Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1068 (2005) 243–248
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

# Direct separation of regio- and enantiomeric isomers of diacylglycerols by a tandem column high-performance liquid chromatography

Weera Piyatheerawong, Yugo Iwasaki *, Tsuneo Yamane

*Laboratory of Molecular Biotechnology, Graduate School of Bio- and Agro-Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan*

Received 7 July 2004; received in revised form 29 November 2004; accepted 27 January 2005

**Abstract**

A novel HPLC-based method for direct separation of the three isomers of mono-acid diacylglycerols (DAGs), i.e., 1,2-DAG, 2,3-DAG and 1,3-DAG, has been established. The method employs a tandem column system, in which two different columns (a conventional silica gel column and a chiral stationary phase column) are connected in series. Two isomeric mixtures of DAGs (i.e., dicapryloylglycerol and dioleoylglycerol) and lipase-catalyzed reaction mixtures were successfully resolved on the tandem column HPLC system without any derivatization prior to the analysis. According to the established analytical method, stereoselectivity of two lipases toward mono-acid triacylglycerols in ethanolysis reaction was investigated. The tested enzymes were immobilized *Candida antarctica* lipase B (CALB) and *Rhizomucor miehei* lipase (RML). Analyses of the enantiomeric purity of 1,2-DAG and 2,3-DAG, generated as intermediates during the reaction, revealed that CALB and RML have *sn*-3 and *sn*-1 stereopreference, respectively.


*Keywords:* Enantiomeric resolution; Diacylglycerols; Chiral stationary phase; Tandem column; Ethanolysis

## 1. Introduction

Diacylglycerols (DAGs) are glycerol derivatives in which two hydroxyl groups are substituted with fatty acids through ester bonds. There are three isomers, which are 1,2-, 1,3- and 2,3-DAGs. For mono-acid DAGs, 1,2- and 2,3-DAGs are enantiomers of each other. DAGs are widely used as emulsifiers in food, cosmetic and pharmaceutical industries, and are utilized in different degree of purity. Frequently, mixtures of monoacylglycerols (MAGs) and DAGs are exploited because they are cheap and give appropriate performance [1]. Besides, pure isomers of DAGs have large potential use as intermediates for organic synthesis of phospholipids, glycolipids, prodrugs and structured lipids [2–5].

Although optically pure 1,2- and 2,3-DAGs are difficult to synthesize by traditional chemical synthetic methods, lipase-catalyzed regio- and stereo-selective reactions would provide alternative routes [6–8]. Asymmetrical deacylation of prochiral mono-acid triacylglycerols (TAGs) at either *sn*-1 or *sn*-3 position by alcoholysis yields 2,3- and/or 1,2-DAGs, respectively (Fig. 1). Therefore, by using this alcoholysis, it will be possible to prepare optically pure 1,2- or 2,3-DAGs. To establish an effective production method of chiral 1,2- or 2,3-DAGs, screening of lipases with desired stereoselectivity is required. Indeed, it is reported that some lipases discriminate *sn*-1 position from *sn*-3 position of TAGs in hydrolysis [6,9,10].

One of the problems for investigating stereoselectivity of lipases toward TAGs is the difficulty in resolution of enantiomeric 1,2- and 2,3-DAGs. At present, two HPLC-based methods for the separation of 1,2- and 2,3-DAGs are available. One of the methods is derivatization of the 1,2- and 2,3-DAGs into diastereomeric naphthylethyl urethanes [11–13] or phenylethyl carbamates [14] using optically pure naphthylethyl isocyanate or phenylethyl isocyanate, respectively, followed by separation of the diastereomers on a conventional silica gel column. The other method is conversion of

* Corresponding author. Tel.: +81 52 789 4144; fax: +81 52 789 4145.
*E-mail address:* iwasaki@agr.nagoya-u.ac.jp (Y. Iwasaki).

