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Enhanced activity and stability of immobilized lipases by treatment with polar solvents prior to lyophilization

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Abstract

Lipases from *Candida rugosa*, *Mucor javanicus* and *Rhizopus oryzae* were respectively adsorbed on Amberlite XAD-7 followed by incubation in 2-propanol and then lyophilization. The activities of the immobilized enzymes were 1.6–3.4 times higher than those of the immobilized enzymes without incubation in the organic solvent before lyophilization for esterification of lauric acid (0.1 M) and 1-propanol (0.1 M) in isooctane at 37 °C. The immobilized *C. rugosa* lipase (Sigma) without the incubation did not show any activity but displayed considerable activity (19.8 μmol h^{-1} mg^{-1}) after the incubation before lyophilization. Besides 2-propanol, acetone, 1-propanol and ethyl acetate were also found to be good solvents for treating *M. javanicus* lipase immobilized on Amberlite XAD-7 and acetone was the best among them. When incubated in isooctane at 25 °C for 120 h, the immobilized *M. javanicus* lipase prepared by incubation in acetone for 1 h before lyophilization retained 70% of its initial activity while the immobilized enzyme without the solvent treatment kept only 50% of its initial activity.

Keywords: Lipase; Immobilization; Adsorption; Organic solvent; Activity; Stability

1. Introduction

Immobilization of enzymes helps their economic reuse and favors the development of continuous biocatalytic processes [1–3]. Adsorption is the simplest method for enzyme immobilization and is especially suitable for preparing immobilized enzymes for use in organic solvents due to the less desorption of the adsorbed enzymes in an organic environment [4,5]. As enzymes generally show a much lower activity in organic solvents than in aqueous solutions [6,7], it is necessary to improve their activity during the preparation of the immobilized enzymes. For free enzymes, it has been reported that the enzyme activity could be significantly improved by dissolving them in aqueous solutions containing some polar organic solvents such as 2-propanol and acetone [8,9] or directly dispersing them in pure organic solvents [10] before lyophilization. For immobilized enzymes, Chang and Hsu [11] reported that the drying of freshly immobilized enzymes with 2-propanol significantly improved their activity and enantioselectivity. The improvement in enzyme performances by treatment with organic solvents is generally ascribed to the conformational change of the enzymes favoring their combination with substrates.

In this work, the lipases from several origins were immobilized on macroporous adsorbents by simple adsorption in aqueous solutions followed by incubation of the wet immobilized enzymes in pure organic solvents prior to lyophilization. The activity and stability of the freeze-dried immobilized enzymes after incubation in organic solvents were found to be significantly improved compared to those of the lyophilized immobilized enzymes without prior incubation in organic solvents.

2. Materials and methods

2.1. Chemicals

The *Candida rugosa* lipase (Type II), *Candida antarctica* lipase immobilized on acrylic resin and olive oil were purchased

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Table 1
Adsorption capacity and activity of *Mucor javanicus* lipase immobilized on different adsorbents

Adsorbent	Amberlite XAD-7	Amberlite XAD-16	Ambersorb 572	Ambersorb 348F
Surface area[a] ($m^2\ g^{-1}$)	450	800	1100	750
Absorption capacity[b] ($mg\ g^{-1}$)	84.7	98.0	128.3	117.1
Lipase activity[c] ($\mu mol\ h^{-1} mg^{-1}$)	33.1	11.1	1.6	1.3

[a] Data were from the Aldrich Handbook of Fine Chemical and Laboratory Equipment.
[b] The adsorption was conducted at 4 °C for 24 h.
[c] The activity was assayed for esterification of lauric acid (0.1 M) and 1-propanol (0.1 M) in isooctane at 37 °C.

from Sigma. The lipase from *Rhizopus oryzae* was obtained from Fluka. The lipase from *Mucor javanicus* (Lipase M "Amano" 10) was from Amano Enzyme Inc., Japan. The lipase from *C. rugosa* (lipase OF) was purchased from Meito Sanyo Co. Ltd., Japan. The adsorbents Amberlite XAD-7, Amberlite XAD-16, Ambersorb 348F and Ambersorb 572 were from Aldrich. All other chemicals were of a reagent or HPLC grade and obtained commercially.

