Enhanced Esterification Activity and Thermostability of Imprinted Poly(Ethylene Glycol)-Lipase Complex

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Although the range of applications for enzymatic reactions in organic solvents is rapidly expanding, this study focused on the enzymatic activity in the esterification of lauric acid with benzyl alcohol, and thermostability of lipase using poly(ethylene glycol) (PEG)-lipase complex and molecular imprinting techniques. The catalytic activity was enhanced through molecular imprinting and the PEG-lipase complex. The imprinting operation was particularly effective for catalytic activity after forming the PEG-lipase complex. The kinetic analysis of the lipase-catalyzed esterification revealed that the increase in esterification rate with imprinted lipases was mainly due to the higher maximum rate achieved by the system. The thermostability of the lipases was significantly improved by imprinting at all temperatures (50~70 °C). After forming a PEG-lipase complex, the imprinted lipase exhibited much higher reactivity and thermostability compared to the native lipase and the imprinted PEG-lipase complex.

Key words
lipase, imprinting, PEG-lipase complex, thermostability

Introduction

Enzymes typically function in aqueous environments in nature. However, as the application of enzymes in chemical synthesis has become more practical, they are increasingly employed in non-aqueous media instead of aqueous media for many poorly water-soluble substrates[1]. Lipases, in particular, are well-known enzymes that function in non-aqueous media[2]. Depending on their source, certain lipases can remain active in hydrophobic organic solvents[3]. However, the disadvantage of enzymatic reactions in organic solvents is that the native enzyme is insoluble in these solvents, and the reaction rates are affected by mass transfer of the substrate[4]. Therefore, enhancing enzymatic reaction rates in organic solvents through simple methods remains challenging from a practical standpoint. Two approaches can be employed to address this challenge: the design of a reaction apparatus, and activation of enzyme functions. The former approach involves the use of microreactors[5]. Microreactors are characterized by a high specific surface area, which often eliminates the effects of mass transfer. The latter approach involves enzyme activation through pretreatment of the enzyme with organic solvents or substrate analogs This method offers several advantages including easy preparation and the absence of additives in the reaction system. One method for enzyme activation involves pretreating an enzyme with an organic solvent before its use in an enzymatic reaction in the organic solvent[6]. Another pretreatment method uses the molecular imprinting technique with a substrate analog, which has been studied in our laboratory[7–9]. This method entails the lyophilization of an enzyme loaded with a substrate analog to form a complex, followed by the removal of the substrate analog through washing the organic solvent[10]. The conformation of the active site of the enzymes is retained even after the removal of the substrate analog[10]. Additionally, when lipases are used as the enzyme, their active sites in the aqueous solution are covered by a flexible region known as the "lid" of the lipase. Interfacial activation by the organic solvent and amphiphiles causes an opening in this lid allowing the active site to become accessible[11]. The imprinted enzymes, especially lipases, have exhibited much higher activity compared to those non-imprinted.

Another method for activating an enzyme is the amphiphile-coating method, in which the enzyme's surface is covered by an amphiphile monolayer that is soluble in most organic media[12]. The primary reason for enzyme activation in this case is the increased solubility of the amphiphile-enzyme complex in organic media. Amphiphiles such as poly(ethylene glycol) (PEG)[4,13] glycolipid[12] and dialkyl glutamic ribitol amide[14] have been reported

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for coating lipase and a-chymotrypsin. Furthermore, imprinted surfactant-coated lipases have been simultaneously prepared by combining imprinting and surfactant coating,[12,15] resulting in improved esterification activity and enantioselectivity. However, it remains quantitatively unclear which effect, surfactant or imprinting, exerts the most influence on the reaction rate. Additionally, the surfactant used in previous studies[12,13,15] had to be synthesized, making the use of this surfactant impractical. No study has investigated the combined effects of imprinting and coating with commercially available PEG on enzyme reaction systems including their kinetics and thermostability.

In this study, we formed a PEG-lipase complex using PEG as a coating material for the enzyme lipase and investigated the effect of imprinting and PEG on the esterification reaction kinetics and thermostability using these enzyme catalysts.

