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Optical resolution of asymmetric triacylglycerols by chiral-phase high-performance liquid chromatography

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Abstract

A simple method for direct optical resolution of some asymmetric triacylglycerols (TGs) has been established. The method employs chiral-phase high-performance liquid chromatography (HPLC). An enantiomeric pair of TGs comprising 1-eicosapentaenoyl-2,3-dicapryroyl-*sn*-glycerol (ECC) and 1,2-dicapryroyl-3-eicosapentaenoyl-*sn*-glycerol (CCE) was resolved on a CHIRALCEL OF™ or on a CHIRALCEL OD™ column. The separation of another pair of asymmetric TGs, 1-docosahexaenoyl-2,3-dicapryroyl-*sn*-glycerol (DCC) and 1,2-dicapryroyl-3-docosahexaenoyl-*sn*-glycerol (CCD), was achieved with the CHIRALCEL OD column. The chiral-phase HPLC method in combination with silver-ion HPLC and high-temperature gas chromatography was used for monitoring two interesterification reactions, whose products were chiral TGs. Interesterification of tricapryloylglycerol with ethyleicosapentaenoate or with ethyldocosahexaenoate was performed using *Rhizomucor miehei* lipase as the catalyst. The products targeted were the asymmetric pair of TGs, ECC and CCE or DCC and CCD. The amounts of *sn*-1-substituted products (ECC or DCC) were greater than their *sn*-3-substituted counterparts (CCE or CCD) throughout the reaction period, suggesting that *R. miehei* lipase had a stereopreference towards the *sn*-1 position over the *sn*-3 position.

Keywords: Triacylglycerol; Structured lipids

1. Introduction

Structured lipids (SLs) are defined as particular molecular species of triacylglycerols (TGs) with specific fatty acid (FA) residues at specific positions in the glycerol backbone [1]. The molecular structure of TGs influences their metabolic fate in organisms (i.e. digestion and absorption) [2–4] as well as their physicochemical characteristics (e.g., melting points and oxidation). Consequently, by designing SLs with a particular chemical structure, it is possible to control the behavior of TGs, thereby improving their nutritional and pharmaceutical properties.

Syntheses of SLs of particular structure require position-specific modifications in the glycerol backbone. Therefore, lipase-catalyzed regio-specific reactions have attracted the attention of researchers for syntheses of SLs [5–13]. However, most of these

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studies on lipase-mediated syntheses were intended for the preparation of ''ABA-type SLs''. ABA-type SLs are defined as SLs having symmetric structures with two identical FA residues at the *sn*-1 and 3 positions and a different FA residue at the *sn*-2 position [1]. Contrary to these symmetric SLs, asymmetric SLs such as AAB-type [with two identical residues at the *sn*-1(2) and 2(3) positions and another residue at the *sn*-3(1) position] and ABC-type (with three different kinds of residues at particular positions) are not well studied from the view point of enzymatic syntheses and biological functions. However, it is known that natural oils and fats have an asymmetrical distribution of FA residues [14,15], implying that the *sn*-1 and 3 positions are not biologically equivalent. In addition, some lipases in animals digest TGs at different rates towards the *sn*-1 and 3 positions [16,17]. From these points arises the necessity to establish synthetic methods for asymmetric SLs.

An obstacle for the establishment of synthetic methods for such asymmetric SLs is the difficulty in quantifying optical isomers of TGs. Several high-performance liquid chromatographic (HPLC) methods (originally developed for stereospecific analyses of the FA distribution in natural oils and fats) may be applicable for the quantification of TG enantiomers [14,15]. However, these methods involve laborious multi-step derivatization of the lipid samples, and therefore require a relatively large amount of sample. For example, the method reported by Ando et al. [15] includes: (i) partial degradation of the TG sample with Grignard's reagent into partial glycerides; (ii) conversion of the generated partial glycerides into 3,5-dinitrophenylurethane derivatives and isolation of the derivatives of 1(3)-monoacylglycerols (MG) on thin-layer chromatography; (iii) optical resolution of the urethane derivatives of 1(3)-MG on a chiral-phase HPLC into fractions of *sn*-1- and *sn*-3-MG derivatives; and (iv) FA composition analysis of the isolated *sn*-1- and *sn*-3-MG derivatives by gas chromatography after converting them into FA methyl esters. Hence, a simple analytical method without any derivatization steps is highly preferable.

The present work demonstrates a direct optical resolution of some asymmetric TGs on chiral-phase HPLC. Using this method, the stereoselectivity of a fungal lipase in an interesterification reaction has been investigated.

2. Experimental

2.1. Chemicals and enzymes

1,2-*O*-Isopropylydene-*sn*-glycerol and 2,3-*O*-isopropylydene-*sn*-glycerol were purchased from Tokyo Chemical Industry (Tokyo, Japan). Caprylic acid (CA), dicyclohexylcarbodiimide and dimethylaminopyridine were from Sigma (St. Louis, MO, USA). Ethyleicosapentaenoate (EtEPA) and ethyldocosahexanoate (EtDHA) were provided by Nippon Suisan (Tokyo, Japan). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were prepared by conventional alkali hydrolysis of EtEPA and EtDHA, respectively. 1,2,3-Tricapryloylglycerol (CCC) was from Wako Pure Chemicals (Osaka, Japan). Ethanol-free dry chloroform was prepared from commercial chloroform by washing with water and distillation over P_2O_5. HPLC-grade *n*-hexane and 2-propanol were from Nacalai Tesque (Kyoto, Japan). Novozym (immobilized *Candida antactica* lipase, non-regiospecific) and lipozyme (immobilized *Rhizomucor miehei* lipase, *sn*-1,3-position-specific) were gifts from Novo Nordisk Bioindustry (Chiba, Japan).

