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Journal of Molecular Catalysis B: Enzymatic 35 (2005) 28–32
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Effect of salts on activity, stability and enantioselectivity of *Candida rugosa* lipase in isooctane

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Received 5 April 2005; received in revised form 6 May 2005; accepted 9 May 2005
Available online 13 June 2005

Abstract

The effect of salts on activity and stability and enantioselectivity of *Candida rugosa* lipase in isooctane was studied. The activity of the lipase lyophilized with LiCl, NaCl or KCl and native lipase for the esterification of lauric acid with 1-propanol in isooctane at $a_w = 0.33$ was 0.057, 0.044, 0.033 and 0.027 g g^{-1} min^{-1}, respectively. The water content played a main role on the effect of lipase activity and lyophilization process did not change the conformation of the lipase. Salt incorporation could keep the conformation of the lipase and prevented the large change of optimum pH condition happening. The stability of the lyophilized lipase was 3.4 times as much as that of native lipase and the lipase thermostability was also improved. The enantioselectivity of lyophilized lipase (20.3) was about 1.6 times as much as that of the native lipase (13.0).

Keywords: Salt; *Candida rugosa* lipase; Lyophilization; Activity; Stability; Enantioselectivity

1. Introduction

Many research studies have shown that salts have a number of effects on properties of protein including activity, conformational stability and solubility. These effects possibly arise from the binding of ions to specific sites on the protein that is able to screen charges on surface amino acid side chains and change the degree of hydration of the protein [1–3].

One effective way to combine salts and enzymes is to lyophilize the solutions involving the salts and the enzymes. Cerasoli et al. [4] pointed the inclusion of salts led to a marked stabilization against unfolding of the enzyme by urea. Triantafyllou et al. [5] demonstrated that adding buffer salts or KCl increased the catalytic activity of lyophilized *Candida antarctica* lipase four-fold over that with no added salt and water played a very important role to affect enzyme activity. Nevertheless, not all ions have the similar favorable effects on the properties of a specific protein. Sometimes, the effects of salts are unexpected. Jernejc and LegiŠa [6] reported that Cu^{2+} ions in a concentration of 0.1 mM completely abolished malic enzyme activity. In the lyophilization process, some factors also strongly affect the properties of enzymes. Ru et al. [7] studied the effects of lyophilization time and water content in organic solvents on enzyme activity and elaborated that activation of both the serine protease subtilisin Carlsberg and the lipase from *Mucor javanicus* resulting from lyophilization in the presence of KCl was highly sensitive to the lyophilization time and water content of the sample.

Though a better understanding of the mechanism is benefit for applying enzymes, unfortunately, little is known about it now. It cannot be concluded that ions must be able to have effects on a specific enzyme, no matter favorable or unfavorable effects. Researchers must try more samples and

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doi:10.1016/j.molcatb.2005.05.002

investigate the mechanism [8–11]. In this paper, the effects of salts on *Candida rugosa* lipase in organic solvent (isooctane) would be studied. The lipase from *C. rugosa* is a relatively cheap commercial enzyme and has been extensively investigated as a biocatalyst in organic solvents [12,13]. The aim of this work is to examine the effect of salts (LiCl, NaCl and KCl) on the activity, stability and enantioselectivity of the lipase, and the effect of other factors on the lyophilization process including lyophilization time and water content.

2. Experimental

2.1. Chemicals

The lipase (E.C. 3.1.1.3) from *C. rugosa* (type VII 700-1, 500 units/mg solid, pH 7.2 (using olive oil, 30 min incubation)) was purchased from Sigma–Aldrich Company. All other chemicals were of a reagent grade and obtained commercially.

2.2. Preparation of lyophilized C. rugosa lipase containing various amounts of LiCl, NaCl or KCl

C. rugosa lipase (50 mg), K_2HPO_4 (50 mg) and various amounts of salts (LiCl, NaCl or KCl) were dissolved in nanopure H_2O (200 ml) each to achieve a final dry preparation of lipase containing various salt concentrations. The pH of the solution was adjusted from 3.0 to 9.0 using a few drops of KOH solution (100 mM) or HCl solution (100 mM). These samples were lyophilized for various hours according to the experimental requirements.

