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Stability Analysis of *Bacillus stearothermopilus* L1 Lipase Fused with a Cellulose-binding Domain

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Abstract This study was designed to investigate the stability of a lipase fused with a cellulose-binding domain (CBD) to cellulase. The fusion protein was derived from a gene cluster of a CBD fragment of a cellulase gene in *Trichoderma hazianum* and a lipase gene in *Bacillus stearothermophilus* L1. Due to the CBD, this lipase can be immobilized to a cellulose material. Factors affecting the lipase stability were divided into the reaction-independent factors (RIF), and the reaction-dependent factors (RDF). RIF includes the reaction conditions such as pH and temperature, whereas substrate limitation and product inhibition are examples of RDF. As pH 10 and 50°C were found to be optimum reaction conditions for oil hydrolysis by this lipase, the stability of the free and the immobilized lipase was studied under these conditions. Avicel (microcrystalline cellulose) was used as a support for lipase immobilization. The effects of both RIF and RDF on the enzyme activity were less for the immobilized lipase than for the free lipase. Due to the irreversible binding of CBD to Avicel and the high stability of the immobilized lipase, the enzyme activity after five times of use was over 70% of the initial activity.

Keywords: stability, lipase, immobilization, cellulose-binding domain

INTRODUCTION

Lipases catalyze the bioconversion (synthesis as well as hydrolysis) of lipids within organisms. To react with water-insoluble lipids, lipases possess the unique feature of acting at an interface between an aqueous and a non-aqueous phase. Lipases also possess stereoselectivity and reaction capability in a non-aqueous phase. In addition to ester hydrolysis and inter-esterification [1], they catalyze alcoholysis, acidolysis, and amidolysis [2]. Hence, lipases have tremendous potential in fields such as food technology, biomedical sciences and chemical industries [3-5]. From the numerous studies of industrial applications of lipases, it has become apparent that lipase immobilization onto a solid support is necessary for the repeated use of lipase at a high temperature [6-8]. Reactions at a higher temperature have several advantages: i) higher conversion rate, ii) lower likelihood of microbial contamination, iii) higher solubility of substrate, and iv) lower viscosity of the reaction medium, thereby favoring mass transfer [9,10]. For the repeated use of lipases for an extended period of time at high temperatures, it is necessary to clarify factors that influence the stability of lipases during the reaction. There have been several studies investigating the effects of immobilization methods and/or carrier types on the thermostability of a lipase [11,12]. However, to our knowledge there have been few systematic approaches to stability analysis of a lipase during long-term reaction.

In the present study, the stability of a lipase immobilized on Avicel (microcrystalline cellulose) *via* a cellulose-binding domain (CBD) was examined. A fusion protein of CBD and lipase was derived from a gene cluster, which was made by fusing a CBD fragment of a cellulase gene of *Trichoderma hazianum* to a lipase gene of *Bacillus stearothermophilus* L1 [13], which is referred to as CBD-BSL lipase hereafter. CBD-BSL lipase was immobilized on Avicel through adsorption, and its desorption was negligible due to the irreversible binding of CBD to Avicel [14]. Factors affecting the lipase stability were divided into reaction-independent factors (RIF), and reaction-dependent factors (RDF). RIF include reaction conditions such as pH and temperature. Substrate limitation and product inhibition, which can occur during an enzyme-catalyzed reaction [15], are examples of RDF. The decrease in lipase activity due to RIF was estimated from the activity measures of CBD-BSL lipase incubated at the reaction pH and temperature prior to the reaction. Lipase activity during the reaction was affected not only by RIF but also by RDF. Hence, the effect of RDF was determined by subtracting the effect of RIF from the decrease in lipase activity during the reaction.

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MATERIALS AND METHODS

Enzyme Production and Purification

CBD-BSL lipase was produced by fed-batch culture of

Saccharomyces cerevisiae harboring the gene cluster for CBD-BSL lipase. Culture broths containing secreted lipases were centrifuged at 1,670 g to remove cells, then the supernatants were freeze-dried. The resulting protein powders were used in oil hydrolysis and lipase immobilization. Protein powders were dissolved to 0.56 mg protein/mL in potassium phosphate solution (10 mM) in which the pH was adjusted for oil hydrolysis reactions. To remove undissolved particles, the solution was filtered through a 0.2-μm nylon filter and stored at 4°C prior to being used.

