Enzymatic Kinetics of Solvent-free Esterification with Bio-imprinted Lipase

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To avoid the use of compounds that burden the environment, a solvent-free enzymatic reaction was the focus of this study. Investigated were the catalytic activities and kinetics of lipases that were pretreated with carboxylic acids for the solvent-free esterification of propionic acid with isoamyl alcohol. The enhancements of the esterification yields and rates by the bio-imprinting effects of carboxylic acids were observed. We found no inhibition of isoamyl alcohol on the solvent-free enzymatic esterification, and obtained a large imprinting effect under a largely excessive amount of isoamyl alcohol to propionic acid. From the kinetic analysis, the imprinting of lipases mainly enhanced the catalytic reaction rate constant rather than the affinity between lipase and propionic acid compared with untreated lipase. The bio-imprinting treatment of lipase is found to be very effective for the yield and kinetics in solvent-free esterification.

Keywords:
lipase, bio-imprinting, solvent-free esterification

Introduction

Lipases have been used in various applications, such as industrial biotransformation, diagnostic and medical processes. For practical application, lipases should be stable and catalytically active under process conditions, such as varying pH, ionic strength, and presence of organic solvent. Lipases have been frequently used in organic media, since their substrates and products are water-insoluble. However, organic solvents are generally harmful and not environmentally benign. Moreover, most water-soluble organic solvents impair enzymatic activity. In this context, a solvent-free enzymatic reaction (oily substrates themselves work as a reaction media without an additional organic solvent) has been studied[1,2] because of the following advantages: (1) substrate concentration can be maximized, (2) harmful organic solvents can be avoided, and (3) load on downstream processing can be suppressed[2].

Many techniques have been developed for enhancing the catalytic activity of enzymes in organic media. One enhancement of enzymatic activity in organic media is the additive approach[3]. Although this method is simple, the product must be separated from the additive. Other methods include the pretreatment of an enzyme before using it in an organic medium. This method has some advantages that its preparation is easy and no additives are required in the reaction system. One pretreatment for rate enhancement is the bio-imprinting method. This method includes the lyophilization of an enzyme loaded with a substrate analogue to form a complex that resembles an enzyme-substrate complex in the aqueous solution and removes the substrate analogue[4]. The treated enzyme retains the structure of the substrate analogues in the reaction medium. Moreover, when lipases were used as the enzyme, they had two conformational states, closed (inactive) and open (active). The active sites of lipases in the aqueous solution are covered by a flexible region (lid) of lipase. Interfacial activation by the organic solvent causes opening of the lid to make the active site accessible[9].

Recently, an increase in the esterification and transesterification activities has been reported with an immobilized lipase that was bio-imprinted with carboxylic acids[5–8]. However, only a few examples of bio-imprinted lipases have been found in solvent-free transesterification[9] and interesterification[10]. In previous papers, bio-imprinting was qualitatively effective in a solvent-free medium as well as in a reaction medium diluted by organic solvents, and there are few kinetic studies for bio-imprinted lipase-catalyzed reaction.

In the present work, a quantitative analysis of a solvent-free esterification catalyzed by a bio-imprinted lipase was carried out to clarify the kinetic aspect of rate enhancement.

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Materials and methods

Materials

Lipase from *Candida rugosa* (Sigma Aldrich L1754, specific activity of 704 olive oil units/mg) was used throughout the experiments. All the remaining reagents were of analytical grade and used without further purification. Carboxylic acids, hexanoic, octanoic, decanoic, and dodecanoic acids were used for treating the lipase.

Preparation of bio-imprinted lipase

A bio-imprinted lipase was prepared as follows: 300 mg of lipase were dissolved in 9 cm^3 of a phosphate buffer solution (10 mmol dm^{-3}) at pH 6.5. Carboxylic acid (3.5 mmol) and Tween 20 (1000 mg) were dissolved in 10 cm^3 of ethanol. One cm^3 of carboxylic acid solution was added to the enzyme solution. The mixed solution was then incubated for 20 min at room temperature (≈ 25 °C), and freeze-dried. The obtained powder was washed with hexane (100 mL) to remove the acid. The resultant lipase was filtered and dried in vacuo. As a control, we prepared the lipase, which was pretreated without carboxylic acid.