2.3. Assay of lipase activity and stability

All lipase preparations and the solvents were equilibrated against saturated aqueous solutions of various salts at 25 °C for 2 days. The water activity of all reactions was adjusted by these saturated aqueous solutions: LiCl ($a_w = 0.12$), $MgCl_2 \cdot 7H_2O$ ($a_w = 0.33$), NaCl ($a_w = 0.75$) and KCl ($a_w = 0.84$). The reaction mixture (10 ml) consisted of lauric acid (0.1 M), 1-propanol (75 μl) and native or lyophilized lipase (protein equivalent to 2–4 mg ml^{-1}) 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 NaOH (0.05 M). Specific activity of the enzyme was defined as the amount of acid consumed per gram of protein per minute. Lipase stability was evaluated by analyzing the residual activity at different incubation times at 30 °C.

2.4. Determination of enzyme enantioselectivity

(*R*,*S*)-Ibuprofen (4 mg and 2 μM) was dissolved in isooctane (3 ml) containing 1-propanol (6 μM) and 40 mg of lyophilized 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.

3. Results and discussion

3.1. Effect of additive content

Generally, lyophilized *C. rugosa* lipase with salts exhibited an improved activity compared to the native lipase without salt. Fig. 1 shows that the maximum activity of the lyophilized lipase with LiCl, NaCl or KCl and native lipase at $a_w = 0.33$ is 0.057, 0.044, 0.033 and 0.027 g g^{-1} min^{-1}, respectively. The salts are considered to be favorable to disperse the enzyme molecules, thereby facilitating mass transport during the process. Nevertheless, with the increasing of the salt content of the lyophilized lipase, the lipase esterification activity decreased because the more salts covered the surface of the lipase and prevented the substrates contacting with lipase. It is not clear why LiCl, NaCl and KCl have different effects on improving lipase activity. The possible reason is that Li^+ is more chemically active to facilitate the contact between the substrates and the lipase.

3.2. Effect of lyophilization time and water activity

Ru et al. [7] investigated the catalytic efficiencies of lyophilized *C. antarctica* lipase powder in organic solvents as a function of freeze-drying duration and water content of the sample. Chowdary and Prapulla [14] studied the influence of water activity on the lipase catalyzed synthesis of butyl butyrate by transesterification and a direct relationship

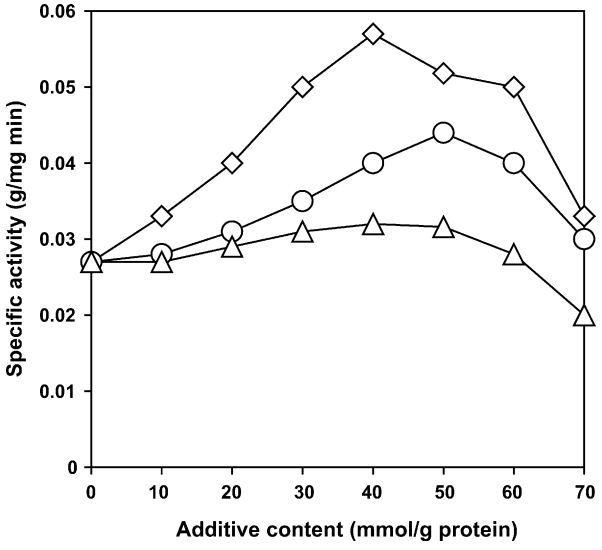

Fig. 1. The esterification activity of *Candida rugosa* lipase as a function of the amount of additive to the preparation at $a_w = 0.84$; (◊) LiCl, (○) NaCl and (△) KCl.

