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Cross-linked enzyme aggregates (CLEAs) with controlled particles: Application to *Candida rugosa* lipase

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Abstract

Aggregation agent type and concentration, lipase and glutaraldehyde concentration, and pH are able to affect the formation of cross-linked lipase. The carrier-free immobilized *Candida rugosa* lipase with a particle size of 40–50 μm showed higher activity than that of the lipase with other particle sizes. The carrier-free immobilized *C. rugosa* lipase can keep 86% original lipase activity (0.018 g g^{-1} min^{-1}). The enantioselectivity of the carrier-free immobilized lipase (23.3) was about 1.8 times as much as that of the native lipase (13.0) in kinetic resolution of ibuprofen racemic mixture.

Keywords: Carrier-free; Immobilization; *Candida rugosa* lipase; Particle size

1. Introduction

Recently carrier-free enzyme immobilization approach has attracted more attention due to clear advantages: highly concentrated enzyme activity in the catalyst, high stability and the low production cost due to the exclusion of an additional carrier [1–4]. Cao et al. [5] reviewed the various carrier-free enzyme immobilization approaches. Generally they are prepared by directly cross-linking different enzyme preparations such as dissolved enzyme, crystalline enzyme, spray-dried enzyme and physically aggregated enzyme, resulting in the formation of cross-linked dissolved enzymes (CLDEs), cross-linked enzyme crystals (CLECs), cross-linked spray-dried enzymes (CSDEs) and cross-linked enzymes aggregated (CLEAs), respectively. Thus, they are discriminated from each other only by the precursors used for cross-linking. Among these techniques, CLEA is a simpler one developed by Cao et al. in 2000 [6]. The procedure of the approach consists of the covalent cross-linking of a precipitated enzyme. The idea is from the concept that protein in general can be precipitated by agents such as inorganic salts or organic solvents without undergoing denaturation. This technique had been successfully applied in the preparation of carrier-free enzyme immobilization of aminoacylase and some lipases [7–9]. Nevertheless, how to control the lipase particle size in the CLEA process and the effect of particle size on the activity of the lipase had rarely been reported. In the paper, *Candida rugosa* lipase was precipitated to be cross-linking aggregate; some factors to influence the cross-linking lipase particle size including aggregation agent types, fraction of the aggregation agent and pH, were reported. The effect of particle size on the activity of the lipase and the enantioselectivity of the cross-linked lipase for kinetic resolution of ibuprofen with 1-propanol in isooctane at 30 °C were investigated.

2. Materials and methods

2.1. Chemicals and enzyme

The lipase (E.C. 3.1.1.3) from *C. rugosa* was purchased from Sigma–Aldrich Company. The concentration of protein in the crude enzyme is 196.2 mg protein g^{-1} determined by UV detector at the wavelength of 280 nm. All other chemicals were of a reagent grade and obtained commercially.

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2.2. General CLEA preparation

C. rugosa lipase (100 mg) was dissolved in phosphate buffer (2 ml, 0.1 M, pH 7). After centrifuge, the transparent enzyme solution was shifted to a crystallizer (50 ml) designed in our lab. $(NH_4)_2SO_4$ (1.2 g), $(NH_4)_2SO_4$ solution (2 ml, 60%, w/v) in phosphate buffer (0.1 M, pH 7) and 10–90 μl glutaraldehyde (70%, w/v in water) were added. The pH of the buffer was adjusted by HCl and NaOH from 2 to 10. The mixture was stirred at 4 °C for 20 h. After then, the mixture was centrifuged. The supernatant was decanted and the residue was washed three more times with buffer, centrifuged and decanted. The enzyme left was lyophilized for overnight. The final enzyme preparation was used to test particle size and specific activity. The particle size was determined by LS Particle Size Analyzer from COULTER for three times. Generally, 70–80% protein crystals share a similar particle size. Therefore, herein, the mean value was used to represent the particle size.

2.3. Assay of lipase activity and stability

The reaction mixture (10 ml) consisted of lauric acid (0.1 M), 1-propanol (75 μl) and crude or immobilized lipase in isooctane. The mixture was incubated at 30 °C for 3 h with continuous shaking at 180 rpm. Reaction samples (1 ml) were withdrawn and mixed with 10 ml ethanol/acetone (1:1, v/v). The remaining acid was determined by titration with 0.05 M NaOH. Specific activity of the enzyme was defined as the amount of acid consumed g^{-1} protein min^{-1} for three times.

2.4. Determination of enzyme enantioselectivity

(*R,S*)-ibuprofen (4.06 mg, 2 μM) was dissolved in isooctane (4 ml) containing 1-propanol (6 μM) and 40 mg of immobilized lipase or 14.1 mg native lipase. The suspension was incubated at 30 °C under continuous shaking (190 rpm). Samples (10 μl) were collected at predetermined time intervals for analysis by HPLC for three times.