Esterification

To examine the bio-imprinting effect of the alkyl chain length of carboxylic acids as substrate analogues, lauric acid and benzyl alcohol were used as substrates for the esterification. The reactions were initiated by adding 1 mg of lipase powder that was pretreated by carboxylic acids into 1 mL of a toluene solution 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. After 30 min, the vial tubes were removed from the thermomixer (Comfort, Eppendorf), and immediately centrifuged for 3 min at 10000 rpm. The benzyl laurate concentration in the supernatant was measured using HPLC.

Solvent-free esterification

Propionic acid and isoamyl alcohol were used as substrates for solvent-free esterification because lipase was actively inhibited by benzyl alcohol[11]. The reactions were initiated by adding 25 mg of the lipase powder that was pretreated by octanoic acid into 0.25 mL of a mixture containing both substrates (propionic acid and isoamyl alcohol) without solvent in a vial tube at 37 °C and 1400 rpm. At various intervals within 24 h, the vial tubes were removed from the thermomixer, and immediately centrifuged for 3 min at 10000 rpm. Ethanol (0.9 mL) was added to the supernatant (0.1 mL) to stop the reaction. Its extent was monitored by measuring the isoamyl propionate concentration using HPLC. The reaction rates were evaluated as the initial rate in the initial period (5 h). The values obtained were averaged over three measurements. The esterification reaction used in this study is presented in Scheme 1. For kinetic study, propionic acid (its concentration 25 ~ 1600 mmol dm^{-3}) was dissolved in isoamyl alcohol.

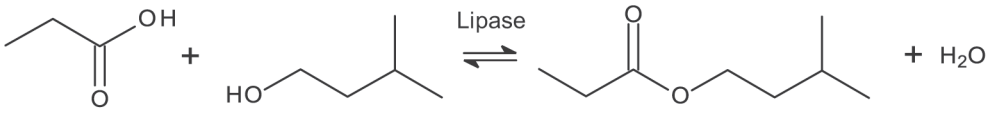
Analysis

The standard benzyl laurate and isoamyl propionate solutions were used for preparing the calibration curves. The concentrations of the benzyl laurate and the isoamyl propionate in the organic solutions were determined by HPLC (Shimadzu LC-10ADvp) with a Wakosil-II 5C18AR column and an eluent solution (ethanol: methanol:water = 15:5:3 for the esterification, and methanol:water = 5:5 for the solvent-free esterification) as a mobile phase (0.2 mL min^{-1} for the esterification, and 1.0 mL min^{-1} for the solvent-free esterification). Esters were detected with an UV detector (255 nm for the esterification, and 212 nm for the solvent-free esterification, Shimadzu SPD10AV).

Results and discussion

Effect of alkyl chain length of carboxylic acids as substrate analogues on esterification

Prior to the solvent-free esterification catalyzed by imprinted lipase, examined was the effect of the imprinting molecules on the esterification of lauric acid with benzyl alcohol diluted in toluene. Fig. 1 shows the concentration of benzyl laurate produced at 30 min with various lipases (untreated, control, and carboxylic acid pretreated). When cyclohexane was used as a diluent, very similar results were obtained (data not shown). Although activity of the control lipase was lower than that of the untreated lipase, the activities of the pretreated lipase with carboxylic acids were much higher than that of the



Scheme 1 – *Esterification reaction to form isoamyl propionate from propionic acid and isoamyl alcohol*

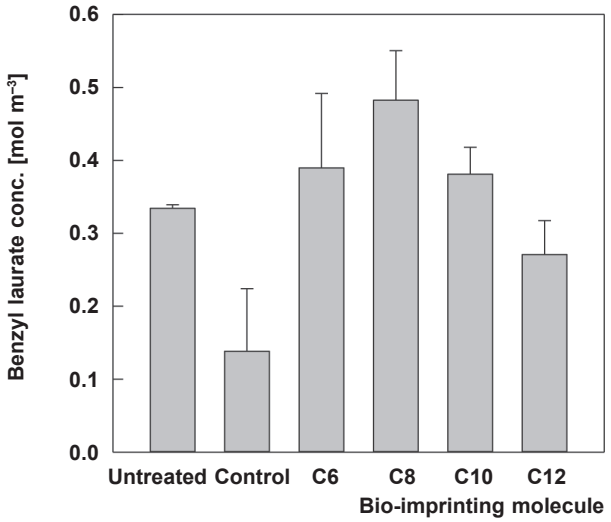


Fig. 1 – *Effect of alkyl chain length of carboxylic acids as bio-imprinting molecule on esterification in toluene*

