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Conversion of degummed soybean oil to biodiesel fuel with immobilized *Candida antarctica* lipase

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Abstract

Crude soybean oil did not undergo methanolysis with immobilized *Candida antarctica* lipase but degummed oil did. Therefore, the substance that was removed in the degumming step was estimated to inhibit the methanolysis of soybean triacylglycerols (TAGs). The main components of soybean gum are phospholipids (PLs), and soybean PLs actually inhibited the methanolysis reaction. In addition, PLs were detected in chloroform/methanol (MeOH) extracts from the immobilized lipase preparation that had been used in the methanolysis of crude soybean oil. These results showed that PLs were at least one of the inhibitory substances in methanolysis of TAGs. The inhibition may due to the interference of the interaction of the lipase molecule with substrates by PLs bound on immobilized preparation. These findings indicated that degummed oil has to be used as a substrate for enzymatic methanolysis. Indeed, three-step methanolysis successfully converted 93.8% degummed soybean oil to its corresponding methyl esters, and the lipase could be reused for 25 cycles without any loss of the activity.

Keywords: Immobilized *Candida antarctica* lipase; Biodiesel fuel; Methanolysis; Degummed soybean oil; Phospholipid

1. Introduction

Enzymatic production of biodiesel fuel (fatty acid methyl esters, FAMEs) has recently attracted great interests because of its waste-free process. A lot of studies on alcoholysis of triacylglycerols (TAGs) with lipases had been reported [1–8], but their reaction systems were insufficient to apply for industrial biodiesel fuel production from the following reasons: (i) reaction efficiency was poor and a large amount of enzyme was necessary; (ii) methanolysis of TAGs efficiently proceeded in the presence of organic solvent, but not in the absence and (iii) though immobilized lipase was used as a catalyst, the enzyme preparation could not be reused.

These reactions reported then were conducted with more than stoichiometric amount of short-chain alcohol. As the solubility of short-chain alcohol is low, a part is insoluble in the reaction mixture. On the other hand, enzymes are unstable in short-chain alcohol in general. We thus hypothesized that low methanolysis was due to the inactivation of lipase caused by the contact between the lipase and insoluble methanol (MeOH) existed as drops in the oil. Indeed, when the methanolysis of vegetable oil was conducted with immobilized *Candida antarctica* lipase, the lipase was inactivated irreversibly in the presence of >1/2 molar equivalent MeOH for the stoichiometric amount [9]. These findings led to a stepwise reaction by two or three successive addition of

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MeOH, and >90% waste edible oil was successfully converted to its corresponding FAMEs [9–11]. Not only the stepwise batch reaction, but also three-step flow methanolysis did not inactivate the lipase and achieved continuous production of biodiesel fuel from waste edible oil for a considerably long period [11].

Besides waste edible oil, biodiesel fuel market can use surplus edible oil and oils from low-quality seeds without their refining. We thus attempted to produce biodiesel fuel from unrefined soybean oil using immobilized *C. antarctica* lipase. This paper describes that degummed soybean oil is efficiently converted to its corresponding FAMEs by three-step methanolysis, although the crude oil does not undergo the methanolysis. In addition, phospholipids (PLs) in the crude oil are indicated to be at least one of inhibitory substances.

2. Experimental

2.1. Materials

Crude, degummed, and refined soybean oils were provided by Yoshihara Oil Mill Ltd. (Osaka, Japan). Crude soybean oil contained 0.7% free fatty acid (FFA; acid value, 1.3 mg KOH/g), 0.08% phosphatidyl choline (PC), and 0.12% water. Saponification value was determined as 191 mg KOH/g. The crude oil was centrifuged at 5000 × *g*, for 15 min and its supernatant was used for experiments. Degummed soybean oil contained 1.1% FFA (acid value, 2.1 mg KOH/g), and 0.06% water. Its saponification value was 192 mg KOH/g. Soybean PLs (PC, 16%; phosphatidyl ethanolamine, 14%; phosphatidyl inositol, 11%; phosphatidic acid, 7%; other components are mainly acylglycerols), and egg yolk PC (purity, >95%) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). L-α-Lecithin (purity, >98%) was purchased from Funakoshi Co. Ltd. (Tokyo, Japan). Immobilized *C. antarctica* lipase (Novozym 435) was donated from Novozymes (Bagsvaerd, Denmark). Other chemicals were of analytical grade. Soybean oil containing PLs was prepared by adding soybean PLs or egg yolk PC to refined soybean oil, followed by sonicating at 100 W for 15–30 min with Kubota Insonator 201 M (Tokyo, Japan).