3. Results and discussion

3.1. Salt effect on particle size

It is possible to form aggregates by changing the hydration state of enzyme molecules or by altering the electrostatic constant of the solution by adding appropriate aggregation agents. Not all salts have the same effect on the lipase aggregation. It is well understood that the different salt exhibits different aggregation effect on the specific lipase. Therefore it is crucial to screen a suitable salt to be aggregation agent in the process of CLEAs. In the part, some salts were applied to be aggregation agents for the preparation of *C. rugosa* lipase (CRL) CLEAs and the results were shown in Table 1. The results presented that LiCl and NaCl had no effect, NH_4Cl could not exhibit strong effect and Na_2SO_4 and $(NH_4)_2SO_4$ claimed a good effect, on the precipitation of CRL. $(NH_4)_2SO_4$ was selected as the aggregation agent in the subsequent work.

Table 1
Salt effects on solubility and particle size of CRL CLEAs

Salts	Salt concentration ($g\,ml^{-1}$)	Solubility (%)	Particle size (μm)[a]
LiCl[b]	0.69	100	–
NaCl[b]	0.36	100	–
NH_4Cl[b]	0.37	83	6.37
Na_2SO_4[b]	0.2	23	16.86
$(NH_4)_2SO_4$	0.6	17	16.45

[a] Mean particle size.
[b] Saturated salt solution.

3.2. Ammonium sulfate ($(NH_4)_2SO_4$) fraction effect on particle size

It was demonstrated that the maximal activity of the insoluble cross-linked enzyme was recovered at the point where the majority of the protein is precipitated out of the solution; the lowest activity was obtained without any precipitant. In the process, the fraction of ammonium sulfate played a key role to control the formation of the particle of the enzyme. The particle size of the protein in the process could also be affected by the fraction of ammonium sulfate. Fig. 1 shows the process of *C. rugosa* lipase precipitation is controlled by ammonium sulfate fraction. At the beginning of precipitation of the lipase, the particle size was very small (1–2 μm) because fewer protein precipitated and could not form bigger particle. The particle size of the *C. rugosa* lipase grew with adding ammonium sulfate until most of protein had precipitated. Generally the mean particle size of the enzyme could be controlled in 16–17 μm when the most of CRL had precipitated.

3.3. Enzyme and glutaraldehyde concentration effect on particle size

It is clear that the concentration of enzyme and glutaraldehyde in the enzyme solution has a strong effect on the particle size of CLEAs. Figs. 2 and 3 show that the particle size becomes bigger

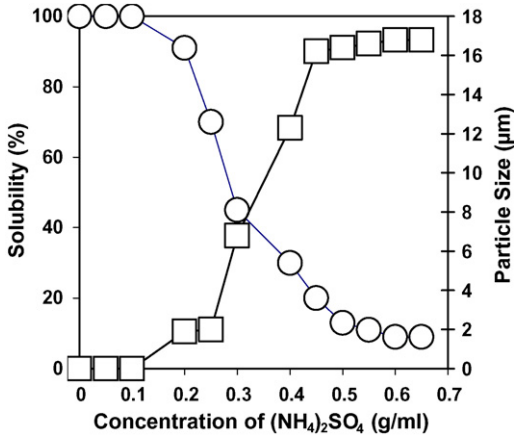

Fig. 1. Plot of the effects of concentration of $(NH_4)_2SO_4$ on particle size of *Candida rugosa* lipase protein aggregates. Various concentration $(NH_4)_2SO_4$ solutions in phosphate buffer (0.1 M, pH 7) and 40 μl glutaraldehyde (70%, w/v in water) were added in 2 ml lipase solution (50 mg/ml).

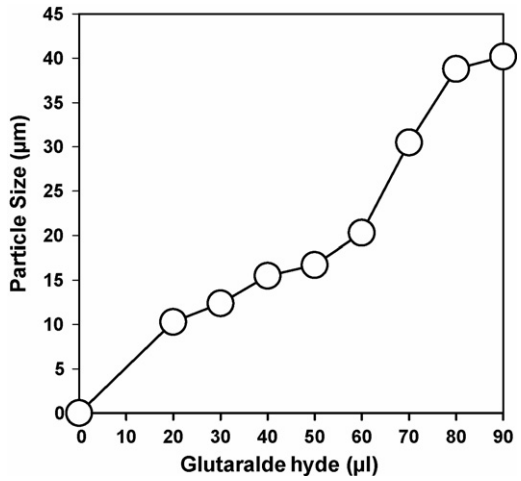

Fig. 2. Plot of the effects of glutaraldehyde amount on particle size of *C. rugosa* protein aggregates. Various amounts of glutaraldehyde (70%, w/v in water) were added in the mixed solutions of 2 ml $(NH_4)_2SO_4$ solution (0.6 g/ml, pH 7) and 2 ml lipase solution (50 mg/ml).

with increasing the concentration of enzyme and glutaraldehyde in the enzyme solution. It is easy to understand that more proteins precipitates at high enzyme concentration solution and formed bigger CLEAs; more amounts of glutaraldehyde are able to make more proteins cross-linked.