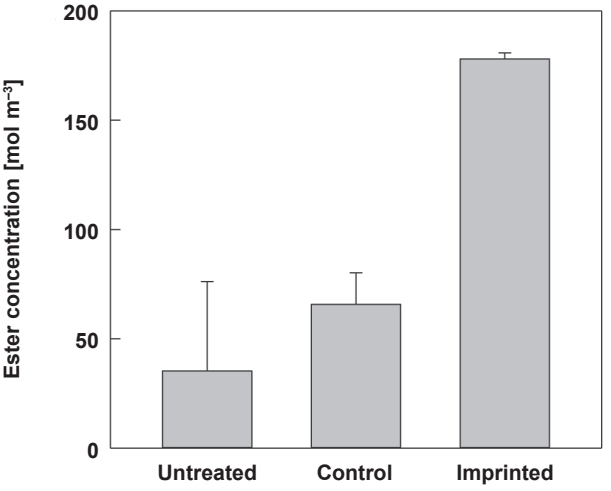


Fig. 2 – *Effect of lipase imprinting on solvent-free esterification (molar ratio of isoamyl alcohol to propionic acid = 3, ester concentrations are at 24 h)*

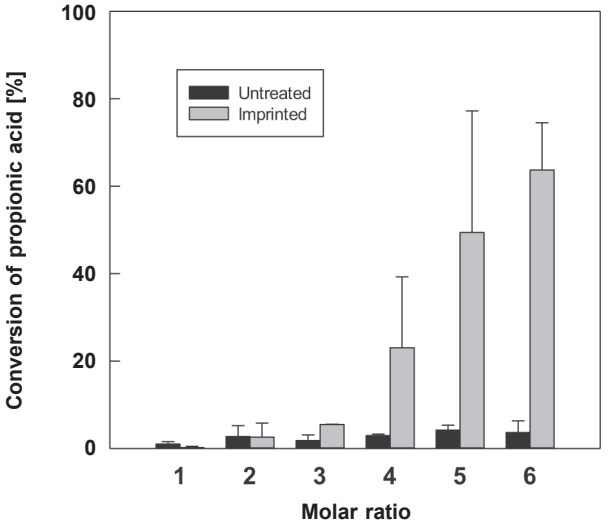


Fig. 3 – *Effect of molar ratio to conversion of propionic acid to isoamyl propionate with untreated and imprinted lipases*

control lipase, suggesting that pretreatment of the carboxylic acids is effective for esterification. Among the carboxylic acids, the highest activity was obtained with lipase that was pretreated by octanoic acid as a substrate analogue. This suggests that the preferable conformational change of lipase for binding with lauric acid is initially induced by octanoic acid and its conformation is kept after removing the octanoic acid in a process that is called bio-imprinting[4]. The enhancement of lipase activity by bio-imprinting has been also explained by the interfacial activation mechanism[5,7,8]. Contact with the hydrophobic substrate such as octanoic acid causes the lid opening to make the active site accessible. The highest activity of octanoic acid was considered to be caused by the balance between the cavity size in lipase and the hydrophobicity of carboxylic acid. In the following experiments, octanoic acid was used as an imprinting molecule.

Solvent-free esterification

Because of the strong inhibition effect of benzyl alcohol on the enzymatic reaction[11], esterification of propionic acid with isoamyl alcohol was selected as a model reaction system. Fig. 2 shows solvent-free esterification with various lipases (untreated, control, and imprinted) at a molar ratio of isoamyl alcohol to propionic acid = 3 after 24 h. Similar results were obtained with other molar ratios. Esterification yield with imprinting lipase is evidently much higher than those of the control and untreated lipases, suggesting that the imprinting treatment to lipase is also effective for solvent-free esterification. In solvent-free transesterification of 1-butyl caprylate and 1-hexanol[9], activation factor of reaction rate spanned from 2.1 to 117.5 depending on lipase source. The activation factor of this system is within this range.