0021-9673/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2005.01.075

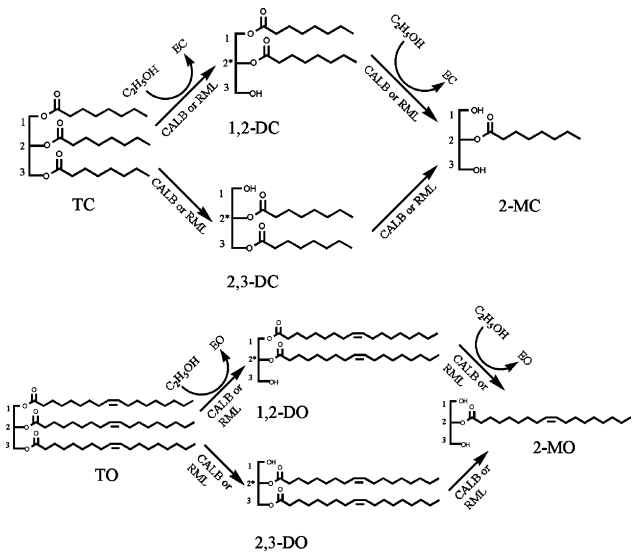


Fig. 1. Lipase-catalyzed ethanolysis of mono-acid TAGs. Tricapryloylglycerol (TC) or trioleoylglycerol (TO) is utilized as the substrate. The reaction initially gives 1,2(2,3)-dicapryloylglycerol [1,2(2,3)-DC] or 1,2(2,3)-dioleoylglycerol [1,2(2,3)-DO], which are further converted to 2-monocapryloylglycerol (2-MC) or 2-monooleoylglycerol (2-MO) as the final product. Ethyl caprylate (EC) or ethyl oleate (EO) is liberated as byproduct of the reaction. The carbons in the glycerol moiety are numbered stereospecifically. Asterisks indicate the chiral centers at the *sn*-2 position.

1,2- and 2,3-DAGs into 3,5-dinitrophenyl urethanes using 3,5-dinitrophenyl isocyanate, followed by separation of the enantiomeric urethanes on a chiral stationary phase column [15]. However, the above methods involve laborious derivatization steps and require relatively large amount of samples. In addition, the possibility of spontaneous isomerization of 1,2- and 2,3-DAGs into 1,3-DAG by acyl migration during the derivatization procedure cannot be excluded. Therefore, a simple analytical method without any derivatization steps is highly favorable.

The aim of the present study is to establish an analytical method in which enantiomeric 1,2- and 2,3-DAGs are directly separated without any derivatization. Separation of the isomeric DAGs mixtures and the lipase-catalyzed reaction mixtures on a tandem column HPLC system is demonstrated. Using the new method, the stereoselectivity of some lipases on TAGs during ethanolysis reaction is investigated.

## 2. Experimental

### 2.1. Chemicals and enzymes

Tricapryloylglycerol (TC), trioleoylglycerol (TO), caprylic acid, HPLC-grade *n*-hexane, HPLC-grade 2-propanol and palladium adsorbed on activated carbon (Pd/C, palladium content was 10%) were purchased from Wako Pure Chemicals (Osaka, Japan). 1,2-Dicapryloyl-*sn*-glycerol (1,2-DC), 1,2-dioleoyl-*sn*-glycerol (1,2-DO), 1,2-dioleoyl-*rac*-glycerol (*rac*-1,2-DO), 1,3-dioleoylglycerol (1,3-DO) and *rac*-1-benzylglycerol were purchased from Sigma (St. Louis, MO, USA). 1,2-Dicapryloyl-*rac*-glycerol (*rac*-1,2-DC) was self-prepared by acylation of *rac*-1-benzylglycerol with caprylic acid in the presence of a lipase, followed by removal of the benzyl group by catalytic hydrogenation with Pd/C. 1,3-Dicapryloyl-glycerol (1,3-DC) was self-prepared by direct acylation of glycerol with caprylic acid using a 1,3-specific lipase as described elsewhere [5]. Lipozyme RM IM [immobilized *Rhizomucor miehei* lipase (RML)] and Novozym 435 [immobilized *Candida antarctica* lipase B (CALB)] were gifts from Novozymes Japan Ltd. (Chiba, Japan). All other chemicals were of analytical quality or better.

### 2.2. Tandem column HPLC system

Two analytical columns, a silica gel column (Ultrasphere 5 μl column, 4.6 mm × 250 mm, Bachman Coulter Inc., Fullerton, CA, USA) and a chiral stationary phase column (CHIRALCEL OD$^{TM}$, (cellulose-tris-3,5-dimethylphenylcarbamate)-impregnated silica, 4.6 mm × 250 mm, Daicel Chemical, Tokyo, Japan [16]) were connected in series. The mobile phase was *n*-hexane–2-propanol (300:7) at flow rate of 1.0 ml/min using an HPLC pump (Model 880-PU, Jasco, Tokyo, Japan) at 25 °C. A 5 μl aliquot of the sample (containing 10–50 μg of 1,2- and/or 2,3-DAGs dissolved in *n*-hexane) was injected using a 10 μl injection loop. The peaks were detected using an evaporative light scattering detector (ELSD; Model ELSD-LT, Shimadzu Corporation, Kyoto, Japan). The drift tube temperature was 50 °C. Nitrogen was used as evaporation gas at 350 kPa.