3.4. pH effect on particle size

One of the important reasons to cause proteins to precipitate is electrostatic balance loss in the enzyme solutions. pH value is able to adjust the electrostatic balance in the enzyme solutions and affect the formation of precipitants. Fig. 4 shows different particle size of the enzyme is formed under the different pH value. The protein did not precipitate at pH 2. The particle size at pH 10 was slightly larger than that at pH 7, a favorite condition for keeping CRL specific activity. It could be concluded that an

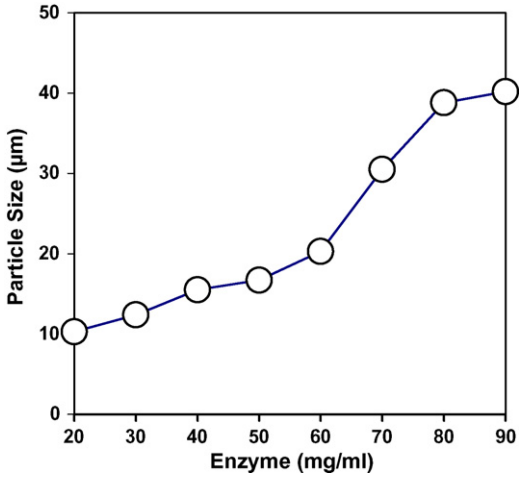

Fig. 3. Plot of effects of enzyme concentration on particle size of *C. rugosa* lipase protein aggregates. A series of 2 ml lipase solution with different concentration were added in the mixed solutions of 2 ml $(NH_4)_2SO_4$ solutions (0.6 g/ml, pH 7) and 40 μl glutaraldehyde (70%, w/v in water).

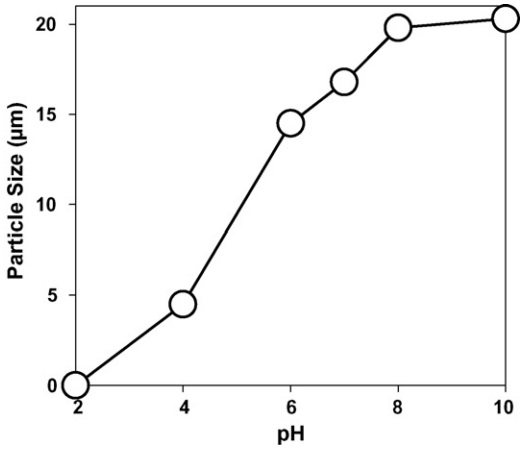

Fig. 4. Plot of the effects of pH on particle size of *C. rugosa* protein aggregates. A series of 2 ml $(NH_4)_2SO_4$ solution (0.6 g/ml) with different pH were added in the mixed solutions of 2 ml lipase solution (50 mg/ml) and 40 μl glutaraldehyde (70%, w/v in water).

alkali environment was favorable to the formation of CLEAs of CRL.

3.5. Particle size effect on lipase activity

According to the Michaelis–Menton theory, the substrates must form Michaelis–Menton complex with enzymes before the catalytic reaction takes place. The particle size of the enzymes can influence mass transfer of the substrates to contact with the inner enzymes of the CLEAs. If the particle size of the immobilized enzymes is too big, the inner enzymes of the CLEAs lose the opportunity to form complex with substrates, in other words, these enzymes waste their activity. If the particle size of the immobilized enzymes is too small, all enzyme particles have a direct contact with organic solvents so it is harmful for enzyme to keep activity. Fig. 5 shows that the enzyme activity is the highest (0.015 g g^{-1} min^{-1}) at the particle size of 40–50 μm. Compared with the native *C. rugosa* lipase activity (0.018 g g^{-1} min^{-1}), the carrier-free immobilized lipase can keep 86% lipase activity.