2.2. Methanolysis

Methanolyses of crude, degummed and refined soybean oils were performed at 30 °C in a 20 or 50 ml screw-capped vessel with shaking at 130 oscillations/min. The reaction for studying the effect of PLs was conducted in a mixture of oil, PLs, 1/3 molar equivalent MeOH for the stoichiometric amount, and 4 wt.% immobilized *C. antarctica* lipase. Three-step methanolysis of degummed or refined oil was conducted as follows: the first-step reaction was started in a mixture of oil and 1/3 molar equivalent MeOH, and 4 wt.% immobilized lipase, and the second and third 1/3 molar equivalent MeOH were added after complete consumption of MeOH. The reactions were repeated by transferring the enzyme into a fresh substrate mixture. The initial velocity in all methanolyses with a fresh immobilized lipase was slower than that in the subsequent reactions. The result was coincided with the report by Samukawa et al. [12]: when a fresh enzyme preparation was used as a catalyst for methanolysis of soybean oil, penetration of the substrate into the preparation was restricted. Hence, the methanolyses were conducted using pretreated lipase preparation. The pretreatment was carried out for 24 h under the same conditions as those in the main reaction.

2.3. Analysis

The FAME content in the reaction mixture was quantified on a Shimadzu 18-A gas chromatograph (Kyoto, Japan) equipped with a DB-5 capillary column (0.15 mm × 10 m, J&W Scientific, Folsom, CA, USA) using tricaprylin as an internal standard. The analysis was performed under the conditions described previously [9]. The composition of FAME, acyl glycerols, and FFA were analyzed by a thin-layer chromatograph/flame-ionization detector (Iatroscan MK-5; latron Laboratories Inc., Tokyo, Japan) after double developments: the first and second developments were performed using solvent mixtures of benzene/chloroform/acetic acid (50:20:0.7, by volume), and *n*-hexane/ethyl acetate (65:5, by volume), respectively. Water content was analyzed by Karl Fisher titration (Moisture Meter CA-07; Mitsubishi Chemical Corp., Tokyo, Japan). PC was quantified with high performance liquid chromatography (HPLC) equipped

with Shim-pack CLC-SIL(M) (4.6 mm × 250 mm, Shimadzu, Kyoto, Japan). The sample was eluted with a mixture of acetonitrile/MeOH/water (3:1:1, by volume) at 40 °C and a flow rate of 1.5 ml/min. The contents of PC were determined using L-α-lecithin as a standard. PLs bound on immobilized enzyme preparation were extracted with 10 ml chloroform/MeOH (2:1, by volume) by vigorous mixing with a vortex mixer for 3 min. PLs were analyzed by thin layer chromatography (TLC). After the sample was developed using a mixture of chloroform/aceton/MeOH/acetic acid/water (100:40:30:20:12, by volume), the spots were visualized by spraying a Dittmer–Lester reagent [13].

3. Results and discussion

3.1. Methanolysis of unrefined oils with immobilized C. antarctica lipase

In methanolysis of a vegetable oil, immobilized *C. antarctica* lipase was irreversibly inactivated in the presence of >1/2 molar equivalent MeOH for the stoichiometric amount [9]. Crude and refined soybean oils were thus alcoholyzed with 1/3 molar equivalent MeOH by the immobilized lipase. The methanolysis was repeated by transferring the lipase to a fresh substrate mixture for five cycles. Both reactions were repeated five more cycles using a mixture of refined oil and 1/3 molar equivalent MeOH as substrates. Table 1 shows the conversions after 6 h. The conversion of refined oil in the first-cycle reaction reached 30.2%, showing that the MeOH added was mostly consumed. However, the conversion of crude oil was only 10.3% and decreased to 7.1% after five cycles. In addition, the conversion decreased further even though the reaction was repeated using a substrate mixture of refined oil and 1/3 molar equivalent MeOH.

Table 1
Methanolyses of crude, degummed, and refined soybean oil with immobilized *C. antarctica* lipase

Substrate	FAME content (%)		
	First cycle	Fifth cycle	Tenth cycle
Crude oil	10.3	7.1	0.5
Degummed oil	28.1	30.3	30.9
Refined oil	30.2	32.2	32.4

A 10 g mixture of oil and 1/3 molar equivalent MeOH for the stoichiometric amount was shaken at 30 °C with 4 wt.% immobilized lipase. The methanolysis was repeated by transferring the lipase to a fresh substrate mixture every 24 h. After five cycles, the three reactions were repeated further five cycles (10 cycles in total) using refined oil and 1/3 molar equivalent MeOH as substrates. The FAME contents at 6 h reactions are shown.