2.2. Immobilization of lipases

A typical procedure for lipase immobilization was as follows. Crude lipase (480 mg) was added into 80 ml phosphate buffer (0.01 M, pH 7.0) and the mixture was stirred for 30 min followed by centrifugation at 5000 rpm for 5 min to collect the supernatant. Into 50 ml of the supernatant was added 500 mg adsorbent and the mixture was shaken (300 rpm) for 1 h at room temperature then put into a refrigerator for 24 h. Then the adsorbent was collected by filtration and the amount of lipase adsorbed was calculated according to the difference of lipase concentrations in the solution before and after the adsorption. Lipase concentration was determined based on the UV absorbance at 280 nm. The collected immobilized enzymes were either directly subjected to lyophilization or added into 50 ml pure organic solvents, shaken at 300 rpm for 2 h at room temperature, collected by filtration and then lyophilized.

2.3. Assay of enzyme activity

The lipase activity was assayed by incubating 100 mg immobilized lipase in 20 ml isooctane containing 0.1 M lauric acid and 0.1 M 1-propanol at 37 °C and 180 rpm for 1–3 h. The consumed acid was analyzed by titration with 0.1 M NaOH after addition of 20 ml mixture of ethanol and acetone (50:50, v/v). The lipase activity was expressed as mmol acid consumed $h^{-1}\ g^{-1}$ protein.

2.4. Assay of enzyme thermostability

Immobilized lipase (100 mg) was added into 10 ml isooctane and the mixture was shaken at 180 rpm at different temperatures (25, 40, 60 and 80 °C) for 2 h, cooled down to room temperature and mixed with 10 ml isooctane containing 0.1 M lauric acid and 0.1 M 1-propanol. The mixture was shaken at 180 rpm and 37 °C for 1–3 h. The lipase activity was assayed following the same procedure as described above.

2.5. Assay of enzyme time stability

Immobilized lipase (100 mg) was incubated in 10 ml isooctane at 25 °C for different times (6, 24, 48, 72, 96 and 120 h) and then mixed with 10 ml isooctane containing 0.1 M lauric acid and 0.1 M 1-propanol. The residual lipase activity was assayed at 37 °C following the same procedure as described above.

3. Results and discussion

3.1. Screening of adsorbents

Four commercially available adsorbents were tested (Table 1). The Ambersorb adsorbents gave much higher adsorption capacity for *M. javanicus* lipase than the Amberlite adsorbents, but the enzyme activity of the latter was much higher than that of the former. The higher enzyme adsorption capacity of the Ambersorb adsorbents might be partially attributed to their larger surface area compared to that of the Amberlite adsorbents. The stronger enzyme adsorption on the Ambersorb adsorbents might severely deform the enzyme resulting in lower enzyme activity. The activity of the enzyme adsorbed on Amberlite XAD-7, a moderately polar acrylic resin, was almost three times higher than that of the enzyme adsorbed on Amberlite XAD-16, a hydrophobic polyaromatic resin. In the subsequent study, Amberlite XAD-7 was used as the adsorbent.

3.2. Effect of lipase origins and treatment of adsorbed enzymes with 2-propanol

Four kinds of lipases were respectively adsorbed on Amberlite XAD-7 followed by incubation in 2-propanol for 2 h before lyophilization (Table 2). It is obvious that the incubation in 2-propanol before lyophilization markedly increased the enzyme activity for all the four lipases. For *C. rugosa* lipase from Sigma, no detectable activity was observed when the enzyme was directly lyophilized after the adsorption. However, an activity similar with that of the lipase of the same origin (lipase OF) was observed when the adsorbed enzyme was incubated in 2-propanol for 2 h before lyophilization. This might indicate that the incubation in the polar organic solvent helped the conversion of the inactive closed form of the enzyme to the active open form [12]. The activity of the immobilized lipase OF from *C. rugosa* treated with 2-propanol before lyophilization was 3.4 times higher than that of the immobilized enzyme directly lyophilized after adsorption. The activities of the immo-