Experimental methods

Materials

Lipase from porcine pancreas (Type II, L3126, Sigma-Aldrich (St. Louis, U.S.A.)) was utilized in all our experiments. We employed poly(ethylene glycol) (PEG) with an average molecular weight of 20,000. A PEG-lipase complex of 20,000 exhibits higher lipase activity in ionic liquids compared to other molecular PEG weights[13]. Lauric acid and benzyl alcohol served as substrates in the esterification. Cyclohexane and toluene were chosen as the media for esterification, while hexane was used in the pretreatment process and dried with 3 Å molecular sieves before use. Benzyl laurate, an esterification product, was used as a standard sample for quantification and was purchased from Tokyo Chem Ind. (Tokyo, Japan). All other reagents were purchased from Wako Pure Chem. Ind. (Osaka, Japan), except for the enzyme. All the reagents were of analytical grade and used without further purification.

Imprinting of lipase

The pretreatment of native lipase or PEG- lipase complex with octanoic acid as an imprint molecule followed a previously described method[5–7]. Lipase (300 mg) was dissolved in 9 cm^3 of a phosphate buffer solution at pH 7. Octanoic acid (3.5 mmol) and Tween 80 (1000 mg) were dissolved in 10 cm^3 of ethanol. To the enzyme solution, 1 cm^3 of octanoic acid solution was added, and the mixed solution was incubated for 30 min at 25 °C. The mixed solution was then freeze-dried. The obtained powder was washed with hexane to remove octanoic acid. The resulting lipases were dried *in vacuo* (Ulvac, GCD-051X, Chigasaki, Japan).

PEG-lipase complex

Our procedure for the preparation of the PEG-lipase complexes closely followed the method described in a previously published paper[13]. Lipase (10 mg) and PEG (16 mg) were dissolved in a phosphate buffer (5 mL, pH 7). Toluene (13 mL) was added to the lipase solution and emulsified at 20,000 rpm for 3 min using a homogenizer (Thermo Fisher Scientific, Polytron PT 2500, Waltham, U. S. A.) in an ice-bath to prepare water-in-oil (w/o) emulsions. The w/o emulsion was immediately frozen in liquid nitrogen, followed by lyophilization for 24 h. PEG-lipase complexes were obtained as white powders.

Esterification

To assess the effect of imprinting and the PEG-lipase complex, lauric acid and benzyl alcohol were used as substrates for esterification. The reactions were initiated by adding 1 mg of lipases (native, imprinted or PEG-lipase complex) of an organic solution (cyclohexane or toluene) containing both substrates (30 mmol dm^{-3} lauric acid and 30 mmol dm^{-3} benzyl alcohol) in a vial at 37 °C and 1000 rpm (Eppendorf, Thermomixer Comfort, Hamburg, Germany). Samples were taken every 30 minutes for up to 2 hours. The vial tubes were removed from the thermomixer, and immediately centrifuged for 3 min at 10000 rpm (Eppendorf, Centrifuge 5418). The benzyl laurate concentration in the supernatant was measured using HPLC. Reaction rates were determined as the initial reaction rate in the linear region during the first 90 minutes of the reaction. All experiments were performed in triplicate and demonstrated reproducibility with a relative standard deviation of 5 %.

Thermostability of lipase

The lipases were incubated in cyclohexane at 50~70 °C. Following the designated incubation period, the cyclohexane solution containing substrates, lauric acid, and benzyl alcohol, was added and the reaction was carried out at 37 °C and 1000 rpm for 10 min in a thermomixer. Samples were collected to measure the initial esterification rate, and the residual activities were determined as the ratio of the initial esterification rates of the incubated lipases to those of lipases without incubation.

