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Dependency of water concentration on ethanolysis of trioleoylglycerol by lipases

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Abstract

The effects of water concentration on ethanolysis of trioleoylglycerol catalyzed by four different lipases were studied. The target product of the ethanolysis was 2-monooleoylglycerol (2-MO). Novozym 435 (a commercially available preparation of immobilized *Candida antarctica* lipase B, CALB) exhibited both the highest product yield and the reaction rate at very low (less than 1 wt.%) free water concentration. Its catalytic activity did not drop even in dry state, i.e. in the system of dry CALB in dry ethanol (water concentration was ca. 0.1 wt.%). In contrast, other three immobilized lipases tested (*Rhizomucor miehei* lipase, *Burkholderia cepacia* lipase and *Thermomyces lanuginosus* lipase) required larger amounts of free water (ca. 7–9 wt.%) for their best performance and exhibited no ethanolysis reaction at low free water concentrations. The CALB's anomalous behavior was also observed in other two different preparations of CALB; i.e., free CALB powder and silica-immobilized CALB as well as Novozym 435. Thus, it was confirmed that no extra water requirement of CALB was an intrinsic property of CALB itself. No extra water requirement of CALB implies that this enzyme is able to keep water molecules tightly to retain its catalytic activity even in dry ethanol (a water-depriving solvent). It is suggested that some structural features, such as a carbohydrate molecule, a lid-like domain, or very tight bonding of the essential water molecule(s), might be involved in this very unique property.

Keywords: Ethanolysis; *Candida antarctica* lipase B; Water concentration; 2-Monoacylglycerol; Triolein

1. Introduction

Lipases are extensively used to catalyze hydrolysis, alcoholysis, esterification and transesterification of carboxylic esters. These reactions typically proceed with high regio- and stereo-selectivities, making lipases an important group of biocatalysts in organic chemistry [1–3]. In a serial research on lipase-catalyzed synthesis of symmetrical triacylglycerols our group reported that the immobilized *Candida antarctica* lipase B (CALB) exhibits very efficient catalytic activity in the ethanolysis of triacylglycerol (TAG) to yield 2-monoacylglycerol (2-MAG) [4,5]. In aqueous system, 2-MAG is unstable, easy to isomerize to 1(3)-monoacylglycerol. However, in ethanol 2-MAG appears rather stable [5].

An important factor that greatly affects the activity of biocatalysts in microaqueous reaction system is the amount of water in the reaction system. Most of enzymes require a certain amount of water to maintain their active conformation. The ability to retain the essential water may differ depending on the type of enzyme. In many cases, the reaction rate is low at very low water content, and increases drastically when more water is present. Therefore, it would be expected that, in a typical ethanolysis reaction, ethanol (water-miscible solvent) deprives crucial water molecules from the catalyst, and that the catalyst loses its activity [6–8]. However, in the case of CALB-mediated ethanolysis, the reaction undergoes efficiently without losing the catalytic activity even in excess of ethanol [5]. From this aspect, we wondered that CALB might be able to preserve necessary water molecule in its structure even though it was exposed to a strongly water-depriving solvent, because generally ethanol is a water-miscible solvent so that it deprives water from water-containing solid particles when they are put into it.

The aim of the present study is to investigate the water requirement of CALB in ethanolysis of trioleoylglycerol (TO) under condition of controlled water content. Its

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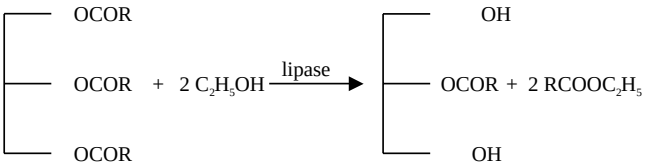
reaction scheme is



where RCO– is oleic acid residue.

We have found an anomalous property of CALB that it requires no extra amount of water as compared with other lipases. Taking the structural features of CALB molecule into consideration, some possible explanations for no extra water requirement of CALB are discussed.