With this mobile phase, some of the polar compounds such as free fatty acid and MAGs were not eluted, and were accumulated in the silica gel column. For washing out these polar compounds, the silica gel column (disconnected from the chiral column) was eluted with a linear gradient of 0–10% 2-propanol in *n*-hexane in 60 min at 1.0 ml/min flow rate. We usually do this cleaning once in 10 injections.

### 2.3. Lipase-catalyzed ethanolysis

Ethanolysis of TC or TO was performed using immobilized RML or CALB as a catalyst (Fig. 1). The reaction initially gives 1,2- and/or 2,3-DAGs, which are further deacylated to 2-monoacylglycerol (2-MAG) as the final product.

In a typical experiment, mixtures consisting of 1.0 g TC or TO, 4.0 g of ethanol, and 0.4 g of water (in the case of RML) were incubated at 35 °C with stirring for 15 min to emulsify the reaction mixtures. In the case of CALB, no exogenous water was added to the mixtures. Then, the reaction was started by addition of 0.5 g (10% of the reactants) of the immobilized lipase. Portion of the reaction mixtures (100 μl) was withdrawn at intervals, mixed with 400 μl of diethyl ether and filtered to remove the catalyst. The water content in the reaction mixtures was optimized according to our previous research [17]. The actual water contents in the

reaction mixtures measured by a Karl–Fischer moisture meter (MKS-1, Kyoto Electronics Co., Ltd., Kyoto, Japan) were 0.24 wt.% in the case of CALB and 7.73 wt.% in the case of RML.

The glyceride composition in the resultant lipid solution such as TAG, 1,3-DAG, 1,2(2,3)-DAGs and MAGs were analyzed with a thin-layer chromatography/flame-ionization detector (TLC/FID) analyzer (Iatroscan MK-5; Iatron Laboratories, Tokyo, Japan) as described elsewhere [17].

For investigating enantiomeric purity of 1,2- and 2,3-DAGs, 40 μl of the sample solution was evaporated at low temperature under vacuum and was redissolved in 40 μl of *n*-hexane. The resulting lipid solution (5 μl) was analyzed by the tandem column HPLC system. The weight-based amount (in μg) of each enantiomer was calculated from the corresponding peak area using standard curves drawn with known amounts of racemic-1,2-DAG. The enantiomeric purity of 1,2(2,3)-DAGs was evaluated from enantiomeric excess (%ee) value, which was calculated using the weight-based amount of each isomer as follows:

$$\%\text{ee} = \frac{[1,2\text{-DAG}] - [2,3\text{-DAG}]}{[1,2\text{-DAG}] + [2,3\text{-DAG}]} \times 100,$$

for CALB-catalyzed reactions,

or

$$\%\text{ee} = \frac{[2,3\text{-DAG}] - [1,2\text{-DAG}]}{[1,2\text{-DAG}] + [2,3\text{-DAG}]} \times 100,$$

for RML-catalyzed reactions.

## 3. Results and discussion

### 3.1. Separation of the lipid mixtures by the tandem column HPLC system

This work is the first report of the direct resolution of enantiomeric 1,2- and 2,3-DAGs. In our previous work, we demonstrated a direct method for analyzing asymmetric TAGs by chiral stationary phase HPLC [18]. Chiral stationary phases are powerful tools of HPLC for effectively resolving a wide range of racemic aliphatic and aromatic compounds. Among them, polysaccharide-based phases such as cellulose esters, phenylcarbamates of cellulose and amylose are some of the most popular ones. Trisphenylcarbamate derivatives of cellulose exhibit remarkably resolving ability for polar racemates and sensitive to molecular geometry [19–21].