Table 2
Adsorption capacity and activities of lipases from different origins adsorbed on Amberlite XAD-7 with or without incubation in 2-propanol before lyophilization[a]

Lipase origin	Adsorption[b] (mg g^{-1})	Activity without incubation, A_0[c] (μmol h^{-1} mg^{-1})	Activity after incubation, A_i[c] (μmol h^{-1} mg^{-1})	A_i/A_0
Candida rugosa (lipase OF)	63.1	5.0	17.1	3.4
C. rugosa (Sigma)	71.4	0.0	19.8	–
Rhizopus oryzae	95.7	206.0	326.6	1.6
M. javanicus	77.3	33.1	64.0	1.9

[a] The wet immobilized enzyme was incubated in 2-propanol at 25 °C for 2 h.
[b] The adsorption was conducted at 4 °C for 24 h.
[c] The activity was assayed for esterification of lauric acid (0.1 M) and 1-propanol (0.1 M) in isooctane at 37 °C.

bilized lipases from *M. javanicus* and *R. oryzae* treated with 2-propanol before lyophilization were also respectively 1.6 and 1.9 times higher than those of the immobilized enzymes directly lyophilized after the adsorption. The incubation of wet immobilized enzymes in polar 2-propanol not only activated the enzyme, but also helped remove most of the water contained in the wet immobilized enzymes, favoring the subsequent lyophilization, which further removed the water that may not be easily removed by simply rinsing with the solvent [11] and stabilized the enzyme preparation. In the subsequent study, the lipase from *M. javanicus* was used as the model enzyme to further investigate the effect of organic solvent treatment.

3.3. Effect of solvent type

The *M. javanicus* lipase adsorbed on Amberlite XAD-7 was incubated in different organic solvents for 2 h before lyophilization. Table 3 shows that not only 2-propanol, but several other polar solvents including acetone, 1-propanol and ethyl acetate also markedly activated the immobilized enzyme and acetone was the best among them. The treatment with DMSO and DMF, the most polar two solvents used, completely deactivated the enzyme. This might be ascribed to the stripping-off of the essential water from the enzyme surface by the extremely polar solvents as well as their stronger interaction with the enzyme molecules severely deforming the enzyme. The treatment of the immobilized enzyme using the solvents with a log P from −0.22 to 0.68 gave an increased enzyme activity compared to the untreated enzyme with an exception of THF. Obviously, the polarity of solvents markedly affected the enzyme activity but was not the only factor of influence. This is clearer when comparing the activities of the immobilized enzyme treated with isooctane and octane, two solvents of the same polarity. The activity of the enzyme treated with isooctane decreased more severely than that of the same enzyme treated with octane. In the subsequent study, acetone was used as the solvent for further investigation.

Table 3
Activity of *M. javanicus* lipase adsorbed on Amberlite XAD-7 followed by incubation in solvents for 2 h at 25 °C before lyophilization[a]

Organic solvent	Log P[b]	Activity (μmol h^{-1} mg^{-1})[c]	Relative activity (%)
No (control)		33.1	100
DMSO	−1.3	0.0	0.0
DMF	−1.0	0.0	0.0
Methanol	−0.76	3.4	10.3
Acetonitrile	−0.33	28.3	85.5
Ethanol	−0.24	17.2	51.9
Acetone	−0.22	82.8	246.5
2-Propanol	0.05	64.0	193.3
1-Propanol	0.25	50.2	151.7
THF	0.49	13.4	40.5
Ethyl acetate	0.68	55.7	168.3
Diethyl ether	0.85	19.6	59.2
Benzene	2.0	23.4	70.7
Toulene	2.5	24.9	75.2
Cyclohexane	3.2	23.1	69.8
Hexane	3.5	14.3	43.2
Heptane	4.0	29.5	89.1
Isooctane	4.5	17.7	53.5
Octane	4.5	25.1	75.8

[a] The adsorption was conducted at 4 °C for 24 h.
[b] Data were from Laane et al. [13].
[c] The activity was assayed for esterification of lauric acid (0.1 M) and 1-propanol (0.1 M) in isooctane at 37 °C.