In the refining process of edible oil, the next step of extraction is degumming. Hence, we next performed methanolysis of degummed soybean oil under similar conditions as stated above (Table 1). The conversion was 28.1% after 6 h in the first cycle, and was 30.3% after five cycles. These results indicated that an inhibitory substance in crude soybean oil is removed in the degumming step.

3.2. Effect of PL on methanolysis of refined soybean oil

The main components of soybean gum are PLs. Methanolysis of refined oil was therefore repeated five cycles in the presence of various amounts of soybean PLs. Figure 1 shows the conversion after 3 h, which indicates the reaction velocity. The conversion in the first-cycle reaction was 22.5%, when soybean PLs were not added. The conversion velocity decreased with increasing the amount of PLs; the conversions in the reactions with 0.2, 0.5, and 1.0% PLs were 18.1, 12.8, and 12.2%, respectively. Furthermore, when >0.5% PLs-containing oil was used as a substrate, the reaction velocity decreased a little by recycling the reaction.

To investigate the restoration of the lipase activity, the above methanolyses were recycled in the absence of soybean PLs after five cycles (Fig. 1). The activity of the enzyme which had been used in the presence of 0.2% PLs was slightly restored by recycling, but the restoration was not observed in the reaction with the enzyme which had been used in the presence of 0.5% PLs. In addition, the activity of the lipase which had been used in the presence of 1.0% PLs decreased even in the subsequent reactions, being consistent with crude soybean oil methanolysis.

Similar experiment was conducted using PL from a different origin, egg yolk PC (Fig. 2). Conversions in the first-cycle reactions with 0.2, 0.5, and 1.0% egg yolk PC were 16.5, 13.5, and 2.6%, respectively, showing that egg yolk PC also inhibited the methanol-

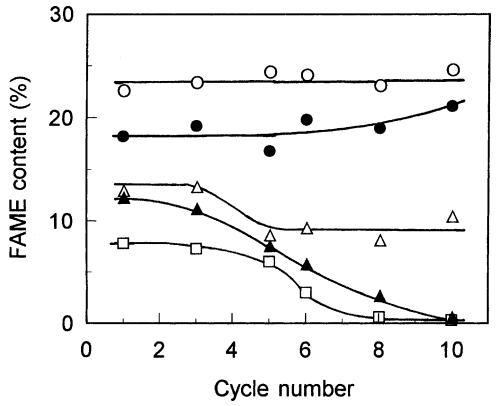

Fig. 1. Effect of soybean PLs on methanolysis of refined soybean oil with *C. antarctica* lipase. The reaction was performed in a 10 g mixture of the refined oil, various amounts of soybean PLs, 1/3 molar equivalent MeOH for the stoichiometric amount, and 4 wt.% immobilized lipase. The methanolysis was repeated by transferring the lipase to a fresh substrate mixture every 24 h. After five cycles, the reactions were repeated further five cycles (10 cycles in total) using refined oil and 1/3 molar equivalent MeOH as substrates. Crude oil methanolysis was conducted similarly as a control. The FAME contents at 3 h reactions are shown. (○), Without soybean PLs; (●), soybean PLs 0.2%; (△) 0.5%; (▲), 1%; (□), crude soybean oil.

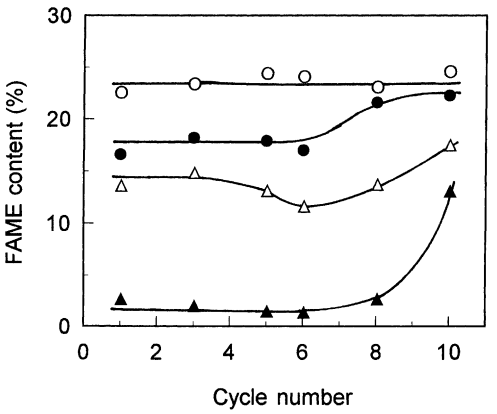

Fig. 2. Effect of egg yolk PC on methanolysis of refined soybean oil with *C. antarctica* lipase. The reaction was performed in a 10 g mixture of the refined oil, various amounts of egg yolk PC, 1/3 molar equivalent MeOH for the stoichiometric amount, and 4 wt.% immobilized lipase. The methanolysis was repeated by transferring the lipase to a fresh substrate mixture every 24 h. After five cycles, the reactions were repeated further five cycles (10 cycles in total) using refined oil and 1/3 molar equivalent MeOH as substrates. The FAME contents at 3 h reactions are shown. (○), Without egg yolk PC; (●), egg yolk PC 0.2%; (△), 0.5%; (▲), 1%.