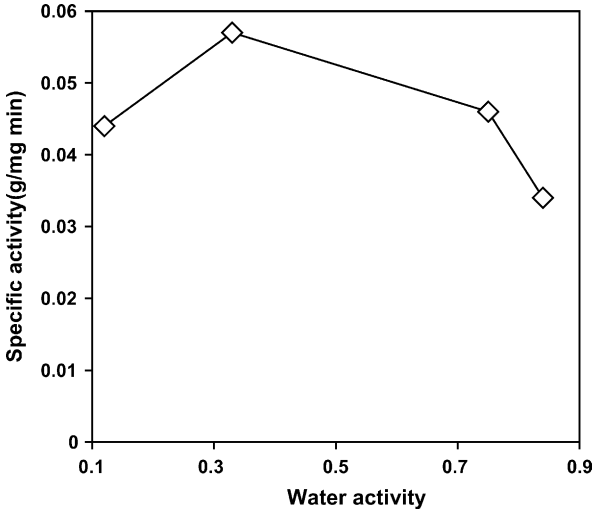

Fig. 2. The esterification activity of *Candida rugosa* lipase lyohphilized with 40% LiCl (w/w) as a function of water activity.

between water activity and reaction rate was observed. Other researcher reported the similar results [15,16]. Actually, it is not clear that the effects on lipase properties were caused by salt incorporation or lyophilization, which possibly can change lipase conformation by the process of dehydration. It is important to differentiate the effects of lyophilization and of salt incorporation on enzyme catalysis in organic solvents.

In the work, the *C. rugosa* lipase lyophilized with LiCl for 48 h and the medium were equilibrated with various saturated aqueous solutions, respectively. The relationship between water activity and reaction activity of the lipase was studied and shown in Fig. 2. The results showed that the water activity had strong effect on the activity of the lipase.

Fig. 3 shows that the activity was able to keep constant under the same water activity and the lipase activity increased

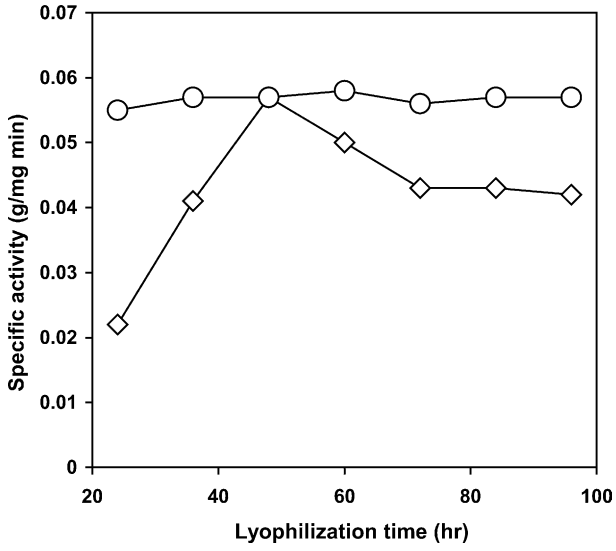

Fig. 3. The esterification activity of *Candida rugosa* lipase as a function of lyophilization time; (◯) $a_w = 0.33$ and (◊) water activity is not controlled.

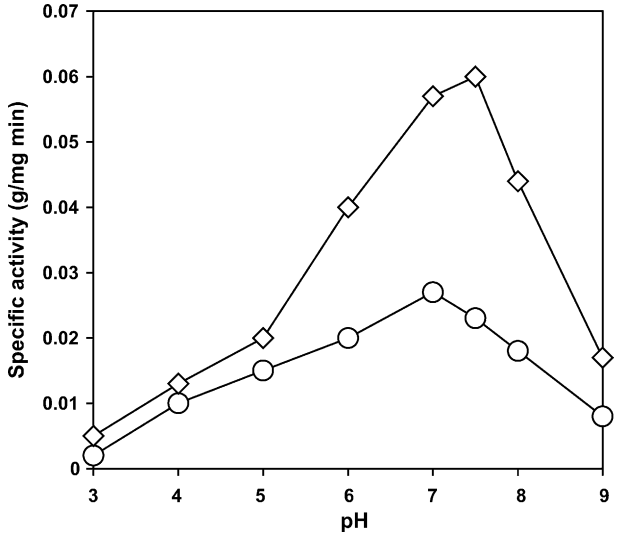

Fig. 4. The esterification activity of *Candida rugosa* lipase as a function of pH at $a_w = 0.33$; (◯) native lipase and (◊) lyophilized lipase with LiCl.

