Effects of Minor Components of Crude Vegetable Oil on the Enzymatic Method to Analyze Positional Fatty Acid Distributions in Triacylglycerols with Candida antarctica Lipase B

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Abstract: Crude soybean and rapeseed oils were subjected to the method to determine FA distributions in TAG using Candida antarctica lipase B, giving similar results to those for refined oils. Minor components in crude oils, such as percentages of FFA or phospholipids were indicated not to affect 1(3)-selective transesterification by the lipase and FA compositional analysis of the resulting 2-MAG fraction significantly. Phospholipids were confirmed not to contaminate the 2-MAG fraction. Oxidized soybean oil with a PV of 10 meq/kg also gave similar results to the ones for refined oil. The method was confirmed to be applicable for crude oils and oxidized oils with a PV smaller than 10 meq/kg without prior purification of TAG.

Key words: crude oil, phospholipids, regiospecific distribution, triacylglycerol, 2-monoacylglycerol, Candida antarctica lipase B

1 INTRODUCTION

An enzymatic method to analyze FA distributions in triacylglycerol (TAG) using immobilized Pseudozyma (Candida) antarctica lipase B (CALB) is advantageous for its applicability to TAG-containing short chain and polyunsaturated (PU) fatty acids (FAs), in contrast to the conventional enzymatic method using pancreatic lipase or Rhizopus oryzae (delemon) lipase¹¹. Compared with other lipases including those from pancreas and R. oryzae, CALB has little FA selectivity and reacts with the majority of FAs to a similar degree². Thus, the method using CALB has been applied to a wide range of fats and oils, such as milk-fat-containing short chain FAs³,⁴, vegetable oils including palm oil with a melting point at ca. 50°C⁵,⁶. It was also applied to analyze the positional distribution of trans FAs in ruminant fat⁷.

In addition, CALB specifically provides fatty acid ethyl esters (FAEE) and 2-monoacylglycerol (MAG) without detectable levels of 1-MAG under analytical conditions. Thus, fractionation of 2-MAG is a simple procedure compared to the chemical method that provides all different kinds of partial acyl glycerol species after Grignard degradation. This contributes to the accuracy of the results. This method is also advantageous because it reveals the positional distribution of several tens of FA species simultaneously, unlike NMR, which reveals that of some PUFA only⁸.

The CALB method mainly consists of three steps (Fig. 1): step 1, sn-1(3)-selective transesterification of target oil with 63-fold molar excess of ethanol; step 2, fractionation of the resulting 2-MAG by silica gel chromatography; step 3, FA composition analysis of 2-MAG by GC after methylation. Propylation or butylation are recommended to determine the contents of short chain FAs, because their methyl esters are detectable by GC, whereas the methyl esters of long chain FAs are not.

Abbreviations: AOCS, American Oil Chemists’ Society; CALB, lipase B from Candida antarctica; DAG, diacylglycerol; FA, fatty acid; FFA, free fatty acid; FAEE, fatty acid ethyl ester; FAME, fatty acid methyl ester; GC, gas chromatography; HPLC, high performance liquid chromatography; JOCS, Japan Oil Chemists’ Society; MAG, monoacylglycerol; OA, oleic acid; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanol amine; PG, phosphatidyl glycerol; PI, phosphatidyl inositol; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol.

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Esters tend to be lost during the experimental procedure due to their volatility and water-solubility. The above method was supposed to be applied to pure TAG to avoid the effect of contaminants. However, a rough estimation of the FA distribution in TAG without prior purification of TAG may be sufficient depending on the levels and requirements of the study. In these cases, it is important to understand what types of oil contaminants could affect the data obtained with the method. Crude oils generally contain amounts of free fatty acids (FFAs) and phospholipids, which are removed in the refining steps to produce commercial vegetable oils.

Lipase, however, reacts with FFAs and phospholipids as well as with acyl glycerols. Thus, CALB could produce FAEE, lysophospholipids or phosphatidyl glycerol from FFAs and phospholipids during step 1 of the above procedure, if they are present in the oil applied to the method. In step 2, 2-MAG is fractionated by elution with diethyl ether after rinsing out FAEE, and small amounts of TAG and diacylglycerol (DAG) remain in the reaction mixture with a solvent containing hexane and diethyl ether (8:2) in silica gel chromatography. Given that phospholipids and lysophospholipids are more polar than 2-MAG, they can be co-eluted in the 2-MAG fraction during step 2 and analyzed as 2-MAG in step 3; this possibility, however, has not been evaluated yet.

In this study, the CALB method was applied to crude vegetable oils and the effect of minor components of vegetable oils on the method was estimated.