Analysis

Standard benzyl laurate solutions were utilized to prepare the calibration curves. The concentrations of the benzyl laurate in the cyclohexane solutions were determined by HPLC (Shimadzu, Prominence, Kyoto, Japan) with a Wakosil-II 5C18AR

(4.6 mm X 150 mm, Wako Pure Chem. Ind.) column and an eluent solution (ethanol: methanol: water = 15:5:3) as a mobile phase (0.6 cm^3 min^{-1}). Esters were detected using an UV detector at 255 nm (Shimadzu, SPD10AV). Retention time was 14 min.

Results and discussion

Effect of pretreatment on esterification

The effect of pretreatment (imprinting and PEG-lipase complex) on the esterification of the lauric acid with benzyl alcohol was examined. Fig. 1 shows the initial esterification rates in cyclohexane and toluene. In a previous paper[16] it was reported that the initial esterification rates exhibited a linear relationship with solvent hydrophobicity (log *P*) in porcine pancreatic lipase-catalyzed esterification of oleic acid with ethanol. Therefore, cyclohexane, possessing a higher log *P* value than toluene, yielded a larger initial reaction rate than toluene, except in the case of imprinted lipase. As described in previous papers[7–9], imprinting was effective for activating the lipase in toluene, but had little effect in cyclohexane. Conversely, the PEG-lipase complex substantially increased the initial reaction rate in cyclohexane, although it had minimal effect in toluene. This suggests that PEG-lipase complexes were not effectively formed in toluene. Therefore, we prepared lipases by combining imprinting and PEG-lipase complex. As shown in Fig. 1, the activity of the lipase imprinted after forming a PEG-lipase complex was clearly higher than that of the

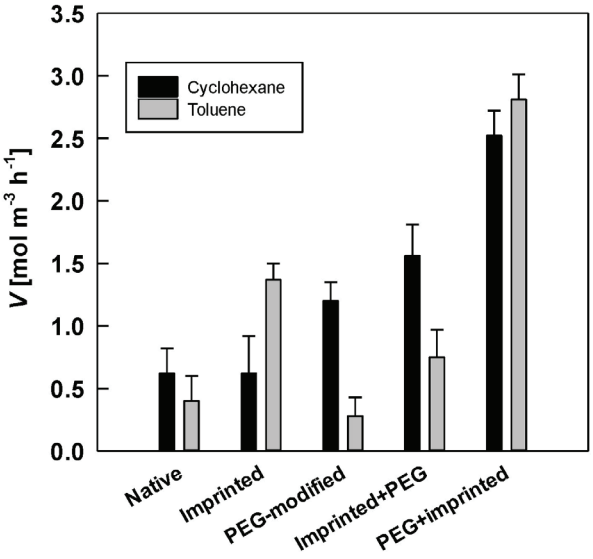

Fig. 1 – *Effect of imprinting and PEG-lipase complex on initial esterification rate in cyclohexane and toluene. Imprinted + PEG denotes that lipase was imprinted and formed a complex with PEG. PEG + Imprinted denotes that lipase was a complex with PEG and imprinted. Lauric acid concentration = benzyl alcohol concentration = 30 mmol* dm^{-3}.