with the lyophilization time increasing and kept consistent after 72 h. It is clear that water content played a main role on the effect of lipase activity and lyophilization did not change the conformation of the lipase. It is concluded that only salt incorporation had effect on the activity of the lipase.

3.3. Effect of pH

It was widely accepted that the activity of a lyophilized enzyme from an aqueous solution with a pH corresponds to the optimal pH of the enzyme in water. Guinn et al. [17] found although alcohol dehydrogenase was most active in water at pH 7.5, the optimum activity in organic solvents for lyophilized lipase was achieved from a solution of pH 2.0. Therefore, it is necessary to find the optimum pH of the aqueous solution, which the *C. rugosa* lipase was involved in.

Fig. 4 shows that the optimum pH (7.5) for the activity of lyophilized *C. rugosa* lipase was quite close to the optimum pH (7.0) for the native *C. rugosa* lipase in aqueous solution. It is concluded that salt incorporation was able to keep the conformation of the lipase and prevent the large change of optimum pH happening. In other words, the stability of the lipase was improved due to the salt induced.

3.4. Effect of LiCl on the stability of C. rugosa lipase

Due to the advantages of biotransformation in organic solvent, enzymes are applied in the organic solvents frequently. Therefore, it is very important to study the stability of enzyme.

Assuming that lipase follows the first-order deactivation model [18]:

$$E \xrightarrow{k_d} E_d \quad (1)$$

Here E and E_d represent active and partially deactivated enzymes, respectively, and k_d the deactivation rate constant. The concentrations of active $[E]$ and partially deactivated $[E_d]$ enzymes can be expressed as:

$$[E] = E_0 \exp(-k_d t) \quad (2)$$

$$[E_d] = E_0[1 - \exp(-k_d t)] \quad (3)$$

Here E_0 represents the initial enzyme concentration and t is the time. Assuming that the active and partially deactivated enzymes have the same Michaelis constant (K_m), the relative activity (R) can be calculated as:

$$R = \frac{k_{cat}[E] + \alpha k_{cat}[E_d]}{k_{cat} E_0} = \alpha + (1 - \alpha) \exp(-k_d t) \quad (4)$$