In the present study, we examined two types of trisphenylcarbamate columns, CHIRALCEL OF$^{TM}$ and CHIRALCEL OD$^{TM}$ that consist of cellulose-tris-4-chlorophenylcarbamate and cellulose-tris-3,5-dimethylphenylcarbamate, respectively, coated on silica gel. Separation of enantiomeric 1,2- and 2,3-DAGs could not be achieved on the CHIRALCEL OF$^{TM}$ column. On the other hand, a baseline separation was achieved on the CHIRALCEL OD$^{TM}$ column. This result was in agreement with the former reports [19,20], indicating that the abilities of phenylcarbamates of polysaccharides to recognize chirality are greatly affected by substituents on the phenyl groups. In the case of CHIRALCEL OD$^{TM}$ column, the substituent is an electron-donating methyl group, which increases the electron density at the carbonyl oxygen atom of the carbamates. Therefore, alcohols are strongly adsorbed on the chiral stationary phase through a hydrogen-bonding interaction. Unfortunately, use of only the chiral stationary phase column was not enough for separating all isomers of DAGs, because the peak of 1,3-DAG (which might be contained in the sample), overlapped with the peak of 2,3-DAG (data not shown). For this reason, we employed the tandem column system, where a conventional silica gel column was connected upstream of the chiral stationary phase column. Since the silica gel column is able to separate 1,3-DAG from 1,2(2,3)-DAGs before they enter into the chiral stationary phase column, there is no need to isolate the fraction of 1,2(2,3)-DAGs in advance.

Once the tandem column system was developed, the optimization of the mobile phase composition was also conducted. According to the previous work, the separation of asymmetric TAGs was successfully achieved with the mobile phase of *n*-hexane and 2-propanol [18]. Therefore, in the present study, we tested several mixtures of *n*-hexane and 2-propanol with different composition as the mobile phase. Table 1 displays the effect of mobile phase composition on separation of isomeric DAGs by the tandem column HPLC system. The best resolution between 1,2-DAG and 2,3-DAG was observed with *n*-hexane to 2-propanol ratio of 300:7. By

Table 1
Effect of mobile phase composition on the separation of isomeric mono-acid diacylglycerols by the tandem column HPLC system

| DAGs | Mobile phase composition (*n*-hexane:2-propanol) | Retention time (min) | | | $\alpha$[a] | $R_s$[b] |
|---|---|---|---|---|---|---|
| | | 1,3-DAG | 2,3-DAG | 1,2-DAG | | |
| DC | 300:10 | 18.21 | 21.54 | 22.26 | 1.03 | 0.89 |
| | 300:7 | 27.41 | 31.76 | 33.46 | 1.05 | 1.14 |
| | 300:5 | 32.99 | 41.58 | 43.38 | 1.04 | 1.06 |
| DO | 300:10 | 13.21 | 15.46 | 16.12 | 1.04 | 0.86 |
| | 300:7 | 17.70 | 21.98 | 23.56 | 1.07 | 1.33 |
| | 300:5 | 21.95 | 27.47 | 28.93 | 1.05 | 1.17 |

[a] $\alpha$ is separation coefficient between 2,3-DAG and 1,2-DAG.
[b] $R_s$ is peak resolution between 2,3-DAG and 1,2-DAG.

means of this mobile phase, separation factor ($\alpha$) and peak resolution ($R_s$) were 1.05 and 1.14, respectively, for 1,2- and 2,3-DC, and 1.07 and 1.33, respectively, for 1,2- and 2,3-DO (Table 1). Changing the 2-propanol content (i.e., rising from 7 to 10 or reducing from 7 to 5) deteriorated the resolution, relating in the reduction of $R_s$ values. The retention times of the lipids were also affected by the mobile phase composition. Taking the resolution efficiency and the retention time (which is reflected to the total analysis time) into consideration, we concluded the mobile phase of *n*-hexane to 2-propanol at the ratio of 300:7 was the optimum.

Fig. 2 shows the chromatograms of authentic isomers of 1,3-DAG, *rac*-1,2-DAGs and *sn*-1,2-DAG on the tandem column HPLC system. 1,3-DC, 2,3-DC and 1,2-DC were eluted at 27.41, 31.76 and 33.46 min, respectively (Fig. 2A–C and Table 2). The peaks were identified by comparing the retention times of the authentic 1,3-DC and *sn*-1,2-DC. In the same way, authentic isomers of 1,3-DO, 2,3-DO and 1,2-DO were eluted at 17.70, 21.98 and 23.56 min, respectively (Fig. 2D–F and Table 2). In addition, the relative standard deviation (RSD) of retention times of isomeric DC and DO were less than 2% (Table 2), indicating that the retention time is reproducible. Moreover, the resolution efficiency and the retention times of DAGs were not significantly affected when another CHIRALCEL OD$^{TM}$ column was used (data not shown), indicating that the column-to-column reproducibility was satisfactory.