Lipase-catalyzed Oil Hydrolysis

Oil emulsion was prepared by mixing olive oil (Sigma Chemical Co., St. Louis, MO, USA) with an aqueous solution of NaCl (20 mM) and $CaCl_2$ (1 mM) containing gum Arabic at 0.5% (w/v) as an emulsifier. The initial concentration of olive oil in the oil emulsion was 1% (w/v). Oil hydrolysis reactions were initiated by the addition of free or immobilized lipase to 35 mL of the oil emulsion in a temperature-controlled vessel. A solution of free or immobilized lipase, which was stored at 4°C, was incubated for 30 min at the reaction temperature prior to being added to the vessel in order to avoid heat shock to the lipases. The extent of the reaction was monitored by titrating the liberated fatty acids with NaOH (0.1 M) solution using a pH-stat titrator (718 Stat Titorino, Metrohm, Switzerland) [16]. One unit of lipase activity was defined as the amount of enzyme that liberates a titratable amount of fatty acid equivalent to 1 μmol of NaOH in 1 min [17].

Enzyme Immobilization

Avicel with an approximately 50 μm particle size (Fluka, Buchs, Switzerland) was used as a support for lipase immobilization. Lipase immobilization was conducted by mixing 2 g of Avicel with 5 mL of the lipase solution for 3 h. After washing twice with potassium phosphate solution (10 mM) with pH adjusted for oil hydrolysis reactions, the immobilized lipases were stored at 4°C prior to being used [18]. The amount of proteins adsorbed on Avicel was determined by measuring the protein concentration of the lipase solution before and after the immobilization by Lowry method.

Stability Determination of CBD-BSL Lipase

To determine the effect of RIF on the activities of free and immobilized CBD-BSL lipases, the lipase solution and the suspension of immobilized lipases were put in glass vials with screw caps. For the immobilized lipase, 10 mg of the lipase-immobilized carrier and 0.1 mL of potassium phosphate solution (10 mM, pH 10) were added to each vial. The vials were then incubated at 50°C. During the incubation, 35 μL of the lipase solution or one vial for the immobilized lipase was taken intermittently, and added to 35 mL of the oil emulsion (1% w/v) for which the pH and temperature were adjusted to 10 and 50°C, respectively. Subsequently the lipase activity was estimated by measuring the amount of NaOH added for 10 min to maintain the reaction pH. The effect of RIF on the lipase activity (ERIF) is defined as the fraction of the activity loss:

$$\mathrm{ERIF}(t) = \left(1 - \frac{\text{enzyme activity at } t}{\text{initial enzyme activity}}\right) \times 100 \quad (1)$$

where t denotes the incubation time after the first 30 min. Initial enzyme activity in Eq. (1) is the lipase activity measured after 30 min incubation.

Oil hydrolysis reactions were conducted to determine the effect of RDF. 35 μL of the lipase solution or 10 mg of the lipase-immobilized Avicel, which was pre-incubated at 50°C for 30 min, was added to 35 mL of the oil emulsion (1% w/v) for which the pH and temperature were adjusted to 10 and 50°C, respectively. The lipase activity was estimated by monitoring the amount of NaOH added to maintain the reaction pH. As described above, the loss in the lipase activity was caused not only by RDF but also by RIF for the reaction time. Hence, the effect of RDF on the lipase activity (ERDF) is defined:

$$\mathrm{ERDF}(t) = \left(1 - \frac{\text{enzyme activity at } t}{\text{initial enzyme activity}}\right) \times 100 - \mathrm{ERIF}(t) \quad (2)$$

where t denotes the reaction time.

Reusability of Immobilized CBD-BSL Lipase

After performing the oil hydrolysis reaction for 10 min at pH 10 and 50°C with 10 mg of the immobilized CBD-BSL lipase, the immobilized enzyme was recovered by centrifugation at 1,000 rpm for 2 min. The loss of enzyme during the recovery step due to the adsorption of enzymes to the surface of centrifuge tubes was estimated from a separate control experiment: i) 10 mg of the immobilized lipase was incubated in 35 mL of potassium phosphate solution (10 mM, pH 10) at 50°C for 10 min; ii) the enzymes recovered by centrifugation at 1,000 rpm for 2 min were transferred to the 35 mL of oil emulsion and the enzyme activity was then measured.

RESULTS AND DISCUSSION

Optimum Conditions for the Oil Hydrolysis by CBD-BSL Lipase

The protein content of the immobilized lipase on Avicel was 1.12 mg/g, and the specific activity of the free and the immobilized lipase were 1,120.9 and 1,494.5 U/mg protein, respectively when measured at pH 10 and 50°C [14]. The specific activity of BSL lipase was 795.6 U/mg protein. The effects of pH and temperature on the activities of the free and the immobilized lipase are shown in Fig. 1. The pH and temperature chosen were the optimal conditions for oil hydrolysis by lipase used in this study.