ysis of refined oil. A significant difference, however, was observed in the reactions in the absence of PLs after five cycles of reactions in its presence; the activity of the lipase which had been used in the reaction with 1.0% egg yolk PC was restored by repeating the reaction with a substrate mixture of refined oil and MeOH.

3.3. Inhibition mode by PL

Soybean PLs and egg yolk PC inhibited methanolysis of refined soybean oil with immobilized *C. antarctica* lipase, although the inhibition level and the recovery from the inhibition were different. To further study on this inhibition, we examined the interaction of PLs with immobilized lipase preparation. The immobilized lipases which had been used in methanolyses of crude oil (Fig. 1), refined oils containing 1.0% soybean PLs (Fig. 1), and 1.0% egg yolk PC (Fig. 2) were recovered from the 10th-cycle reaction mixtures by filtration. Total lipids were then extracted from the lipase preparations with chloroform/MeOH (2:1, by volume), and the resulting extracts were subjected to TLC. PLs were detected in the extracts from the enzyme preparations which were used in the reactions of crude oil and soybean PLs-containing oil. But a less amount of PL (PC) was detected in the extract from enzyme preparation which was used in the reaction of egg yolk PC-containing oil (data not shown). These results suggested that PLs bound on the immobilized preparation and interfered the interaction of the lipase molecule with substrates. In addition, egg yolk PC released from the immobilized carrier more easily than soybean PLs. This fact may be explained by a difference in PL compositions of soybean PLs and egg yolk PC as stated in Section 2, and/or by unknown components in crude oil and soybean PLs preparation, which tightened the binding of PLs with immobilized carrier.

3.4. Three-step methanolysis of degummed soybean oil

Three-step methanolyses of degummed and refined soybean oils were conducted and the time courses were shown in Fig. 3. In the first-step reaction, the conversion of degummed oil at 1 h was 5.9% and that of refined oil was 11.6%, showing that the initial

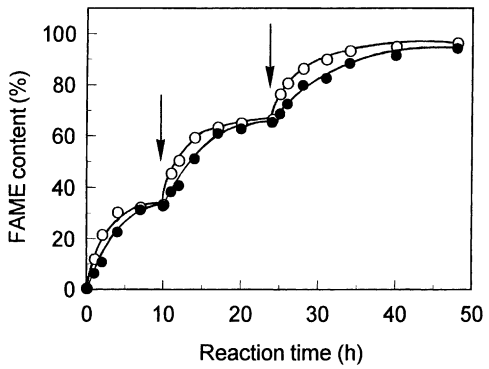

Fig. 3. Three-step methanolyses of degummed and refined soybean oils with immobilized *C. antarctica* lipase. The reaction was started in a 30 g mixture of oil, 1/3 molar equivalent MeOH, and 4 wt.% immobilized lipase. Arrows indicate the addition of 1/3 molar equivalent MeOH. (○), Refined oil; (●), degummed oil.

velocity of degummed oil methanolysis was about half of that of refined oil. However, the conversions of the two oils reached 30.5% after 6 h. The second- and third-step reactions were started by adding 1/3 molar equivalent MeOH after 10 and 24 h, respectively. The initial velocities of the second- and third-step methanolyses of degummed oil were also half of those of refined oil. After the three-step reaction (48 h in total), the conversion of refined oil reached 95.9% and that of degummed oil reached 93.8%. The compositions of the reaction products from two oils were almost similar: the FFA content was $<0.3\%$, and remaining acylglycerols were mainly partial acylglycerols. These results showed that degummed oil was efficiently converted to its corresponding FAMEs.

To investigate the stability of the immobilized lipase in methanolysis of degummed oil, the three-step reaction was repeated every 48 h. The conversion was maintained during 25 cycles (50 d). In addition, the initial velocity in the 25th cycle was almost the same as in the first cycle. These results showed that the stepwise methanolysis with *C. antarctica* lipase is effective for the production of biodiesel fuel from degummed soybean oil.

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