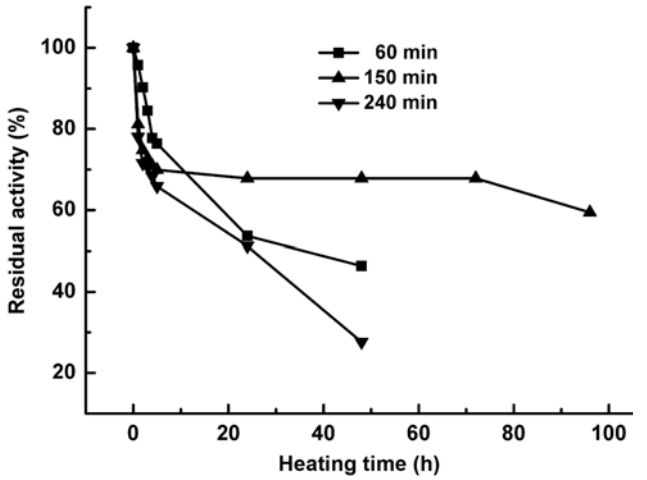

Fig. 2. Effect of cross-linking time on the thermal stability of lipase CLEAs, the contents of lipase and *p*-benzoquinone were 3.2 mg mL^{-1} and 10 mM in the immobilization mixture, respectively. The mixture was stirred at 150 rpm for different time period. The temperature for cross-linking reaction was 25 °C and the pH was controlled at a value of 6.5.

3. Effect of Cross-linker Content on the Coupled Yields, Activity and Thermal Stability of Lipase CLEAs

Table 2 gives the correlation of cross-linker content with the coupled yield and activity of CLEAs. When cross-linker content varied from 5 to 20 mM, there was no clear rise for the coupled yield of lipase when the content was rather high (20 mM). However, the specific activity of lipase in CLEAs varied distinctly. The specific of CLEAs prepared with 5 mM of *p*-benzoquinone is 32.72 U mg^{-1}, 1.30 fold of that of CLEAs prepared with 20 mM of *p*-benzoquinone.

Similar to the specific activity, thermal stability of CLEAs preparation shows notable differences from each other. Fig. 3 shows that the residual activity of CLEAs prepared using 5 mM of *p*-benzoquinone was 65.2% of its initial activity, 2.83 fold of that of CLEAs prepared with 20 mM of *p*-benzoquinone. It has been reported that multipoint covalent attachment of proteins on supports clearly increased the thermal stability of immobilized enzymes. However, the stabilizing effect increases with the number of covalent bonds

Table 2. Effect of content of cross-linker on the coupled yield and activity of lipase CLEAs

Content (mM)	Coupled yield (%)	Specific activity (U mg^{-1})
5	73.62	32.72
10	72.93	26.29
20	76.06	25.20

The content of lipase was 3.2 mg mL^{-1} in the immobilization mixture. The mixture containing different content of *p*-benzoquinone was sttired at 150 rpm for 150 min. The temperature for cross-linking reaction was 25 °C, crosslinking time was 150 minutes, and the pH was controlled at a value of 6.5.

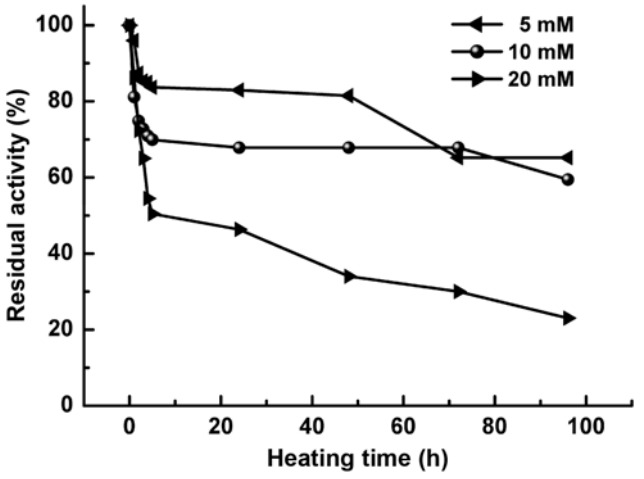

Fig. 3. Effect of *p*-benzoquinone cross-linker content on the thermostability of CLEAs preparations.