3.4. Effect of incubation time in acetone

The *M. javanicus* lipase immobilized on Amberlite XAD-7 was incubated in acetone for different times before lyophilization. Fig. 1 shows that the optimal incubation time was 1 h, corresponding to a maximal enzyme activity which was 2.7 times higher than that of the immobilized enzyme directly lyophilized after the adsorption. Even when the incubation time was as short as 0.5 h, the enzyme activity was as high as 2.4 times of that of the control. When the incubation time was as high as 8 h, the enzyme activity was still 1.9 times higher than that of the control. Therefore, the adsorbed enzyme was quite resistant to the polar solvent, possibly due to the effective protection of the surrounding water layer. It is worth mentioning that we tried to improve the activity of the commercial immobilized *C. antarctica* lipase by putting them in these organic solvents for different times but did not observe an obvious activity improvement (data not shown). In the subsequent study, the incubation time of the immobilized enzyme in acetone was kept at 1 h.

3.5. Enzyme thermostability in organic solvents

Fig. 2 shows that the stabilities of both enzyme preparations with and without incubation in acetone before lyophilization decreased with increasing temperature. However, the immobilized enzyme with incubation in acetone for 1 h was more

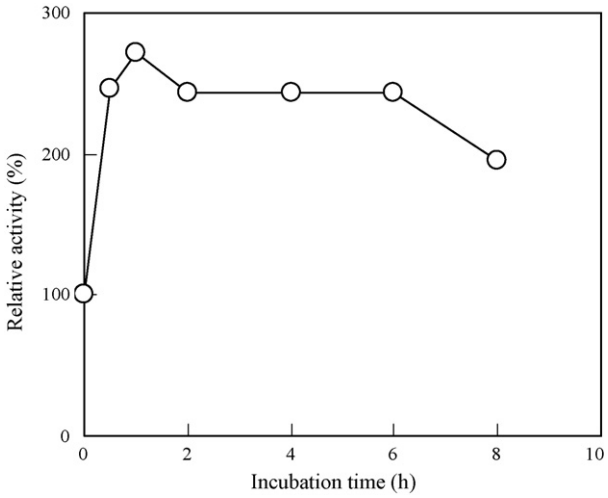

Fig. 1. Activity of *Mucor javanicus* lipase immobilized on Amberlite XAD-7 as a function of incubation time in acetone at 25 °C before lyophilization. The enzyme adsorption was conducted at 4 °C for 24 h. The enzyme activity was assayed for esterification of lauric acid (0.1 M) and 1-propanol (0.1 M) in isooctane at 37 °C.

resistant to temperature. For example, when kept in isooctane at 40 °C for 2 h, the enzyme treated with acetone retained almost all its initial activity but the enzyme without the solvent treatment maintained only 70% of its initial activity. When kept in isooctane at a temperature as high as 80 °C for 2 h, the immobilized enzyme with solvent treatment lost 60% of its initial activity but the untreated enzyme preparation lost 80% of its initial activity. It seems that the treatment of immobilized enzyme with acetone before lyophilization helped strip off some unessential water from the enzyme molecules, making the enzyme molecules become more rigid and more resistant to thermo-deactivation, which needs the participation of water [14,15].