2. Experimental

2.1. Materials

Lipozyme RM IM (immobilized *Rhizomucor miehei* lipase (RML); particle diameter 0.2–0.6 mm), lipozyme TL IM (immobilized *Thermomyces lanuginosus* lipase (TLL); particle diameter 0.3–1.0 mm), Novozym 435 (immobilized *C. antarctica* lipase B; particle diameter 0.3–0.9 mm) and Novozym CALB L (aqueous solution of CALB) were gifts from Novozymes Japan Ltd. (Chiba, Japan). Amano PS-C-I (immobilized *Burkholderia cepacia* lipase (BCL); particle diameter 0.2–0.5 mm) was donated from Amano Enzyme Inc. (Nagoya, Japan). Submicron silica particle (type KE-P30; diameter 0.27–0.35 μm) was a gift from Nippon Shokubai Co. Ltd. (Osaka, Japan). TO and ethanol were purchased from Wako Pure Chemicals (Osaka, Japan) and used after drying by adding dry molecular sieves particles (type 3A1/8, Wako Pure Chemicals, Osaka) in their bottles. The purchased TO contained less than 0.036 wt.%, and the purchased ethanol contained 0.25 wt.% of water content, respectively. The water contents of the dried TO and ethanol were less than the detection limit of Karl–Fischer moisture meter (0.02 wt.%). Karl–Fischer's reagent was purchased from Mitsubishi Chemical Corp. (Tokyo, Japan). All other chemicals were of analytical quality or better. Pure water was obtained via a Milli Q system (Millipore, Japan). For investigation into the ethanolysis of TO at very low water content, Novozym 435, Lipozyme RM IM and Lipozyme TL IM were pre-dried by putting them into dry ethanol and replace it rapidly by fresh dry ethanol three times consecutively to reduce water content.

2.2. Ethanolysis reaction of trioleoylglycerol

In a typical experiment, dried TO (3.3 g) was mixed for 30 min with dried ethanol (13.2 g) and pure water was added at various quantities in 20 ml reaction bottle at 500 rpm, 35 °C for emulsification of the reaction mixture, then the reaction was started by the addition of 2.0 g (10% of reactants) immobilized lipase. To avoid moisture from air inside the reaction bottle and surrounding area, the volume of the reaction mixture was set to cover more than 95% of the space inside the bottle and the bottle was closed tightly with a rubber cap which was not opened all the time. The samples were withdrawn at intervals via a needle penetrated through the rubber cap. After the reaction, the mixture was left for 20 min for precipitation of catalyst and then upper clear liquid was used for free water content measurement by a Karl–Fischer moisture meter (MKS-1, Kyoto Electronics Co. Ltd., Kyoto, Japan). The data were expressed as free water content in the reaction mixture (wt.%).

2.3. Thin-layer chromatography/flame-ionization detector (TLC/FID) analysis

The samples withdrawn from the reaction mixture were dissolved into diethyl ether, and filtered to remove the catalyst. The resulting lipid solution was analyzed with TLC/FID analyzer (Iatroscan MK-5; Iatron Laboratories, Tokyo, Japan) using Chromarod S III quartz rods. The rods loaded with the samples were eluted for 10 cm with *n*-hexane/diethyl ether (9:1) and then the upper 8 cm were scanned for the quantification of ethyl oleate, TO and oleic acid. 1,2-Dioleoylglycerols (DO) and monooleoylglycerols (MO) (which may contain 1(3)-MO and 2-MO isomers remaining close to the rod origin after the first development) were separated by a second development with benzene/chloroform/acetic acid (100:5:1).

For separation of 2-MO from 1(3)-MO (which cannot be separated by the above-mentioned method), the lipid solution was applied to Chromarod S III quartz rods treated with boric acid. The rod was developed 8 cm with chloroform and subsequently developed again with chloroform/methanol/ammonium hydroxide (70/0.04/0.01) for 10 cm.