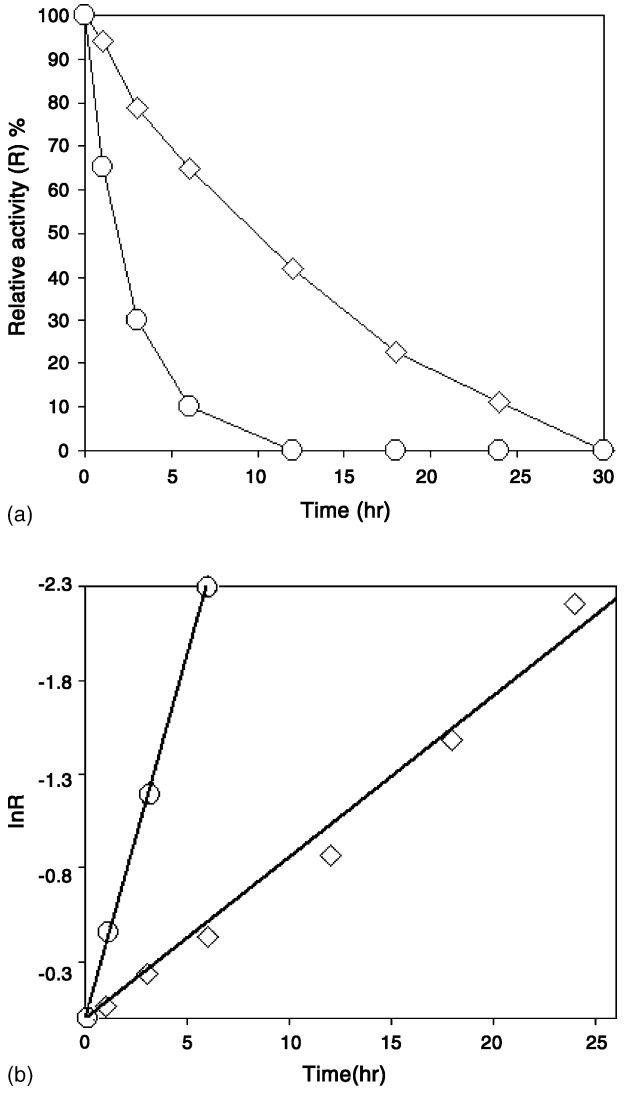

Fig. 5. (a) Lipase stability as a function of time; 100% activity values correspond to the initial activities of 0.057 g g^{-1} min^{-1} for lyophilized lipase and 0.018 g g^{-1} min^{-1} for native lipase, respectively; (◯) native lipase and (◇) lyophilized lipase with LiCl. (b) Plot of ln R against time according to equation (6) for determination of deactivation constants. (◯) Native lipase; (◇) lyophilized lipase with LiCl.

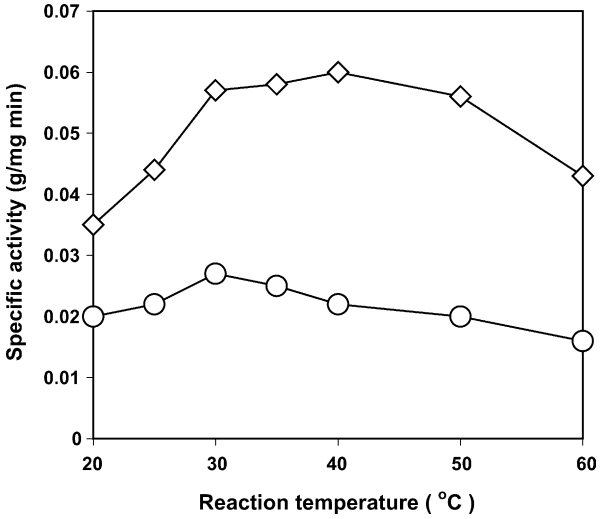

Fig. 6. The effect of reaction temperature on the lipase specific activity at a_w=0.33; (◯) native lipase and (◇) lyophilized lipase with LiCl.

Here k_{cat} is the catalytic activity constant and α represents the ratio of the specific enzyme activity of the final state (E_d) to the initial state (E).

For determination of the deactivation rate constant (k_d), equation (4) is transformed to the following form:

$$\ln(R - \alpha) = \ln(1 - \alpha) - k_d t \quad (5)$$

In the work, there is no activity in the final state (Fig. 5a), therefore, $\alpha = 0$.

$$\ln R = -k_d t \quad (6)$$

The plot of ln R against t (Fig. 5b) shows excellent linear correlations, indicating that the first-order deactivation model was applicable. The calculated k_d values in the native lipase and the lyophilized lipase from Fig. 5b were 0.38 and 0.086 h^{-1}, respectively. The stability of lyophilized lipase was 3.4 times as much as that of native lipase.

3.5. Effect of reaction temperature

Fig. 6 shows that the activity of lipases lyophilized with LiCl for 48 h at $a_w = 0.33$ and native lipase shared the same trend with the changing of reaction temperature. Normally, the optimum reaction temperature for native *C. rugosa* lipase is around 30 °C and at high temperatures lipase activity decreases. Interestingly, the lyophilized lipase was able to keep rather high activity at higher temperature (50 °C). It is evident that salt induction improved *C. rugosa* lipase thermostability due to the improved conformational stability as discussed above.