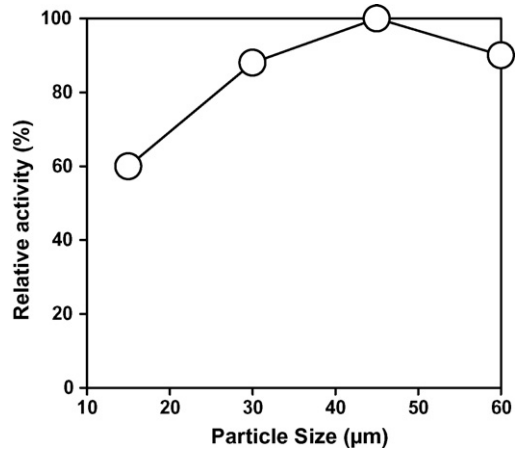

Fig. 5. Plot of the effects of particle size on the enzyme activity. Hundred percent activity corresponds to the activity of lipase with a particle size of 40–50 μm (0.015 g g^{-1} min^{-1}).

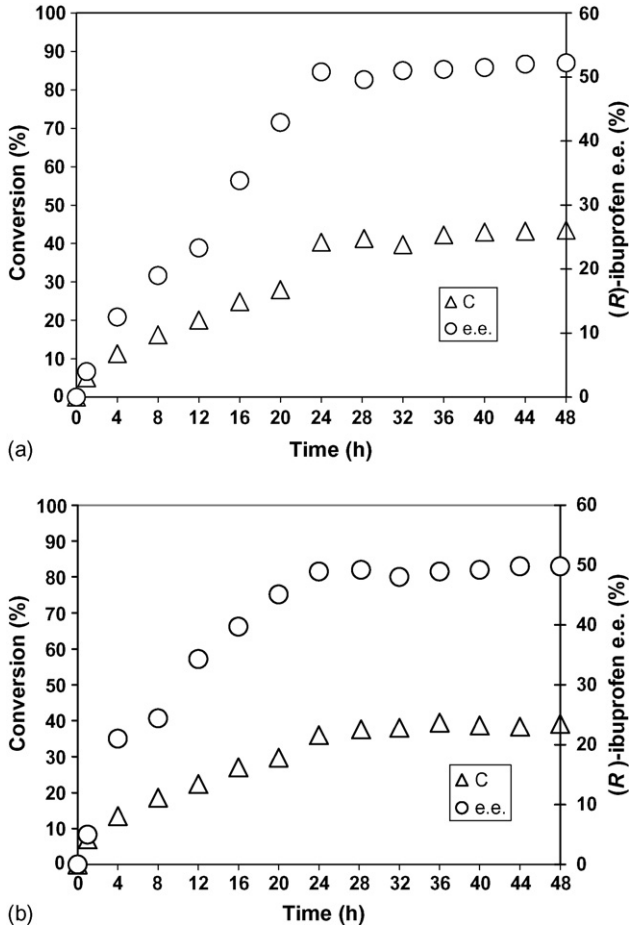

Fig. 6. Time courses of substrate conversion (C) and enantiomeric excess (e.e.) of esterification of (*R,S*)-Ibuprofen with 1-propanol in isooctane at 30 °C. (a) Native lipase and (b) cross-linked lipase.

3.6. Enantioselectivity of the cross-linked lipase

The reaction scheme and time course of esterification of (*R,S*)-ibuprofen with 1-propanol in isooctane is shown in Fig. 6(a) and (b), respectively. The E value (23.3) of the cross-linked lipase was 1.8 times as much as that of the native lipase (13.0), where $E = \ln[(1 - C)(1 - \text{e.e.}_s)]/\ln[(1 - C)(1 + \text{e.e.}_s)]$, C is the substrate conversion and e.e._s is the enantiomeric excess of the remaining substrate. After cross-linking, the lipase was partially purified, which should be partially responsible for the improved enantioselectivity. Additionally, cross-linking is able to maintain the stability of the conformation of the enzyme molecules, favoring the improvement of the enzyme enantioselectivity. It should be mentioned that the particle size has no significant effect on the enantioselectivity of the cross-linked CRL.

4. Conclusions

Compared to other kind of immobilized enzyme, CLEA exhibits many advantages such as no solid support, easy preparation and only pure protein which might perform high activity and enantioselectivity. Many factors can affect the particle size of the protein in the precipitation process of *C. rugosa* lipase, including aggregation agent type and concentration, enzyme and glutaraldehyde concentration, and pH. Among these factors, enzyme and glutaraldehyde concentration plays a significant role in affecting particle size compared with other factors. The particle size of the protein has an effect on maintenance of enzyme activity. Therefore it is important to control these factors in the precipitation process to prepare carrier-free enzyme with suitable particle size. Under suitable conditions, the carrier-free immobilized *C. rugosa* lipase can keep lipase activity quite well and enantioselectivity of the lipase is able to be improved after immobilized by CLEA.

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