2.4. Preparation of free CALB and its physical immobilization on submicron silica particle

Novozym CALB L was dialyzed against pure water to remove impurities and then purified by precipitation with 60% saturated ammonium sulfate. The precipitate was redissolved into water, dialyzed against water, and lyophilized. The resulting powder was used as free CALB. SDS-PAGE analysis of the resulting powder showed only one major band, confirming that the preparation was nearly pure.

For immobilization onto submicron silica particle, the above prepared lipase powder (25 mg) was dissolved in pure water (10 ml) and subsequently mixed with submicron silica particle (0.5 g). The mixture was incubated at 4 °C for 1 h with stirring, and then added cold ethanol (200 ml). After centrifugation of the mixture, the pellet was recovered. The pellet was lyophilized and used as silica-immobilized CALB.

3. Results

3.1. Effect of water concentration during ethanolysis of trioleylglycerol

Fig. 1 shows the time course of 2-MO formation during ethanolysis by various lipases at different free water contents. In the case of CALB (Fig. 1A) at free water content of 0.1 wt.%, the reaction proceeded rapidly and reached a plateau at 180 min. However, at higher free water content (2.2 or 4.3 wt.%), the reaction became slower. In contrast, the other three immobilized lipases displayed clear difference (Fig. 1B–D). These three lipases preferred free water content of around 7–9 wt.% for the best performance, and were almost inactive at less than 1 wt.% of free water content. In all the experiments, the concentration of 1(3)-MO was very low (<1 mol%). Thus, no acyl migration from 2-MO to 1(3)-MO in the ethanolysis was reconfirmed.

Fig. 2 summarizes the final content (when the reaction reached plateau) of 2-MO in the ethanolysis. The optimum free water content for CALB was 0.4 wt.% that was much lower than the ones for the other lipases. These results suggest that CALB is less sensitive to low water content than the other three lipases, which might be related with the difference in ability to retain its active conformation in dry ethanol.

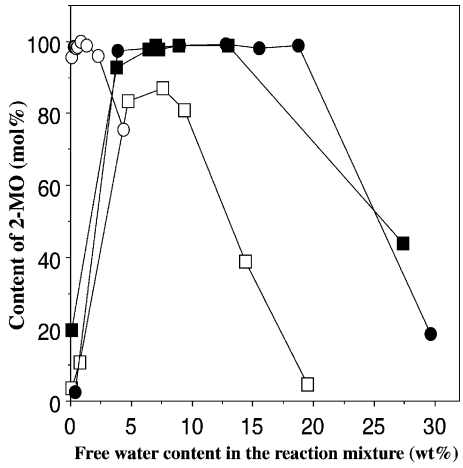


Fig. 2. Final 2-MO yield by ethanolysis of TO by immobilized lipases at different water contents: (○) CALB, (□) RML, (●) BCL and (■) TLL. The data at the lowest water content for CALB, RML and TLL were obtained using the dried CALB, RML and TLL, respectively.