3.6. Effect of LiCl on enantioselectivity

The time courses of esterification of (*R*,*S*)-ibuprofen with 1-propanol in isooctane catalyzed by native lipase

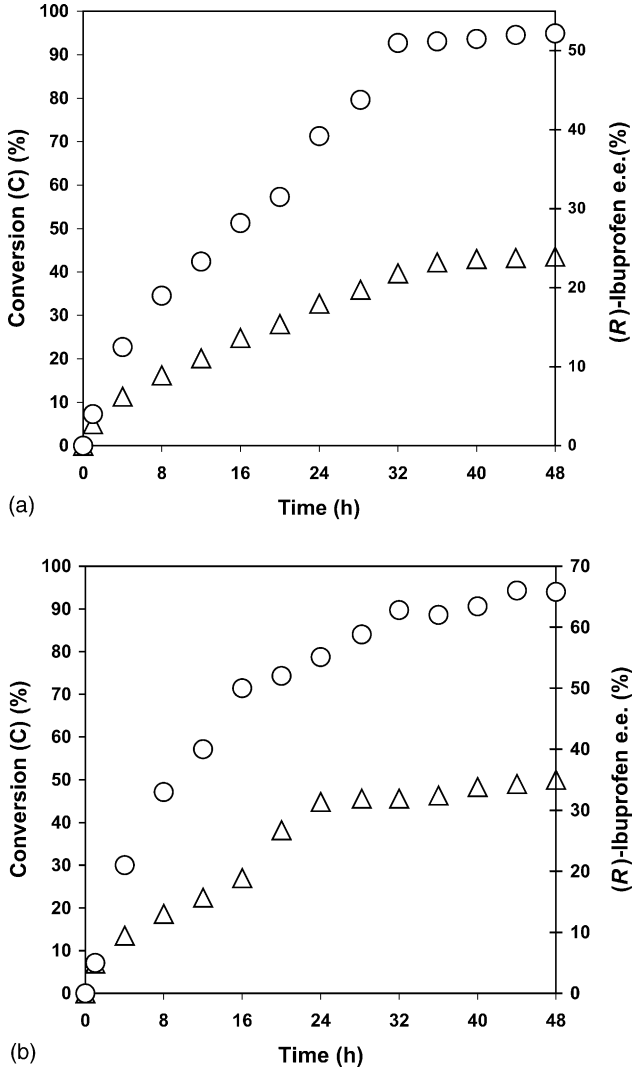

Fig. 7. Time courses of substrate conversion (◊, *C*) and enantiomeric excess (◯, ee) of esterification of (*R*,*S*)-ibuprofen with 1-propanol in isooctane at 30 °C; (a) native lipase and (b) lyophilized lipase.

and lyophilized lipase are shown in Fig. 7a and b, respectively. The *E* value of the lyophilized lipase (20.3) was 1.6 times as much as that of the native lipase (13.0), where $E = \frac{\ln[(1-C)(1-ee_s)]}{\ln[(1-C)(1+ee_s)]}$, *C* substrate conversion and ee_s is enantiomeric excess of the remaining substrate. After lyophilizing, the lipase was partially purified, which should be partially responsible for the improved enantioselectivity.

4. Conclusions

In general, salts (LiCl, NaCl and KCl) had effects on the activity of *C. rugosa* lipase because the diffusion of the enzyme in organic solvent was improved. The water content played a main role on the effect of lipase activity and lyophilization process did not change the conformation of the lipase. Salt incorporation could keep the conformation of the lipase and prevented the large change of optimum pH happening. The stability, thermostability and enantioselectivty of the lyophilized lipase were improved compared to native lipase. Therefore, lyophilizing suitable salts and enzymes is an important way to modify enzyme characteristics and makes enzymes to be adapted in organic environments.

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