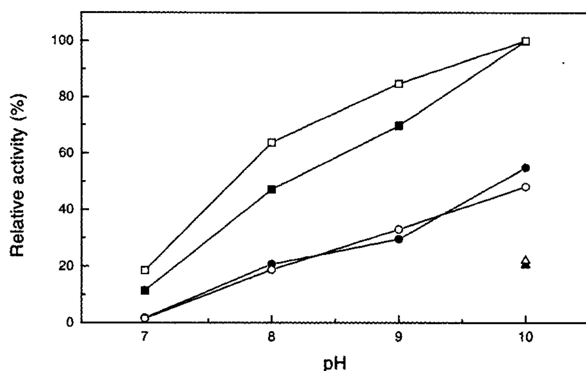

Fig. 1. Effects of pH and temperature on the activities of free (open points) and immobilized (closed points) CBD-BSL lipase: (■, □) 50°C; (●, ○) 40°C; (▲, △) 25°C. The lipase activity was determined from the oil hydrolysis reaction, which was initiated by the addition of the free or immobilized lipase to 35 mL of the oil emulsion (1% w/v). The lipase activity was estimated by monitoring the amount of NaOH added to maintain the reaction pH for 10 min.

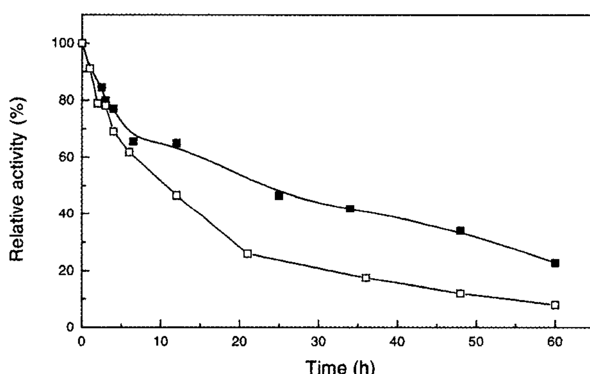

Fig. 2. Reaction-independent stability of free (□) and immobilized (■) CBD-BSL lipase. Oil-free stock solutions of free and immobilized CBD-BSL lipases were incubated at 50°C. At a certain time interval, the lipase solution was taken out and its activity was estimated from the oil hydrolysis reaction for 10 min at 50°C and pH 10.

Table 1. Effects of RIF on the lipase activities for oil hydrolysis[a]

Incubation time (h)	Effect of RIF (%)	
	Free enzyme	Immobilized enzyme
0	0.0	0.0
0.5	9.0	4.2
1.0	18.2	8.9

[a] The effects of RIF were calculated using Eq. (1) and the experimental data shown in Fig. 2.

Reaction-independent Stability of CBD-BSL Lipase

The effect of the incubation time on the activity of CBD-BSL lipases is shown in Fig. 2. Free lipase activity decreased to less than half of the initial activity within 12 h of incubation at 50°C. After 60 h of incubation, the remaining activity was less than ten percent of the initial activity. The activity of the immobilized lipase also gradually decreased over time during incubation, but was always over twice as high as that of free lipase when subject to the longer incubation periods (12~60 h). The effects of RIF (Eq. (1)) on the lipase activities were calculated using the experimental data shown in Fig. 2, and summarized in Table 1.

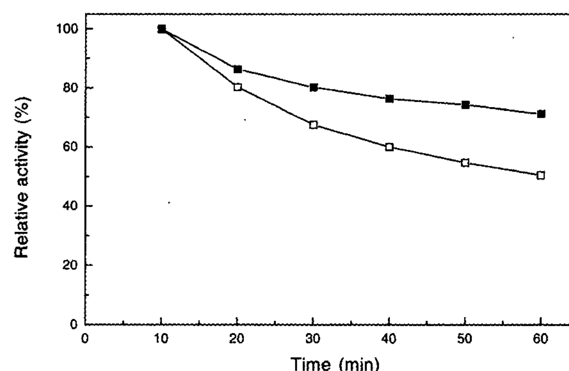

Fig. 3. Reaction-dependent stability of free (□) and immobilized (■) CBD-BSL lipase. The change in the lipase activity during the relatively long-time (60 min) reaction was measured to determine the RDF.

Table 2. Effects of RDF on the lipase activities for oil hydrolysis[a]

Reaction time (h)	Effect of RDF (%)	
	Free enzyme	Immobilized enzyme
0	0.0	0.0
0.5	23.6	15.6
1.0	31.3	17.3

[a] The effects of RDF were calculated using Eq. (2) and the experimental data shown in Fig. 3.