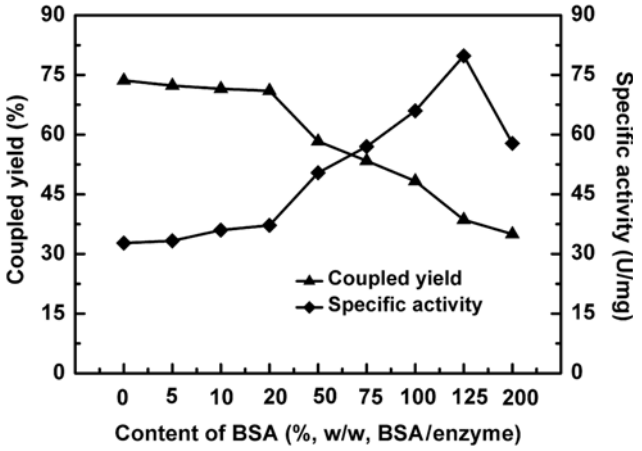

Fig. 4. Effect of content of BSA on the coupled yield and specific activity of lipase CLEAs using *p*-benzoquinone as cross-linking agent. Crosslinking time was 150 minutes.

between enzymes and the support until some critical value (a limit) is achieved, and further increase in the number of bonds does not lead to further stabilization [23]. In the present work, excessive groups of enzyme would be covalently linked with each other in the CLEAs obtained by using a high content of cross-linker, which might damage enzyme structure and deactivate enzyme [1].

4. Effect of BSA Content on the Coupled Yield and Activity of Lipase CLEAs

To further enhance the properties of lipase CLEAs using *p*-benzoquinone, BSA was added to the immobilization mixture. Fig. 4 shows that BSA reagents exert a considerable influence on the coupled yield and specific activity of CLEAs. When the content of *p*-benzoquinone was 5 mM and amount of BSA was 125% of that of lipase (w/w), the specific activity was 79.8 U mg^{-1}, 2.44 fold of that of CLEAs without BSA. This increment in the catalytic activity of lipase CLEAs possibly resulted from the macromolecular crowding [24] derived from the macromolecules beside the enzyme protein in the CLEAs. The crowding could accelerate the refolding of enzyme protein [25,26] and increase the enzymatic activity [20,27].

5. Kinetic Parameters of Lipase CLEAs Preparations

To explore the effects of cross-linking agent and BSA on the CLEAs kinetic properties, the kinetic parameters of free lipase and CLEAs preparations were compared with each other. In this work, *p*-nitrophenyl acetate (p-NPA) was used as substrate to examine the biocatalytic activity of the immobilized lipase preparation. Michaelis-Menten parameters, Km and kcat, interpreted from the Lineweaver-Burk plots, are shown in Table 3. The data indicate that a suitable amount of BSA improved the catalysis efficiency (Kcat/Km) of CLEAs when *p*-benzoquinone was used as cross-linking agent. When 10% of BSA (w/w) was added to the immobilization mixture, Kcat/Km was $2.0*10^4$ $min^{-1}\cdot mM^{-1}$, 1.45- and 2.82-fold of that of free enzyme and CLEAs just using *p*-benzoquinone as cross-linking agent. This increase in Kcat/Km from appreciable amount of BSA was fairer than that [28] using glutaraldehyde as cross-linking agent. Excessive amount of BSA was not useful for improving the catalysis efficiency.