In a solvent-free esterification of ethyl hexanoate[2], substrate inhibition was observed. To clarify the inhibiting and denaturation effects of isoamyl alcohol, the effect of the molar ratio (isoamyl alcohol/propionic acid) on the conversion of the propionic acid was examined at 24 h (Fig. 3). The increase in the isoamyl alcohol concentration caused a significant imprinting effect, and no negative effect of isoamyl alcohol on the solvent-free enzymatic esterification was observed. Because the large imprinting effect was obtained in a huge amount of excess isoamyl alcohol, the effect of propionic acid concentration on the initial esterification rates was examined under this condition (Fig. 4). Generally, lipase catalytic reaction is believed to obey Ping-Pong Bi-Bi mechanism[15]. Initial esterification rate, r_p, under a larger excess of isoamyl alcohol over propionic acid was approximated by the following equation;

$$r_p = \frac{r_{p,max}[S]}{K_m + [S]} \quad (1)$$

where [S] is the initial concentration of the propionic acid, and $r_{p,max}$ and K_m are respectively the apparent maximum rate and the Michaelis constant. The kinetic parameters were determined by a non-linear regression program included in the Sigmaplot 14 software. The solid lines in Fig. 4 were calculated by these kinetic parameters. The calculated lines agree well with the experimental results.

The values of the parameters obtained for the untreated and imprinted lipases are listed in Table 1. Here, the decrease in the K_m values by the imprinting treatment means the increase in the affinity between the propionic acid and the lipase, suggesting that the conformational changes of catalytic site and lid are initially induced by octanoic acid, and this lid-open conformation was retained after removing the octanoic acid[4]. However, the difference in the K_m values was not very large and the contribution of the increase in the maximum rates by the imprinting treatment is large. Generally, hydrophilic organic solvent appeared to strip water off the enzyme and inactivate the enzymatic activity[12]. Since isoamyl alcohol is relatively hydrophilic (log P = 1.42), the imprinting treatment may affect prevention of water stripping. Previous studies[13,14] show few results of the kinetic parameters of enzymatic solvent-free esterification. A detailed analysis of the parameters is needed for an optimum process design.

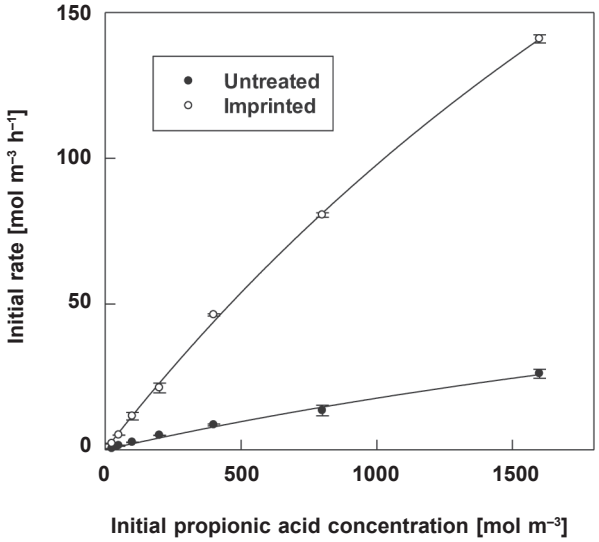


Fig. 4 – *Effect of initial propionic acid concentration on initial esterification rate with untreated and imprinted lipases*

Table 1 – *Apparent kinetic parameters of solvent-free esterification*

	Untreated	Imprinted
$r_{p,max}$ [mol m^{-3} h^{-1}]	108 ± 32	530 ± 39
K_m [mol m^{-3}]	(5.08 ± 1.90) · 10^3	(4.42 ± 0.42) · 10^3

Conclusions

The catalytic activities of lipases pretreated with carboxylic acids were investigated for the solvent-free esterification of propionic acid with isoamyl alcohol, and the enhancements of its yields and rates by bio-imprinting effects of substrate analogues were observed. These effects were caused by the conformational changes of catalytic site and lid induced by octanoic acid. No inhibition of isoamyl alcohol on solvent-free enzymatic esterification was observed, and a large imprinting effect was obtained under a largely excessive amount of isoamyl alcohol to propionic acid. From the kinetic parameter analysis, it was found that the imprinting of lipase mainly enhanced the catalytic reaction rate constant rather than the affinity between the lipase and propionic acid compared with that of the untreated lipase. Bio-imprinting treatment of lipase is very effective for the yield and kinetics in the solvent-free esterification.