Reaction-dependent Stability of CBD-BSL Lipase

CBD-BSL lipase activity during one-hour oil hydrolysis reaction is shown in Fig. 3. The ratio of the enzyme activity to initial activity is plotted versus the reaction time. Assuming the effects of RIF (ERIF) on the lipase activities were linearly proportional to the reaction time up to 1 h, the effects of RDF (ERDF) were calculated from Equation (2) using the experimental data shown in Fig. 3. The values of ERDF for the 1 h reaction are summarized in Table 2 with the values of ERIF. The effects of RDF on the enzyme activity were less for the immobilized lipase than for the free lipase. It was found from Thin Layer Chromatography (TLC) analysis of the reaction mixtures that sufficient amounts of triglycerides in oil remained after 1 h of reaction using either the free lipase or the immobilized lipase (data not shown). Hence,

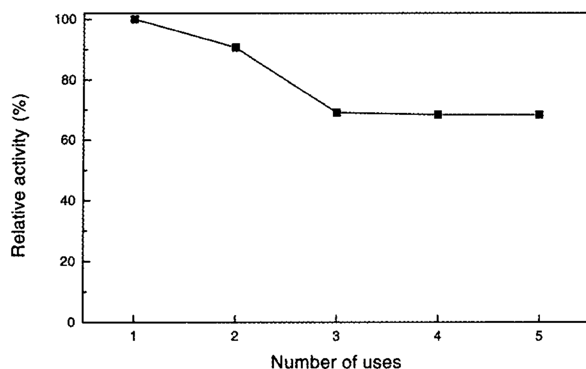

Fig. 4. Relative activity of CBD-BSL lipase immobilized on Avicel with multiple uses for oil hydrolysis. After performing the oil hydrolysis reaction for 10 min at pH 10 and 50°C, the immobilized enzyme was recovered by centrifugation at 1,000 rpm for 2 min and re-used. The enzyme loss due to the adsorption to the surface of a centrifuge vial was measured from a separate control experiment.

substrate limitation cannot explain the difference in the effects of RDF between the free lipase and the immobilized lipase. As immobilized lipase activity was always higher than that of free lipase, larger amounts of fatty acids (products of the lipase-catalyzed oil hydrolysis) should be liberated in the reaction system with the immobilized lipase than with the free lipase. Fatty acids are known to inhibit the lipase-catalyzed oil hydrolysis [16]. Therefore the inhibition of the lipase activity by the produced fatty acids should be larger for the immobilized lipase. The lower ERDF observed for the lipase immobilized on Avicel means that Avicel interfered with the inhibition of lipase activity by fatty acids, which is probably owing to the adsorption of fatty acids to Avicel.

It has been known that the loss of an enzyme immobilized by adsorption is probably coming from its weak binding. A cellulose binding domain (CBD) binds to cellulose *via* adsorption, but their binding is often irreversible depending on the kinds of CBD. CBD-BSL lipase used in this study is a fusion protein of a cellulose binding domain of *Trichoderma hazianum* cellulase and *Bacillus stearothermophilus* L1 lipase. Hence, CBD-BSL lipase can bind to cellulose *via* a specific binding of CBD to the cellulose. Avicel, which is microcrystalline cellulose, could therefore immobilize CBD-BSL lipase. In addition to the insignificant loss owing to the irreversible binding of CBD to Avicel, the enhanced reaction-dependent and reaction-independent stability of the immobilized CBD-BSL lipase suggests that a cellulose-binding domain may be potentially used as a highly versatile linker for the stable enzyme immobilization.

Reuse of Immobilized CBD-BSL Lipase

Fig. 4 shows the relative activities of immobilized CBD-BSL lipase remaining after multiple uses. Over 70% of the initial activity was maintained after 5 uses.

CONCLUSION

The stability of a lipase fused with a cellulose-binding domain (CBD) of a cellulose was studied. The fusion protein was derived from a gene cluster of a CBD fragment of a cellulose gene of *Trichoderma hazianum* and a lipase gene of *Bacillus stearothermophilus* L1. Due to the CBD, this lipase can be immobilized to a cellulose material. Avicel (microcrystalline cellulose) was used in this study as a support for lipase immobilization. Factors affecting lipase stability were divided into reaction-independent factors (RIF), and reaction-dependent factors (RDF). RIF includes the reaction conditions such as pH and temperature, whereas substrate limitation and product inhibition are examples of RDF. As pH 10 and 50°C were found to be optimum reaction conditions for the oil hydrolysis by this lipase, the stability of the free and the immobilized lipase was studied under these conditions. The effects of both RIF and RDF on the enzyme activity were less for the immobilized lipase than for the free lipase. We believe that Avicel decreases the inhibition of the lipase activity by fatty acids, likely resulting from the adsorption of fatty acids to Avicel. In addition to the irreversible binding of CBD to Avicel, these findings suggest that a cellulose-binding domain can be a highly versatile linker for the stable enzyme immobilization.

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