It is often the case that ELSD gives non-linear relationship between amounts of substance and peak areas [22,23]. Therefore, logarithmic transformation of the calibration data to give a linear equation is necessary. As shown in Table 2, we obtained a linear equation for each of the DAG isomers with a coefficient of determination ($r^2$) more than 0.97, revealing that the calibration was reliable. The resolution of the isomeric mixtures of DAGs on the tandem column HPLC system is shown in Fig. 3. 1,3-, 2,3- and 1,2-DAGs of DC and DO were well resolved from each other (Fig. 3A and B). Furthermore, a complete separation of the six DAG species was also possible (Fig. 3C). These results suggest that the tandem column HPLC system is practical and suitable for separating all isomers of DAGs.

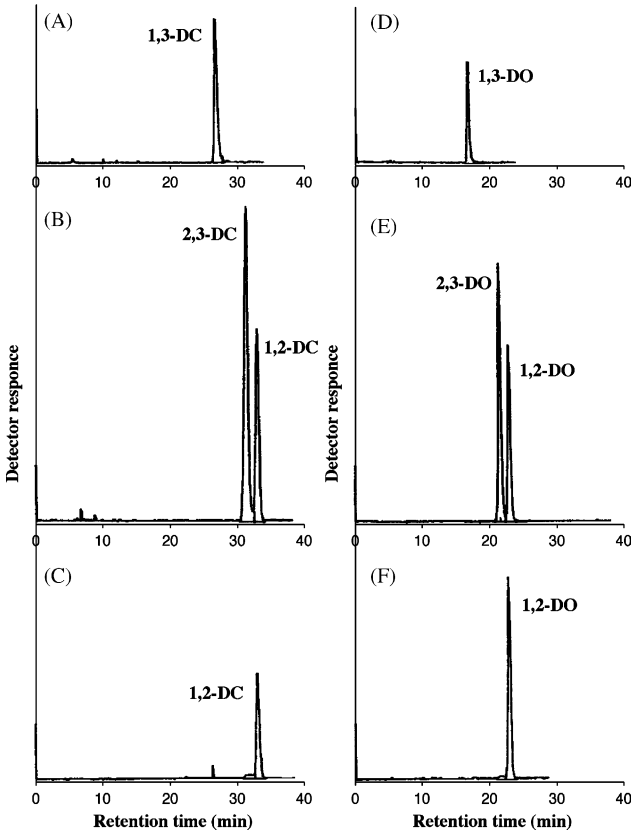


Fig. 2. Separation of authentic isomers of mono-acid DAGs on the tandem column HPLC system. Chromatograms of authentic isomers of 1,3-DC (A), *rac*-1,2-DC (B) and *sn*-1,2-DC (C), and authentic isomers of 1,3-DO (D), *rac*-1,2-DO (E) and *sn*-1,2-DO (F) are shown.

As examples of more complex lipid samples, the chromatogram of lipase-catalyzed reaction mixtures on the tandem column HPLC system is shown in Fig. 3D. The mixtures of CALB-catalyzed ethanolysis of TO are demonstrated. The peaks of 1,2- and 2,3-DO were well resolved from each other and separated from the other lipid species such as ethyl oleate (EO) and TO. In this case, 1,3-DO was under detection limit of ELSD (<5 μg). The absence of 1,3-DO was also confirmed by TLC/FID analyzer. The absence of 1,3-DO excluded the possibility of isomerization of 1,2(2,3)-DO to 1,3-DO by

Table 2
Chromatographic data for isomeric mono-acid diacylglycerols on the tandem column HPLC system

| | DC | | | DO | | |
|---|---|---|---|---|---|---|
| | 1,3-DC | 2,3-DC | 1,2-DC | 1,3-DO | 2,3-DO | 1,2-DO |
| Retention time (min) | 27.41 | 31.76 | 33.46 | 17.70 | 21.98 | 23.56 |
| Repeatability of retention time ($n$ = 6, RSD%) | 0.45 | 0.71 | 0.73 | 0.85 | 1.22 | 1.53 |
| Calibration range (μg) | 5–50 | 5–50 | 5–50 | 5–50 | 5–50 | 5–50 |
| Calibration points | 6 | 9 | 9 | 6 | 9 | 9 |
| Equation, where $y$ = log(peak area) and $x$ = log(weight) | $y = 1.878x + 4.367$ | $y = 1.813x + 3.661$ | $y = 1.872x + 3.308$ | $y = 1.657x + 5.028$ | $y = 1.732x + 4.194$ | $y = 1.736x + 4.065$ |
| Coefficient of determination ($r^2$) | 0.9827 | 0.9899 | 0.991 | 0.9725 | 0.9886 | 0.9872 |

Mobile phase, *n*-hexane:2-propanol = 300:7; flow rate = 1 ml/min.