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References

1. *Hammerer, F., Loots, L., Do, J., Therien, J. P. D., Nickels, C. W., Friščić, T., Aulair, K.,* Solvent-free enzyme activity: Quick, high-yielding mechano-enzymatic hydrolysis of cellulose into glucose, Angew. Chem. Int. Ed. **57** (2018) 2621. doi: https://doi.org/10.1002/anie.201711643
2. *Gawas, S. D., Lokanath, N., Rathod, V. K.*, Optimization of enzymatic synthesis of ethyl hexanoate in a solvent free system using response surface methodology (RSM), Biocatalysis **4** (2018) 14. doi: https://doi.org/10.1515/boca-2018-0002
3. *Liang, Y., Wu, Q., Lin, X.*, Effect of additives on the selectivity and reactivity of enzymes, Chem. Rec. **17** (2017) 90. doi: https://doi.org/10.1002/rcr.201600016
4. *Gupta, M. N.*, Non-aqueous Enzymology: Issues and Perspectives, Gupta, M. N. (ed.), Methods in Non-aqueous Enzymology, 2000, Birkhäuser Verlag, Bazel (2000). doi: https://doi.org/10.1007/978-3-0348-8472-3
5. *Gao, J., Yin, L., Fang, K., Zhou, L., Ma, L., He, Y., Wang, L., Jiang, Y.*, Lipase immobilization through the combination of bioimprinting and cross-linked protein-coated microcrystal technology for biodiesel production, Ind. Eng. Chem. Res. **55** (2016) 11037. doi: https://doi.org/10.1021/acs.iecr.6b03273

6. *Weiser, D., Sóti, P. L., Bánóczi, G., Bódai, V., Kiss, B., Gellért, Á., Nagy, Z. K., Koczka, B., Szilágyi, A., Marosi, G.*, Bioimprinted lipases in PVA nanofibers as efficient immobilized biocatalysis, Tetrahedron **72** (2016) 7335. doi: https://doi.org/10.1016/j.tet.2016.06.027
7. *Mukherjee, J., Gupta, M. N.,* Dual bioimprinting of *Thermomyces lanuginosus* lipase for synthesis of biodeiesel, Biotechol. Rep. **10** (2016) 38. doi: https://doi.org/10.1016/j.btre.2016.02.005
8. *Matsumoto, M., Matsui, E.,* Enhanced activities and thermostability of lipase pretreated with carboxylic and perflurocarboxylic acids in transesterification, J. Chem. Tech. Biotechnol. **93** (2018) 3219. doi: https://doi.org/10.1002/jctb.5678
9. *González-Navarro, H., Braco, L.,* Improving lipase activity in solvent-free media by interfacial activation-based molecular bioimprinting, J. Mol. Catal. B **3** (1997) 111. doi: https://doi.org/10.1016/S1381-1177(96)00038-0
10. *Yilmaz, E.,* Improving the application of microbial lipase by bio-imprinting at substrate-interfaces, World J. Microb. Biotechnol. **18** (2002) 37. doi: https://doi.org/10.1023/A:1013917611796
11. *Singh, M., Singh, S., Singh, R. S., Chisti, Y., Banerjee, U. C.,* Transesterification of primary and secondary alcohols using *Pseudomonas aeruginosa* lipase, Biores. Technol. **99** (2008) 2116. doi: https://doi.org/10.1016/j.biortech.2007.05.041
12. *Gorman, L. N. S., Dordick, J. S.*, Organic solvents strip water off enzymes, Biotechnol. Bioeng. **39** (1992) 392. doi: https://doi.org/10.1002/bit.260390405
13. *Matsumoto, M., Kida, K., Kondo, K.,* Effects of polyols and organic solvents on thermostability of lipase, J. Chem. Tech. Biotechnol. **70** (1997) 188. doi: https://doi.org/10.1002/(SICI)1097-4660(199710)70:2<188::AID-JCTB745>3.0.CO;2-X
14. *Matsumoto, M., Kida, K., Kondo, K.,* Enhanced activities of lipase pretreated with organic solvents, J. Chem. Tech. Biotechnol. **76** (2001) 1070. doi: https://doi.org/10.1002/jctb.491
15. *Garcia, T., Coteron, A., Martinez, M., Aracil, J.,* Kinetic modelling of esterification reactions catalysed by immobilized lipases, Chem. Eng. Sci. **51** (1996) 2841. doi: https://doi.org/10.1016/0009-2509(96)00162-5