## 2 Experimental Procedures

### 2.1 Chemicals and materials

Crude and refined soybean and rapeseed oils were provided by J-Oil Mills, Inc. (Tokyo, Japan). Their fatty acid compositions are given in Table 1. Crude soybean oil showed an AV of 0.9 mg KOH/g, a PV of 0.2 meq/kg, a chlo-
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**Table 1** Major fatty acid composition of oils.

| FA (%)     | 16:0 | 16:1 | 18:0 | 18:1 | 18:2 | 18:3 | 20:0 | 20:1 |
|------------|------|------|------|------|------|------|------|------|
| Refined soybean oil | 10.3 ± 0.1 | 0.1 ± 0.0 | 4.1 ± 0.1 | 23.3 ± 0.3 | 52.1 ± 0.2 | 5.8 ± 0.3 | 0.3 ± 0.0 | 0.2 ± 0.0 |
| Crude soybean oil | 9.6 ± 0.1 | 0.1 ± 0.0 | 3.7 ± 0.2 | 22.9 ± 0.1 | 53.9 ± 0.1 | 6.9 ± 0.1 | 0.3 ± 0.0 | 0.1 ± 0.0 |
| Refined rapeseed oil | 4.1 ± 0.1 | 0.2 ± 0.0 | 1.3 ± 0.1 | 63.2 ± 0.2 | 18.4 ± 0.1 | 7.1 ± 0.2 | 0.6 ± 0.0 | 0.9 ± 0.0 |
| Crude rapeseed oil | 3.8 ± 0.0 | 0.2 ± 0.0 | 1.6 ± 0.0 | 62.5 ± 0.1 | 19.2 ± 0.2 | 9.5 ± 0.1 | 0.5 ± 0.0 | 1.2 ± 0.0 |

2.4 Reactions

Standard conditions for enzymatic transesterification were as follows: 0.5 g (ca. 0.57 mmol) of oil and 5.0 g (0.11 mol) of ethanol were put into a screw-capped glass vial. Novozym 435 (0.22 g) was added. Vials were shaken at 150-170 strokes per minute using a shaker for 3 h at 30°C. Reaction mixtures were recovered by filtration using absorbent cotton and kept at -20°C until further analysis. The lipid composition of the resulting reaction mixtures was analyzed with a TLC-FID analyzer as described previously[3]. 2-MAGs formed by enzymatic transesterification were purified by silica gel chromatography as described previously[3].

2.5 GC analysis of FA composition

TAGs and enzymatically prepared 2-MAG fractions were methylated with the JOCS method 2.4.1.2-2013[2]. FA methyl ester (FAME) compositions were analyzed by capillary GC, equipped with a TC-70 capillary column (0.25 mm × 30 m, 0.25 μm, GL Science, Tokyo, Japan) using helium gas as a carrier at a flow rate of 1.0 mL/min. The temperature of the column was held at 80°C for 2.0 min, increased at 35°C/min to 160°C, and subsequently increased at 2.0°C/min to 185°C, to 230°C at 10°C/min, and kept at 230°C for 9 min. The temperatures of the injector and the FID detector were set at 250°C. The split ratio was 50:1. All reactions and analyses were conducted three times and mean values are presented.

2.6 HPLC analysis of phospholipid contents

Phospholipid content was analyzed according to the AOCs official method 7c-07[14], using HPLC (HITACHI 7000, Hitachi, Tokyo, Japan) equipped with an evaporative light scattering detector (ELSD 2000, Alltech, Nicholasville, USA), and a Lichrospher 100 Diol column (5 μm, 4.0 × 125 mm, Merck, Darmstadt, Germany). Standard mixtures were prepared by mixing the standard reagents of PA (>99%), PC (>99%), PE (>98%), and PI (>99%), using PG (>98%) as an internal standard. Ten milligrams of sample were dissolved into 1 mL of a mixture of chloroform and methanol(2:1, by vol.) containing 0.262 mg PG. Fifty microliters of the solution were subjected to HPLC.
3 RESULTS AND DISCUSSION

3.1 Sn-1 (3)-selective transesterification of crude oil with ethanol by immobilized Candida antarctica lipase B

When refined and crude soybean oils were subjected to Candida antarctica lipase B (CALB) transesterification (Fig. 1, step 1), the resulting lipid composition was similar; the one of refined oil was FAEE 65.2%, 1,2-DAG 8.2%, MAG 26.6%, whereas the one of crude oil was FAEE 65.1%, 1,2-DAG 6.8%, MAG 28.1%. TAG, 1,3-DAG, and FFA could hardly be detected by TLC-FID analysis. MAG was mainly 2-MAG, and little 1-MAG was detected when analysis by borate-impregnated TLC with chloroform: acetone: acetic acid (96:4:0.1, by vol.) as a developing solvent was performed. Fractionation of the resulting 2-MAG (Fig. 1, step 2) and the following GC analysis (Fig. 1, step 3) showed very similar FA compositions (Table 2). Refined and crude rapeseed oils gave similar results in step 2 (64-67% FAEE, 5-7% 1,2-DAG, 28-29% MAG) and step 3 (Fig. 1, Table 2). We have previously shown that FA derived from the sn-2 position was partially detected in the FAEE fraction under experimental conditions, which suggests the occurrence of a direct attack of CALB to the sn-2 position, and/or the acyl transfer from sn-2 to sn-1 (3). However, little 1(3)-MAG was detected in the reaction mixture. Thus, 1(3)-MAG, if produced, is likely to immediately be converted to FAEE and glycerol by the lipase, and not to accumulate in the reaction mixture for a long time interval to reach to the detectable level. In contrast, the 2-MAG fraction contained little FA derived from the sn-1 (3) position, suggesting that the acyl transfer from sn-1 (3) to sn-2 was little.