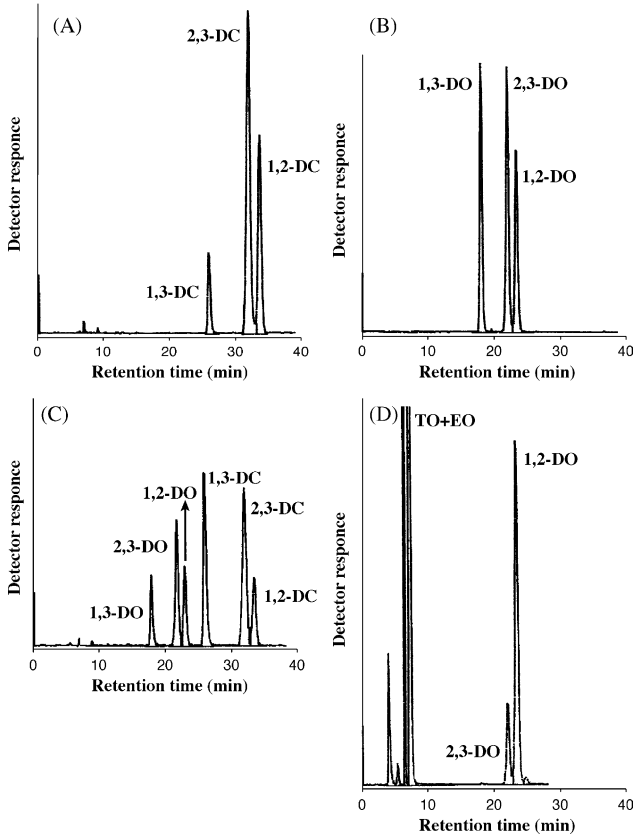


Fig. 3. Resolution of isomeric mixtures of mono-acid DAGs and lipid mixtures on the tandem column HPLC system. Chromatograms of authentic mixtures of 1,3-DC, 2,3-DC and 1,2-DC (A), authentic mixtures of 1,3-DO, 2,3-DO and 1,2-DO (B), two authentic mixtures of DC and DO (C) and the mixtures of CALB-catalyzed ethanolysis of TO at 30 min (D) are shown.

spontaneous acyl migration during the analytical procedure. This result indicates that the tandem column HPLC system is suitable for separating DAGs in the mixture of various lipid species.

### 3.2. Monitoring of stereoselectivity of lipases during ethanolysis

The lipase-catalyzed deacylation of prochiral mono-acid TAGs by ethanolysis initially yields chiral 1,2- and/or 2,3-DAGs as intermediates, which are further converted to 2-MAG as the final product [24,25]. Some lipases are known to have stereoselectivity toward the *sn*-1 or *sn*-3 position in hydrolysis [9,10], but no information was available in alcoholysis. Investigation of stereoselectivity of some lipases during the ethanolysis is one of the purposes in this paper. The optimum water content for high reaction rate was employed according to our previous work [17]. The glyceride composition was analyzed using the TLC/FID analyzer and the enantiomeric purity of 1,2- and 2,3-DAGs was analyzed by the tandem column HPLC analysis. None of these analytical methods required any purification and derivatization steps, allowing rapid analysis with minimal amounts of samples.

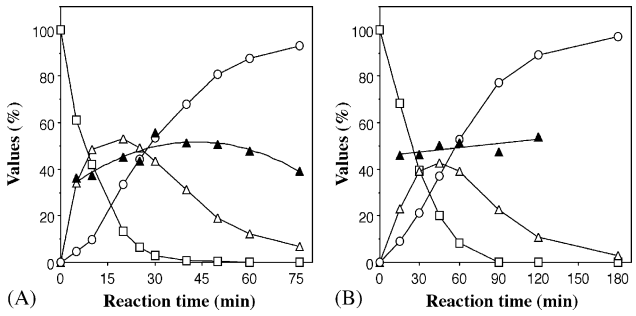


Fig. 4. Time courses of CALB-catalyzed ethanolysis of TC (A) and TO (B). Open square, TAGs (mol%); open triangle, 1,2(2,3)-DAGs (mol%); open circle, MAGs (mol%); closed triangle, %ee = $\frac{[1,2\text{-DAG}]-[2,3\text{-DAG}]}{[1,2\text{-DAG}]+[2,3\text{-DAG}]} \times 100$.