When glutaraldehyde was used as cross-linking agent, catalysis efficiency of CLEAs was higher than that of CLEAs obtained using

Table 3. Kinetic parameters of the lipase CLEAs preparations

Lipase CLEAs preparations	Km (mM)	Kcat (10^3, S^{-1})	Kcat/Km (10^4, min^{-1} mM^{-1})
Free enzyme	5.20	1.20	1.38
CLEAs-GA	16.70	4.99	1.79
CLEAs-BQ	20.68	2.44	0.71
CLEAs-BQ-10BSA	1.57	0.51	2.00
CLEAs-BQ -100BSA	3.51	0.55	0.94

Conditions for preparing lipase CLEAs preparations: CLEAs-GA, glutaraldehyde was used as cross-linking agent and content was 5 mM; CLEAs-BQ, content of *p*-benzoquinone was 5 mM; CLEAs-BQ-10BSA, content of *p*-benzoquinone was 5 mM and amount of BSA to that of enzyme was 10% (w/w); CLEAs-BQ-100BSA, the content of *p*-benzoquinone was 5 mM and amount of BSA to that of enzyme was 100% (w/w)

p-benzoquinone; however, it was still not lower than that of CLEAs obtained with lipase and BSA. In addition, Km of CLEAs was 1.57 mM, significantly decreasing when 10% of BSA was added into the immobilization mixture compared to other lipase preparations. The lowest Km suggests that the substrate-enzyme affinity of CLEAs obtained with lipase and 10% of BSA was highest.

6. Storage Stability of Free Lipase and CLEAs Preparations

Fig. 5 illustrates the storage stability of free lipase and CLEAs preparations at 4 °C for long terms of storage. CLEAs of lipase and 10% of BSA retained more than 55.0% of its initial activity at 4 °C after 30 days. The retained activity was more than 2.13- and 1.77-times of that of free enzyme and CLEAs using glutaraldehyde as cross-linking agent, respectively. Moreover, the cross-linking method also exerted a great influence on CLEAs storage stability. CLEAs

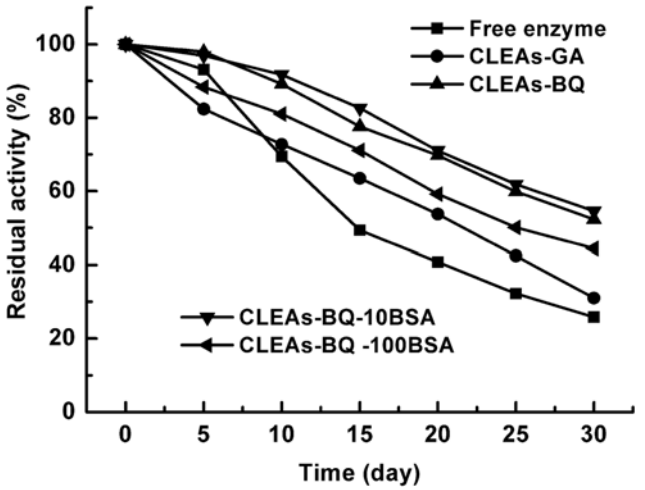

Fig. 5. Storage stability of free lipase and CLEAs preparations, conditions for preparing lipase CLEAs preparations: CLEAs-GA, glutaraldehyde was used as cross-linking agent and content was 5 mM; CLEAs-BQ, content of *p*-benzoquinone was 5 mM; CLEAs-BQ-10BSA, content of *p*-benzoquinone was 5 mM and amount of BSA to that of enzyme was 10% (w/w); CLEAs-BQ-100BSA, content of *p*-benzoquinone was 5 mM and amount of BSA to that of enzyme was 100% (w/w). Crosslinking time was 150 minutes.