3.2 Effect of FFA in vegetable oil on 1(3)-selective transesterification of TAG with ethanol by immobilized CALB

Crude soybean oil and crude rapeseed oil typically contain ~0.5% FFA, ~2% phospholipids as well as chrolophylls that contribute to their greenish color. In order to estimate the effects of these minor components, OA was added to refined soybean oil as described in section 2.2, and subjected to enzymatic transesterification (Fig. 1, step 1). The glyceride composition of the reaction mixture was FAEE 68.7%, 1,2-DAG 4.4%, MAG 26.9%. The FAEE content in OA-added oil was slightly high compared to that in refined oil (65.2%), whereas the MAG content was similar. The esterification of FFA with alcohol by CALB was previously shown to be approximately 10 times faster compared to the transesterification of TAG with alcohol. It was therefore estimated that OA added to oil was esterified faster than acyl glycerols and increased the FAEE content compared with other tested oils. Because OA might not affect 2-MAG production, it is natural that the FA composition of 2-MAG resulting from OA-added soybean oil was similar to that of refined soybean oil (Table 2). However, OA might probably be contained in the FAEE fraction. Therefore, it is recommended that the FA composition at the sn-1 (3) position of TAG is not determined by direct analysis of FAEE composition, but it is calculated based on the mass balance of the FA composition of TAG and the one of the sn-2 position, as previously described.

3.3 Effect of phospholipids in vegetable oil on 1(3)-selective transesterification of TAG with ethanol by immobilized CALB

Soybean lecinthin-added refined oil was also applied to 1(3)-selective transesterification (Fig. 1, step 1) to estimate the effect of phospholipids on the CALB reaction. The resulting glyceride composition after the reaction (FAEE 65.5%, 1,2-DAG 6.4%, MAG 28.1%) and the FA composition of the resulting 2-MAG fraction were similar to those of refined soybean oil (Table 2). Contents of phospholipids in oil samples were monitored by HPLC (Fig. 2). The content of phospholipids was ca. 1.5% in the lecinthin-add-
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Interestingly, phospholipids were hardly detected in the 2-MAG fraction obtained by silica gel chromatography, and not to contaminate the procedure. Lipase activity is often inhibited in oxidized oils. Therefore, it is likely that the reaction is slower if severely oxidized oils are used as samples, and that ca. 30% 2-MAG is not obtained after 3 h of enzymatic reaction. The results obtained in this study indicate that oxidation under the level of 10 meq/kg PV does not markedly affect the CALB method to analyze the positional distribution of FA in TAG.

### 4 Conclusion

Crude soybean and rapeseed oils were subjected to the CALB method to analyze the positional distribution of FA in TAG after 1(3)-selective transesterification with 10-fold ethanol. The produced amounts of FAEE and 2-MAG were almost theoretical, approximately 65% and 28%, respectively, and were similar to the amounts produced from refined oils. FA compositions of 2-MAG obtained from crude oils were, again, similar to those of refined oils.

Minor components of crude oil were characterized by FFA and lecithin containing phospholipids. When 1.5% FFA or 3% lecithin were added to refined oil and subjected to CALB transesterification, the produced amount of 2-MAG and the FA composition of the 2-MAG fraction were similar to those of refined oil. The phospholipid amount in lecithin-added oil was 1.5%, which was reduced to ca. 1% after CALB transesterification. Phospholipids, however, were not detected in the 2-MAG fraction obtained by silica-gel chromatography using diethyl ether as an elution solvent. In addition, oxidized oil with a peroxide value of 10 meq/kg did not alter results significantly compared to the ones for refined oil. We therefore suggest that crude oils can be applied to the CALB method to estimate the positional distribution of FA in oil seeds under which lipoxygenases are active. In addition to crude oils, oxidized soybean oil of ca. 10 meq/kg (PV 10) gave results similar to the ones for refined oil in enzymatic transesterification (FAEE 65.5%, 1,2-DAG 6.4%, MAG 28.1%), and FA composition of the resulting 2-MAG fraction (Table 2). Thus, oxidized compounds in crude oils and oxidized oil (PV 10) derived most probably from unsaturated FAs, did not affect the enzymatic reaction significantly, conducted in the presence of a 10-fold amount of EtOH (5.0 g) compared to oil (0.5 g); specifically, oxidized oil was diluted 11 times with EtOH for the effect of oxidized compounds to be minimum.

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