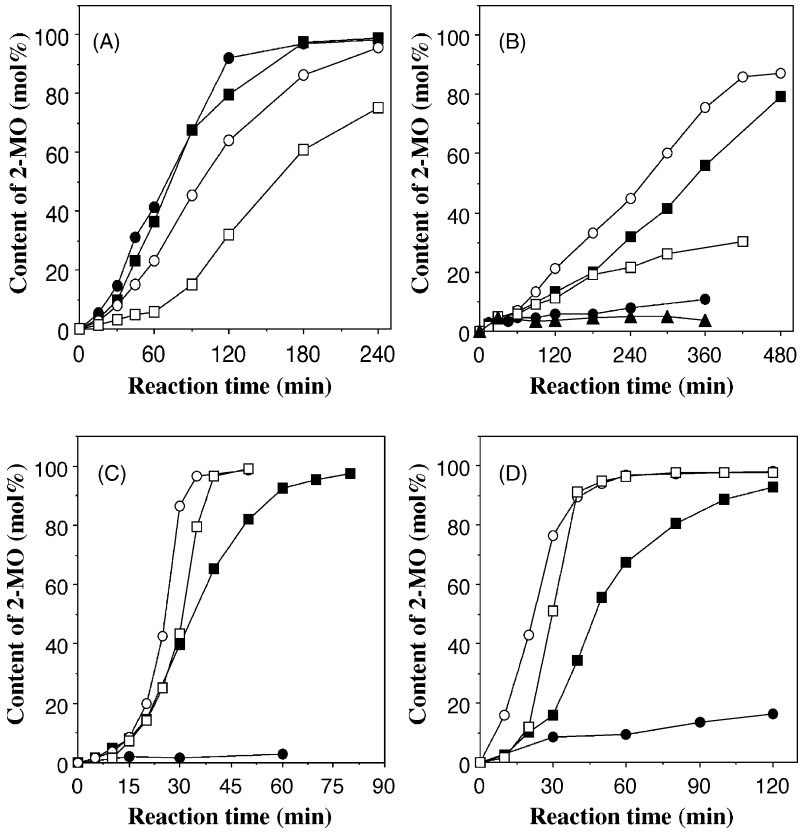


Fig. 1. Time course of 2-MO formation during ethanolysis of TO by immobilized lipases at different water contents. The reaction was performed at ethanol/TO molar ratio = 77/1 (weight ratio = 4/1) and 35 °C: (A) CALB, (B) RML, (C) BCL, and (D) TLL. The legends represent water content in the reaction mixture. (A) (●) 0.1 wt.%, (■) 1.3 wt.%, (○) 2.2 wt.% and (□) 4.3 wt.%; (B) (▲) 0.05 wt.%, (●) 0.8 wt.%, (■) 4.7 wt.%, (○) 7.5 wt.% and (□) 14.4 wt.%; (C) (●) 0.3 wt.%, (■) 3.9 wt.%, (○) 9.0 wt.% and (□) 12.7 wt.%; (D) (●) 0.1 wt.%, (■) 3.7 wt.%, (○) 8.9 wt.% and (□) 12.9 wt.%. The data at the lowest water content for CALB, RML and TLL were obtained using the dried CALB, RML and TLL, respectively.

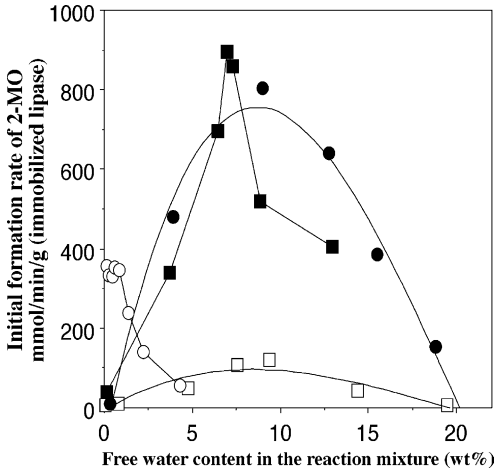


Fig. 3. Initial formation rate of 2-MO by the immobilized lipases in ethanolysis of TO at different water contents: (○) CALB, (□) RML, (●) BCL and (■) TLL. The data at the lowest water content for CALB, RML and TLL were obtained using the dried CALB, RML and TLL, respectively.

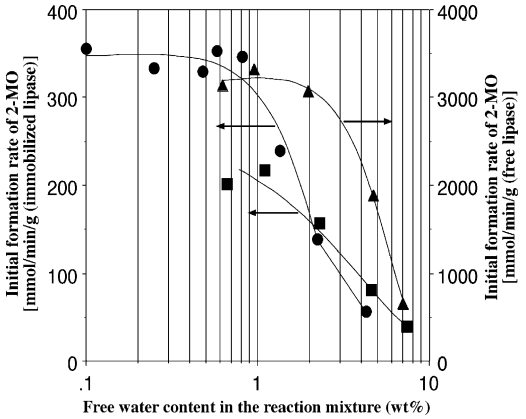


Fig. 4. Initial formation rate of 2-MO in ethanolysis of TO at different water contents: (■) silica-immobilized CALB, (▲) free CALB and (●) Novozym 435. The data at the lowest water content for Novozym 435 was obtained using the dried Novozym 435.