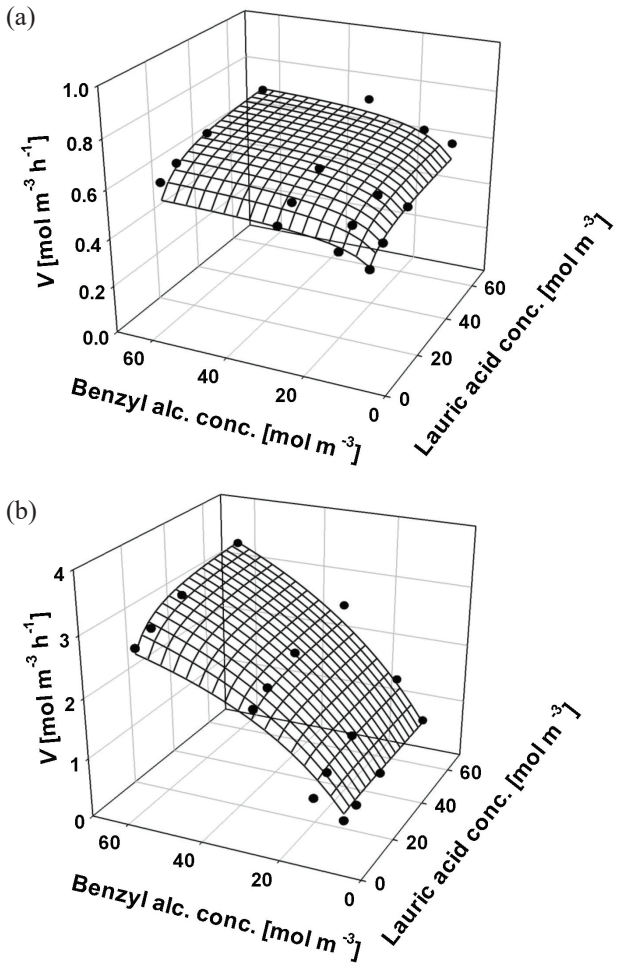

Fig. 2 – *Effects of substrate concentrations on initial rate of esterification. (a) native lipase and, (b) PEG + imprinted lipase. Solid lines were calculated using Eq. (1).*

complexes of imprinted lipase with PEG. The degree of increase in activity exceeded the combined activity of the imprinted lipase and the PEG-lipase complex alone, indicating a synergistic effect. The lipase imprinted after the PEG-lipase complex demonstrated a 4.1-fold and 7.0-fold increase in activity in cyclohexane and toluene compared to the native lipase. In subsequent experiments, cyclohexane was used as the reaction solvent, which gave higher reaction rates and lower toxicity[17].

Effect of PEG-lipase complex and imprinting on esterification kinetics

Fig. 2 shows the effect of substrate concentrations on the initial rate of esterification of both native and treated lipases. No substrate inhibitions were observed. The kinetic rate equation for esterification by lipase can be written using the Ping-Pong Bi-Bi mechanism without substrate inhibition as shown in Eq. 1[18,19].

$$V = \frac{V_m[\mathrm{LA}][\mathrm{BA}]}{K_{\mathrm{m,LA}}[\mathrm{BA}] + K_{\mathrm{m,BA}}[\mathrm{LA}] + [\mathrm{LA}][\mathrm{BA}]} \quad (1)$$

Table 1 – *Kinetic parameters for esterification reaction*

Lipases	V_m [mol m^{-3} h^{-1}]	$K_{m,LA}$ [mol m^{-3}]	$K_{m,BA}$ [mol m^{-3}]
Native	0.801±0.036	3.21±0.70	3.06±0.69
PEG complex	2.04±0.16	8.31±1.75	10.3±2.0
Imprinted	0.780±0.032	2.89±0.61	2.88±0.61
Imprinted/PEG complex	5.59±0.96	20.3±5.8	45.4±11.9
PEG complex/Imprinted	7.22±1.12	5.27±2.20	61.3±14.2

Lauric acid concentration = benzyl alcohol concentration =30 mmol dm^{-3}

where V_m is the maximum rate, [LA] and [BA] are the concentrations of lauric acid and benzyl alcohol, and $K_{m,LA}$ and $K_{m,BA}$ are the Michaelis constants of lauric acid and benzyl alcohol, respectively. Table 1 lists the kinetic parameters determined using a non-linear least-squares method integrated into Sigma Plot 14.0. In Fig. 2, the solid lines calculated with these kinetic parameters agree well with the experimental data. In previous reports[7–9], imprinting led to a decrease in Michaelis constants, $K_{m,LA}$ and $K_{m,BA}$, without changing the maximum rate V_m. However, in our present system, the Michaelis constants were not significantly different from those in the native system, despite the decrease in Michaelis constants. On the other hand, the PEG-lipase complex increased the Michaelis constants, suggesting that the PEG coating on the lipase's surface probably inhibited the interaction between the substrate and the lipase. The PEG-lipase complex significantly increased the maximum rate. This increase in the maximum rate can be attributed to the following factors: The net effect of the PEG molecules shifts the equilibrium between the closed and open forms of the lid in lipases from *Candida rugosa* toward the open form[20]. The preferable conformation change of the lipase (lid open) was initially induced by the contact with the PEG molecule, and imprinting subsequently facilitated substrate accessibility to the active site.