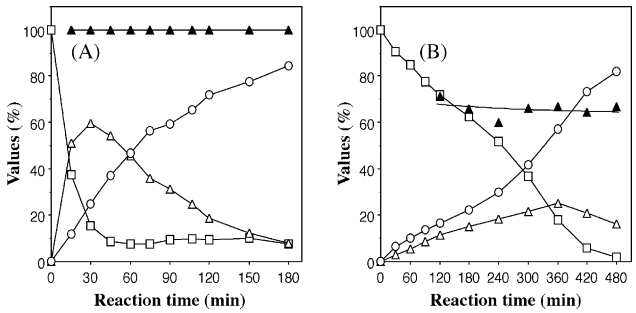


Fig. 5. Time courses of RML-catalyzed ethanolysis of TC (A) and TO (B). Open square, TAGs (mol%); open triangle, 1,2(2,3)-DAGs (mol%); open circle, MAGs (mol%); closed triangle, %ee = $\frac{[2,3\text{-DAG}]-[1,2\text{-DAG}]}{[1,2\text{-DAG}]+[2,3\text{-DAG}]} \times 100$.

Fig. 4A and B show the time courses of CALB-mediated ethanolysis of TC and TO, respectively. In the cases of the both substrates, 1,2(2,3)-DAGs were accumulated (40–55 mol%) for a while, and then they were deacylated to MAGs as the final product. HPLC analysis of the intermediates revealed that the amount of 1,2-DAG was larger than 2,3-DAG (%ee of 35–55).

The time courses of the RML-catalyzed ethanolysis of TC and TO are shown in Fig. 5A and B. TC was deacylated to accumulate 1,2(2,3)-DC, and then converted to MC. In contrast, the reaction with TO took longer time, and the amount of the accumulated 1,2(2,3)-DO was small. Contrary to the case of CALB, the amount of 2,3-DAG was larger than those of 1,2-DAG. Moreover, in the case of TC (Fig. 5A), the %ee value was almost 100% and steady along with reaction time.

The enantiomeric purity of the 1,2(2,3)-DAGs may be affected by two different types of "selectivity" of the lipase. One is stereoselectivity toward either *sn*-1 or *sn*-3 position during the deacylation of the prochiral TAGs, and the other is enantioselectivity toward either 1,2-DAG or 2,3-DAG during the further deacylation of the intermediates. Therefore, stereoselectivity of a lipase should be evaluated at the initial stage of the reaction, where the formation of MAG is negligible. As shown in Fig. 4A and B, CALB generated 1,2-DAGs more than the other enantiomeric counterpart in the initial stage, suggesting that CALB has stereopreference

toward the *sn*-3 position over the *sn*-1 position of TC and TO under the investigated conditions. Considering similarly the results of Fig. 5A, it is suggested that RML has obviously strong stereoselectivity toward the *sn*-1 position of TC.

In the case of RML-catalyzed reaction with TO, the formation of MO was not negligible in the initial stage (Fig. 5B). We tried to find reaction conditions, in which the formation of MO can be reduced, by changing several reaction parameters. However, the formation of MO was still as fast as that of 1,2(2,3)-DO, suggesting that the generated 1,2(2,3)-DO was rapidly deacylated into MO. In this situation, the observed enantiomeric purity of 1,2(2,3)-DO might have been influenced both by stereoselectivity on either *sn*-1 or *sn*-3 position of TO and by enantioselectivity on 1,2-DO or 2,3-DO. Therefore, it was not possible to conclude the stereoselectivity from the observed enantiomeric purity. From the above reason, we could not evaluate the stereoselectivity of RML on TO, although the %ee value of 1,2(2,3)-DO was more than 65%.

The stereoselectivity of CALB and RML in ethanolysis of TC (i.e., *sn*-3 selectivity of CALB and *sn*-1 selectivity of RML) observed in this work is in agreement with the results of previous reports, where the stereoselectivity of lipases in hydrolysis was examined [9,10] and stereoselectivity of RML was investigated in acidolysis [18]. An exception is the selectivity of CALB toward TO. Rogalska et al. [9] reported that CALB showed *sn*-3 selectivity against TC, and that *sn*-1 selectivity on TO in hydrolysis. In our ethanolysis study, however, the enzyme showed *sn*-3 preference on both TC and TO. Although the reason for the different selectivity of CALB in hydrolysis and in ethanolysis is not clear, so-called "solvent effect" of ethanol, which may prevent reversal of selectivity of the enzyme, might be involved.