3.2. Effect of water concentration on the initial ethanolysis rate

The quantity of water in microaqueous reaction system not only affects the final reaction product but also obviously affects the initial reaction rate. As seen in Fig. 3, CALB displayed high initial reaction rate at very low free water content (lower than 1 wt.%), while the other three lipases required some amount of added water to express high initial rate (around 7–9 wt.%). This indicates again that CALB does not need any extra amount of free water to retain its catalytic activity and keeps the essential water molecules extremely tightly.

3.3. Effect of water concentration on the ethanolysis by free enzyme suspended and self-prepared immobilized CALB

It is known that the catalytic activity of lipases in organic solvent depends very much on the type of enzyme preparation used. For this reason, we wondered whether the very low water requirement of CALB, observed so far, was due to an intrinsic property of CALB itself, or due to a synergistic effect of the immobilization support and the enzyme. To confirm this, we investigated the water requirements of free CALB and silica-immobilized CALB. As seen in Fig. 4, where *x*-axis is logarithmic scale, both the free CALB and silica-immobilized CALB demonstrated very low water requirement, which was similar to Novozym 435.

3.4. Water content of immobilized lipases and the ethanolysis in nearly anhydrous condition

For ethanolysis of TO without addition of extra water, most soluble free water came from the immobilized lipase's particles because usually, commercially available immobilized lipases contain some amount of water. Hence, the amounts of water included in the enzyme preparations were measured and listed in Table 1. We found that CALB, RML and TLL were able to undergo the ethanolysis without addition of extra water even though the reaction rate was very slow in the cases of RML and TLL. To observe the ethanolysis in nearly anhydrous condition, these immobilized lipases were dehydrated with dry ethanol repeatedly until their water content were reduced to nearly zero (<0.05 wt.%) and then used in ethanolysis. The results are included in Figs. 2 and 3. Surprisingly, CALB displayed still efficiently ethanolysis of TO even in very low water content (0.1 wt.%, Figs. 1A and 4). Its activity was the highest among the obtained values. In contrast, RML and TLL exhibited no ethanolysis of TO (0.05 and 0.1 wt.%, Fig. 1B and D). This result suggests that although CALB was dehydrated by dry ethanol beforehand, it was still able to retain active conformation probably by keeping essential water molecules in its structure.

Table 1
Amounts of water contained in the enzyme preparations

Enzyme	Amount of water contained (wt.%)
Free CALB	11.4 ± 0.3
Silica-immobilized CALB	3.9 ± 0.3
Novozym 435	2.2 ± 0.3
Lipozyme RM IM	6.4 ± 0.3
Lipozyme TL IM	4.2 ± 0.3
Amano PS-C-I	1.6 ± 0.3

4. Discussion

From the data obtained in this study, it is most probable that the anomalous behavior of CALB in the ethanolysis may be related to its structural features. The crystal structure of CALB seems to be in an open conformation with

an accessible active site. It has a small amount of water molecules bound tightly to the active site, which lacks contact with external solvent molecules [9]. If these water molecules are essential for the catalysis, our results suggest that these water molecules retained in its active site structure are not lost even in dry ethanol. Moreover, CALB also has carbohydrate molecules located in its structure and makes hydrogen bonds with amino acid and water molecules [9]. Since the carbohydrate molecules have many OH groups, these molecules might be able to keep essential water by forming strong hydrogen bonds.

In addition, the active site conformation of the lipases could be involved in the mechanism. The active site of CALB is an elliptical, steep funnel [10]. On the other hand, the active site of RML is a shallow bowl located near the protein surface [10]. Therefore, in RML, the ethanol molecules probably easily access to strip out the crucial water molecules from this site resulting in loss of its catalytic activity.