Effect of PEG-lipase complex and imprinting on thermostability of lipase

Fig. 3 shows the time-courses of the residual activities of the lipases in cyclohexane at 70 °C. In all the lipases, enzyme activity declined rapidly at 20 min and then remained constant. Similar behaviors were observed at other temperatures (60 and 50 °C), indicating that enzyme inactivation conforms to a first-order reversible rate equation. The residual activity a_t, is determined by the following equation which is based on reversible first-order deactivation kinetics[21].

$$\frac{a_t - a_\infty}{1 - a_\infty} = \exp\left\{-\left(k_d + k_r\right)t\right\} \qquad (2)$$

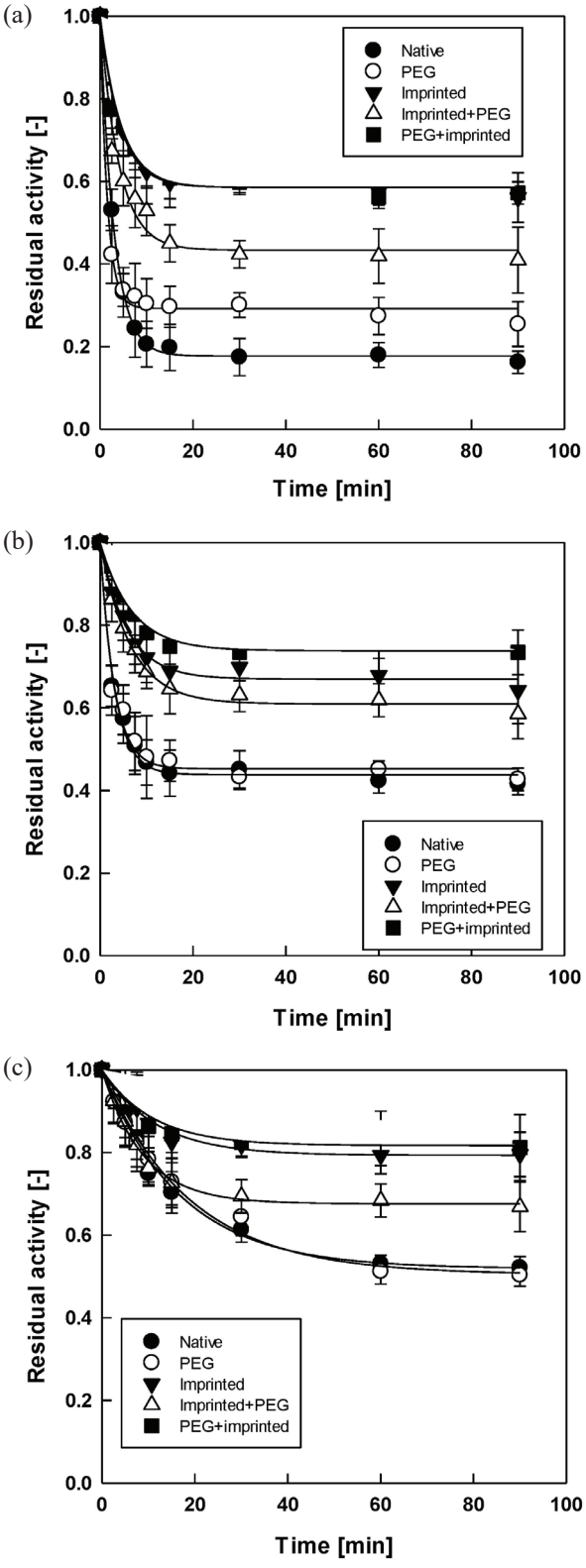

Fig. 3 – *Thermostability of treated lipases at (a) 70 °C, (b) 60 °C and (c) 50 °C. Solid lines were calculated using Eq. (2). Imprinted + PEG denotes that lipase was imprinted and formed a complex with PEG. PEG + Imprinted denotes that lipase was a complex with PEG and imprinted.*

where k_d and k_r are lipase deactivation and refolding rate constants, respectively, a_∞ is the residual activity at equilibrium and t is the elapsed time.