The shift in selectivity of CALB in hydrolysis might be involved through the chain length effect on the three points attachment necessary for stereospecific recognition of the long- and short-chain TAGs molecules [26]. The chiral recognition center might undergo enantiomorphic organization during the stage when the interfacial enzyme–substrate complex is being formed, which may be precisely the stage at which the induced-fit process takes place [10]. However, in organic solvent, especially water-depriving solvent such as ethanol, the enzymes are generally more rigid [27]. In such an environment, the enzyme possibly will be unable to form enzyme–substrate complex in more than one direction, resulting in unaffected selectivity on the TAGs with different chain lengths. Further studies are necessary to clarify the effect of various solvents on alcoholysis.

The present study provides a simple method for resolution of enantiomeric 1,2- and 2,3-DAGs. The method will be useful for evaluation of stereoselectivity of lipases. Since it is in principle a non-destructive method, it can be used for preparation of enantiomerically pure 1,2- and 2,3-DAGs from their mixtures.

## Acknowledgments

The authors are grateful to Novozymes Japan Ltd. (Chiba, Japan) for generous supply of the immobilized lipases. The Ministry of Science and Technology (MOST, Thailand) is acknowledged for financial support to W. Piyatheerawong.

## References

[1] R.M.M. Diks, J.A. Bosley, in: U.T. Bornscheuer (Ed.), Enzymes in Lipid Modification, Wiley-VCH, Weinhein, 2000, p. 3.
[2] L.L.M. Van Deenen, G.H. de Haas, Biochem. Biophys. Acta 70 (1963) 538.
[3] H.P. Wehrli, Y. Pomeranz, Chem. Phys. Lipids 3 (1969) 357.
[4] A. Garzon-Aburbeh, J.H. Poupaert, M. Claesen, P. Dumont, J. Med. Chem. 29 (1986) 687.
[5] R. Rosu, M. Yasui, Y. Iwasaki, T. Yamane, J. Am. Oil Chem. Soc. 76 (1999) 839.
[6] U.T. Bornscheuer, R.J. Kazlauskas, Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations, Wiley-VCH, Weinheim, 1999, p. 336.
[7] F.D. Gunstone, J. Sci. Food Agric. 79 (1999) 1535.
[8] Y. Iwasaki, T. Yamane, J. Mol. Catal. B: Enzym. 10 (2000) 129.
[9] E. Rogalska, C. Cudrey, F. Ferrato, R. Verger, Chirality 5 (1993) 24.
[10] E. Rogalska, S. Nury, I. Douchet, R. Verger, Biochem. Soc. Trans. 25 (1997) 161.
[11] P. Michelson, E. Aronsson, G. Odham, B. Akesson, J. Chromatogr. 350 (1985) 417.
[12] P. Laakso, W.W. Christie, Lipids 25 (1990) 349.
[13] J.J. Agren, A. Kuksis, Lipids 37 (2002) 613.
[14] E. Rogalska, S. Ransac, R. Verger, J. Biol. Chem. 265 (1990) 20271.
[15] Y. Itabashi, A. Kuksis, L. Marai, T. Takagi, J. Lipid Res. 31 (1990) 1711.
[16] Y. Okamoto, M. Kawashima, K. Hatada, J. Chromatogr. 363 (1986) 173.
[17] W. Piyatheerawong, Y. Iwasaki, X. Xu, T. Yamane, J. Mol. Catal. B: Enzym. 28 (2004) 19.
[18] Y. Iwasaki, M. Yasui, T. Ishikawa, R. Irimescu, K. Hata, T. Yamane, J. Chromatogr. A 905 (2001) 111.
[19] E. Yashima, J. Chromatogr. A 906 (2001) 105.
[20] Y. Okamoto, E. Yashima, Angew. Chem. Int. Ed. 37 (1998) 1021.
[21] S. Ahuja, Chiral Separations by Chromatography, American Chemical Society, Washington, DC, 2000, p. 244.
[22] A. Stolyhwo, H. Colin, G. Guiochon, J. Chromatogr. 265 (1983) 1.
[23] A. Stolyhwo, H. Colin, M. Martin, G. Guiochon, J. Chromatogr. 288 (1984) 253.
[24] R. Irimescu, K. Furihata, K. Hata, Y. Iwasaki, T. Yamane, J. Am. Oil Chem. Soc. 78 (2001) 285.
[25] R. Irimescu, Y. Iwasaki, C.T. Hou, J. Am. Oil Chem. Soc. 79 (2002) 879.
[26] I. Douchet, G. Haas, R. Verger, Chirality 15 (2003) 220.
[27] J. Schmitke, C.R. Wescott, A.M. Klibanov, J. Am. Chem. Soc. 118 (1996) 3360.