CALB also does not exhibit typical lid domain but it has a short helix with high mobility, which might be act as a lid [9]. However, the other three lipases (BCL, TLL and RML) have large lid domains and exhibit interfacial activation, which are associated with a large conformational change of the lid [11–13]. It is believed that enzymes are generally more rigid in organic solvent than in water [14]. For this reason, it can be assumed that CALB, which requires a small conformational change during catalysis, becomes more active than the other lipases in dry ethanol. Correspondingly, it was reported that RML, which exhibits interfacial activation in aqueous solution, is unable to display this property in ethanol [15]. Finally, a possibility cannot be ruled out that in CALB, ethanol molecule can play similar function of water molecule partly at least because ethanol molecule has also one OH group.

In the present study, both the final reaction product and initial reaction rate are also decreased at high water content (Figs. 2 and 3). It can be explained by a limitation of substrate transport from reaction medium to the vicinity of enzyme due to water. Actually, electronic microscopy observation of immobilized enzyme at different water activity showed that water surrounded the particle of the biocatalyst, forming a layer preventing lipophilic substrate access to the enzyme, and led to particle aggregation [16]. Although we did not use the same resin, a similar effect can be assumed. However, at low water content, it may not have such a limitation at least in the case of CALB. This suggestion was supported by the previous report. Mei et al. [17] studied the distribution of CALB and polystyrene (a model substrate) within Novozym 435 bead by using infrared microspectroscopy. The result indicated that no physical barrier to CALB or to substrate diffusion throughout the bead because the average bead's pore size was 10 times larger than CALB or polystyrene molecules and the immobilization of CALB within the bead did not cause a change in its conformation.

For the ethanolysis of TO at nearly anhydrous condition, our results showed different from previous report concerning RML. Valivety et al. [18] reported that RML immobilized on either anion exchange resin or macroporous polypropylene remained active at water activity below 0.0001 in the syntheses of dodecyl decanoate by esterification of dodecanol and decanoic acid in *n*-hexane or of octyl decanoate by transesterification from octanol and dodecyl decanoate in *n*-hexane. The rate of dodecyl decanoate synthesis under exhaustive drying conditions was over 30% of that at the optimum (water activity = 0.55). They suggested three possible type of explanations for the substantial activity retained by their immobilized RML even after equilibrium at very low water activity: (i) RML may remain active in the complete absence of any water molecules, (ii) some essential water on RML may be very tightly bound in a thermodynamic sense, and (iii) the rate of desorption of essential water may be extremely slow. Although CALB in our ethanolysis reaction system will be explained also by similar reasons, the exact molecular mechanism is different because RML showed no reaction rate at nearly anhydrous condition in our system (Figs. 1B and 3, at the lowest water content, i.e. 0.05 wt.%).

In this study, we were not able to carry out the ethanolysis reaction by CALB below 0.1 wt.% of the free water concentration due to experimental difficulties. We do not know how many water molecules are bound with the enzyme in such an anhydrous condition, and whether or not these water molecules if any are essential for its catalysis. Further studies are necessary to clarify the exact molecular mechanism of exhibiting anomalously high catalytic activity of CALB in almost dry ethanol. Protein engineering approach will be informative in this respect.

5. Conclusions

This study determined the requirement of water concentration on the ethanolysis reaction by several lipases. The results indicate that:

(i) Immobilized CALB did not need any extra water to express both the high yield of reaction product and the high initial reaction rate compared with other three immobilized lipases (Figs. 2 and 3).
(ii) CALBs catalytic activity did not drop even in dry state, i.e. the system of dry CALB in dry ethanol.
(iii) Addition of excess water lowered CALB's rate of ethanolysis reaction.
(iv) This very unique and anomalous behavior was observed in the three different preparations of CALB, i.e., Novozym 435, free CALB and silica-immobilized CALB.

Therefore, it is strongly suggested that no extra water requirement of this enzyme and high activity in nearly anhydrous state come from CALB molecule itself.

Acknowledgements

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