Table 2 – *Parameters for deactivation kinetics*

70 °C	Native	PEG-complex	Imprinted	Im+PEG*	PEG+Im**
a_∞	0.177±0.004	0.292±0.008	0.586±0.011	0.434±0.018	0.586±0.012
k_d/min	0.276±0.006	0.458±0.040	0.0982±0.0087	0.140±0.016	0.0927±0.0092
k_r/min	0.0594±0.0023	0.189±0.021	0.139±0.016	0.107±0.017	0.131±0.017
60 °C					
a_∞	0.439±0.010	0.453±0.013	0.670±0.009	0.610±0.008	0.739±0.010
k_d/min	0.181±0.014	0.181±0.019	0.0570±0.0046	0.0603±0.0032	0.0386±0.0039
k_r/min	0.141±0.014	0.150±0.021	0.116±0.012	0.0944±0.067	0.109±0.014
50 °C					
a_∞	0.519±0.019	0.565±0.015	0.793±0.009	0.676±0.007	0.817±0.012
k_d/min	0.0286±0.0023	0.0264±0.0016	0.0179±0.0018	0.0368±0.0019	0.0167±0.0026
k_r/min	0.0309±0.0040	0.0269±0.0028	0.0687±0.0092	0.0768±0.0053	0.0757±0.0152

* Im + PEG denotes that lipase was imprinted and formed a complex with PEG.

** PEG + Im denotes that lipase was a complex with PEG and imprinted.

Three parameters, k_d, k_r and a_∞ were calculated for the data in Fig. 3 using Sigma Plot 14.0. The parameter values obtained at each temperature are listed in Table 2. In Fig. 3, the solid lines calculated using Eq. (2), closely approximate the experimental data, affirming the justifiability of employing Eq. 2. Table 2 reveals that the thermostability of both the native lipase and the PEG-lipase complex is significantly enhanced by imprinting at all temperatures. On the other hand, the PEG-lipase complex did not produce a significant improvement in thermostability. The improved thermostability due to imprinting indicates the importance of retaining the imprinted structure for thermostability, and the subsequent PEG modification after imprinting is presumed to slightly impair the retention of the imprinted structure. Our prior studies on imprinting for transesterification in cyclohexane and ionic liquids have shown slight stabilization of lipase, although the extent was not notably large[7,8]. This result suggests that carboxylic acid was not the ideal substrate analog in the transesterification reaction, and that the ester should have been considered as the imprinting molecule. Stabilization of lipase by imprinting not only significantly decreased the deactivation rate constant, but also led to a very high retention of activity after 90 minutes. Although longer-term stability will be investigated in the future, our finding indicate that that imprinting lipase-catalyzed esterification significantly improves its thermostability.

Conclusions

The effects of imprinting and the PEG-lipase complex on catalytic activities and thermostability of lipase were investigated for esterification in organic solvents. Both imprinting and the PEG-lipase complex enhanced the enzyme's activity. The activity of the imprinted PEG-lipase complex increased 5.9-fold in cyclohexane and 27-fold in toluene when compared to the native lipase, respectively. Kinetic analysis of lipase-catalyzed esterification revealed that while the PEG-lipase complex and imprinting had a minor effect on Michaelis constants, the increase in the esterification rate primarily resulted from an enhanced maximum rate. Furthermore, imprinting significantly improved the thermostability of the lipases at every temperature. In contrast, the PEG-lipase complex did not significantly improve thermostability. After forming a PEG-lipase complex, the imprinted lipase exhibited significantly improved reactivity and thermostability compared to both the native lipase and the imprinted PEG-lipase complex. These results hold promising prospects for practical applications in the future.

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