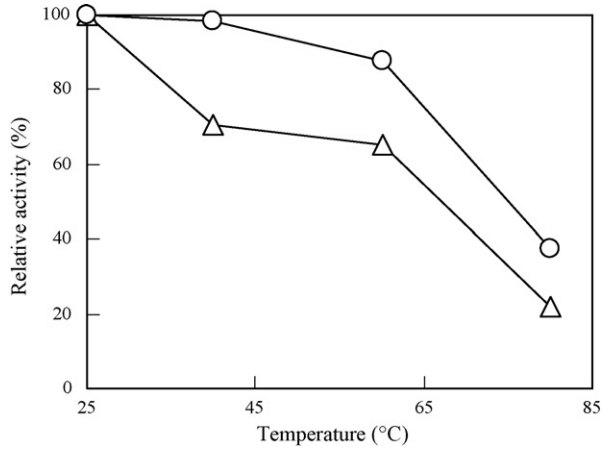

Fig. 2. Stability of *M. javanicus* lipase immobilized on Amberlite XAD-7 as a function of incubation temperature. The immobilized lipase was incubated in isooctane for 2 h. The lipase activity was assayed for esterification of lauric acid (0.05 M) and 1-propanol (0.05 M) in isooctane at 37 °C. The relative activity was expressed as the percentage of the activity of the immobilized enzyme after incubation in isooctane for 2 h to that of the same enzyme preparation without the incubation. (◯) Immobilized enzyme lyophilized after incubation in acetone for 1 h; (△) immobilized enzyme lyophilized directly after adsorption.

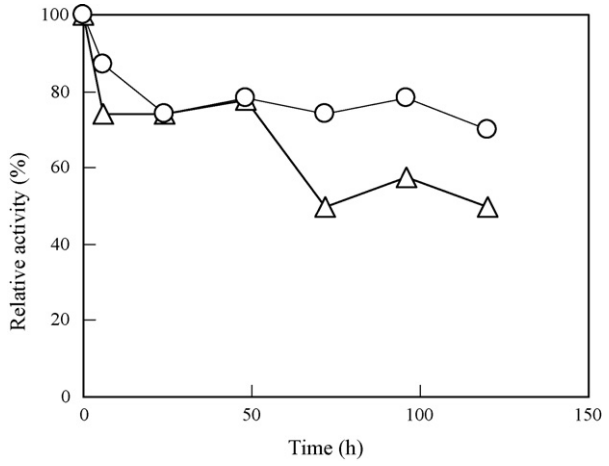

Fig. 3. Stability of *M. javanicus* lipase immobilized on Amberlite XAD-7 as a function of incubation time in isooctane at 25 °C. The lipase activity was assayed for esterification of lauric acid (0.05 M) and 1-propanol (0.05 M) in isooctane at 37 °C. The relative activity was expressed as the percentage of the activity of the immobilized enzyme after incubation in isooctane to that of the immobilized enzyme without the incubation. (◯) Immobilized enzyme lyophilized after incubation in acetone for 1 h; (△) immobilized enzyme lyophilized directly after adsorption.

3.6. Enzyme stability as a function of incubation time in organic solvents

Fig. 3 shows that the activities of both enzyme preparations with and without incubation in acetone before lyophilization decreased with increasing the incubation time in isooctane at 25 °C. The immobilized enzyme with acetone treatment was more stable than the immobilized enzyme without being treated with acetone. This became more obvious at a longer incubation time. For instance, after incubation in isooctane for 48 h, both enzyme preparations retained about 78% of their initial activities. However, when the incubation time was raised to 120 h, the immobilized enzyme with acetone treatment still kept 70% of its initial activity but the immobilized enzyme without acetone treatment showed only 50% of its initial activity. Therefore, the incubation of the adsorbed enzyme in acetone before lyophilization helped improve not only the enzyme activity but also the enzyme thermo-stability and time stability.

4. Conclusions

The lipases from several origins were immobilized by adsorption on solid adsorbents followed by incubation in pure organic solvents before lyophilization. The activity of the immobilized enzymes was markedly increased compared to that of the immobilized enzymes prepared by direct lyophilization after the adsorption. 2-Propanol and acetone were the best two solvents for improving the enzyme performances among the solvents tested. No detectable activity was observed when the immobilized *C. rugosa* lipase from Sigma was directly lyophilized after adsorption on Amberlite XAD-7 but considerable activity was observed when the same enzyme preparation was incubated in 2-propanol before lyophilization. The enzyme thermostability and time stability in organic solvents were also obviously

improved due to the treatment with polar organic solvents prior to lyophilization. The improvement in enzyme activity and stabilities was mainly ascribed to the conversion of the inactive "closed" form of the enzymes to the active "open" form as a result of the pretreatment with polar solvents.

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