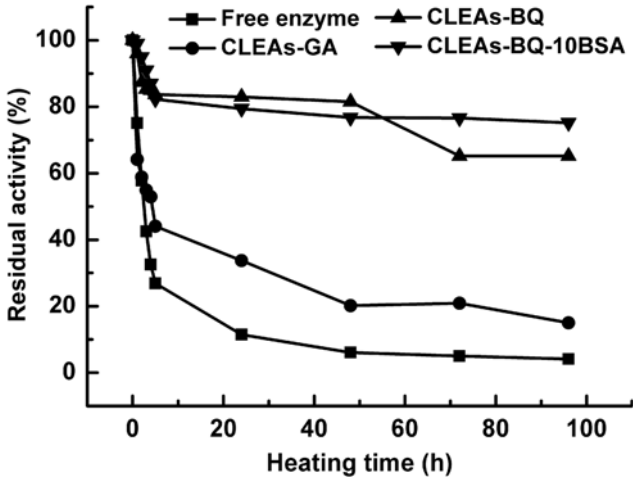

Fig. 6. Thermo-stability of free lipase and CLEAs preparations, conditions for preparing lipase CLEAs preparations: CLEAs-GA, glutaraldehyde was used as cross-linking agent and content was 5 mM; CLEAs-BQ, content of *p*-benzoquinone was 5 mM; CLEAs-BQ-10BSA, content of *p*-benzoquinone was 5 mM and amount of BSA to that of enzyme was 10% (w/w). Crosslinking time was 150 minutes.

using *p*-benzoquinone as cross-linking agent retained 52.3% of its initial activity, 1.69-fold of that using glutaraldehyde.

These results indicate that lipase CLEAs using *p*-benzoquinone as cross-linking agent had good storage stability, especially for CLEAs obtained with lipase and 10% of BSA macromolecular reagents. The decrease in the activity of CLEAs using *p*-benzoquinone as cross-linking agent was slower than that using glutaraldehyde as cross-linking agent. From this it may be concluded that the strong linkage between enzymes from *p*-benzoquinone stabilizes the enzyme protein structure, nor does that from glutaraldehyde [16].

7. Thermo-stability of Free Lipase and CLEAs Preparations

Besides the catalytic activities, catalysis efficiency and storage stability of enzyme preparations, thermo-stability of CLEAs were also carefully examined. As shown in Fig. 6, *p*-benzoquinone and BSA used in the cross-linking technique presented significant improvement of thermo-stability of lipase CLEAs. After heating at 50 °C for 96 h, free lipase almost lost its catalytic activity. Residual activity of lipase CLEAs using glutaraldehyde as cross-linking agent was also only 15.1% of its initial activity. However, the residual activity of CLEAs of lipase and BSA using *p*-benzoquinone as cross-linking agent was 75.18% after heating at 50 °C for 96 h, about 5.01-fold higher than that of the lipase CLEAs using glutaraldehyde as cross-linking agent.

The typically enhanced thermo-stability of lipase can be interpreted as intra- and intermolecular crosslinks, which leads to more rigid molecules that can resist conformational changes [29]. In fact, the covalent bonds created during the crosslinking reaction are stable even at higher temperature. However, lipase is marginally stable in their CLEAs using glutaraldehyde as cross-linking agent, the prominent cause of their irreversible inactivation being cross-linkage by Schiff-base forming reactions [10]. *p*-Benzoquinone can crosslink enzyme by crosslinking the hydroxyl, sulfhydryl and amino groups of enzyme more fully and completely than glutaraldehyde, which would produce rigid and stable CLEAs.

CONCLUSIONS

The present study demonstrated a simple and successful routine to obtain robust and thermally stable lipase CLEAs using *p*-benzoquinine as cross-linker and bovine serum albumin (BSA) as crowding macromolecules. CLEAs prepared using *p*-benzoquinone cross-linker exhibit even better thermal stability than that using glutaraldehyde cross-linker. When 10% of BSA (w/w) was added in to the immobilization mixture, Kcat/Km was 2.0×10^4 min^{-1} mM^{-1}, 1.45- and 2.82-fold of that of free enzyme and CLEAs just using *p*-benzoquinone as cross-linking agent. Most of all, after heat treatment for 96 h at 50 °C, the CLEAs prepared with this facile method kept 75.18% of their initial activity, 5.01-fold more than just that of the CLEAs using glutaraldehyde cross-linker. The strategy is extremely simple and may be of general use to produce rigid and stable CLEAs which can be utilized to enhance the enzymatic preparation of some chemicals and products in biocatalysis.

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