2.2. Preparation of authentic standards

2,3-*O*-Isopropylidene-*sn*-glycerol (397 mg, 3 mmol) and 908 mg EPA (3 mmol) were reacted in the presence of 140 mg of Novozym and 3% water. The mixture was incubated with stirring at 40°C for 1 h at atmospheric pressure, and then under reduced (7 mmHg) pressure for 23 h to remove the condensed water. After the reaction, the immobilized enzyme was removed by filtration, giving 1-eicosapentaenoyl-2,3-*O*-isopropylidene-*sn*-glycerol in 95% yield. The isopropylidene group of the resultant ester was deprotected with 10 ml of 90% trifluoroacetic acid at −20°C for 30 min, neutralized with 40 ml of ice-cold 2 *N* NaOH solution, and then the glycerides were extracted with 50 ml of chloroform–methanol (4:1). The resultant lipid solution was dried with anhydrous sodium sulfate, and the solvent was removed in a rotary evaporator, giving

1-eicosapentaenoyl-*sn*-glycerol in 84% yield. The chiral monoacylglycerol (188 mg, 0.5 mmol), 216 mg (1.5 mmol) of CA, 244 mg (2 mmol) of dimethylaminopyridine and 413 mg (2 mmol) of dicyclohexylcarbodiimide were reacted in 10 ml ethanol-free dry chloroform at room temperature for 24 h. After the removal of the crystallized urea product by filtration and then removal of the solvent, the lipid was chromatographed on a silica gel column with *n*-hexane–diethylether (9:1) to purify the TG fraction in 40% yield. The resultant TG fraction was further purified by HPLC using a silver-ion column (described below) to afford 1-eicosapentaenoyl-2,3-dicapryloyl-*sn*-glycerol (ECC).

Similarly, 1,2-dicapryloyl-3-eicosapentaenoyl-*sn*-glycerol (CCE), 1-docosahexaenoyl-2,3-dicapryloyl-*sn*-glycerol (DCC) and 1,2-dicapryloyl-3-docosahexaenoyl-*sn*-glycerol (CCD) were prepared using appropriate protected glycerols and FAs. The structures of these authentic TGs can be seen in Fig. 1.

2.3. High-temperature gas chromatography (HTGC)

The molecular species of glycerides were analyzed with a gas chromatograph (Model GC14, Shimadzu, Kyoto, Japan) equipped with an on-column injector (Model OCI-14, Shimadzu) and a capillary column (Type HT5, 0.53 mm×6 m, 0.1 μm film thickness, SGE, Australia) as described previously [18]. Using HTGC, molecular species of glycerides were separated depending on their molecular weight. The glyceride composition was calculated from the peak areas.

2.4. Silver-ion HPLC

Silver-ion HPLC was carried out as described previously [18,19] with a Chromspher 5 lipid column (silver-modified cation-exchange ligand-covered spherical silica, 4.6 mm×150 mm, Chromapack, The Netherlands). This method enables the separation of asymmetric TGs (i.e. ECC plus CCE, or DCC plus CCD) from their symmetric positional isomers [i.e. 1,3-dicapryloyl-2-eicosapentaenoyl-*sn*-glycerol (CEC) or 1,3-dicapryloyl-2-docosahexaenoyl-*sn*-glycerol (CDC)]. We employed this analysis to estimate the ratio of ECC plus CCE versus CEC, or the ratio of DCC plus CCD versus CDC. The method was also applied for the purification of the ECC–CCE or DCC–CCD fractions as samples for chiral-phase HPLC.

2.5. Chiral-phase HPLC

Two chiral-phase HPLC columns, CHIRALCEL OF [cellulose-tris(4-chlorophenylcarbamate)-impregnated silica, 4.6 mm×250 mm, Daicel Chemical, Tokyo, Japan] and CHIRALCEL OD [cellulose-tris-(3,5-dimethylphenylcarbamate)-impregnated silica, 4.6 mm×250 mm, Daicel Chemical] were used [20]. The mobile phase was *n*-hexane–2-propanol (200:1 or 400:1) (specified below) at 1.0 ml/min flow-rate using an HPLC pump (Model 880-PU, Jasco, Tokyo, Japan) at 25°C. A 5 μl aliquot of the sample (containing 5–30 μg of ECC–CCE or DCC–CCD dissolved in *n*-hexane) was injected using a 20 μl injection loop. The peaks were detected spectrophotometrically at 210 nm with a UV detector (UVIDEC-100 V, Jasco).

2.6. Lipase-catalyzed interesterification and monitoring the reaction

Interesterification of CCC with EtEPA was performed using the 1,3-specific *R. miehei* lipase as a catalyst (Fig. 1). The product targeted in this synthesis was the asymmetric SLs, ECC or CCE. An important aspect of this synthesis was to substitute only one CA residue of the CCC molecule with an EPA residue, avoiding the formation of 1,3-dieicosapentaenoyl-2-capryloyl-*sn*-glycerol (ECE). This can be achieved by the use of CCC in molar excess (CCC–EtEPA, 3:1, the stoichiometric ratio is 1:1). Another point is to shift the equilibrium towards the formation of the target TG by removing ethylcaprylate (EtCA, which is generated during the reaction) under vacuum. We employed the conditions optimized by Han and Yamane [21] as below.

A lipid mixture of CCC (6 mmol, 2.82 g) and EtEPA (2 mmol, 0.66 g) was reacted with 0.3 g of lipozyme at 40°C. The reaction was conducted at atmospheric pressure (760 mmHg) for 8 h, and then at 3 mmHg for 16 h. Prior to the reaction, CCC and the enzyme were kept in a desiccator containing saturated LiCl solution in order to adjust the water

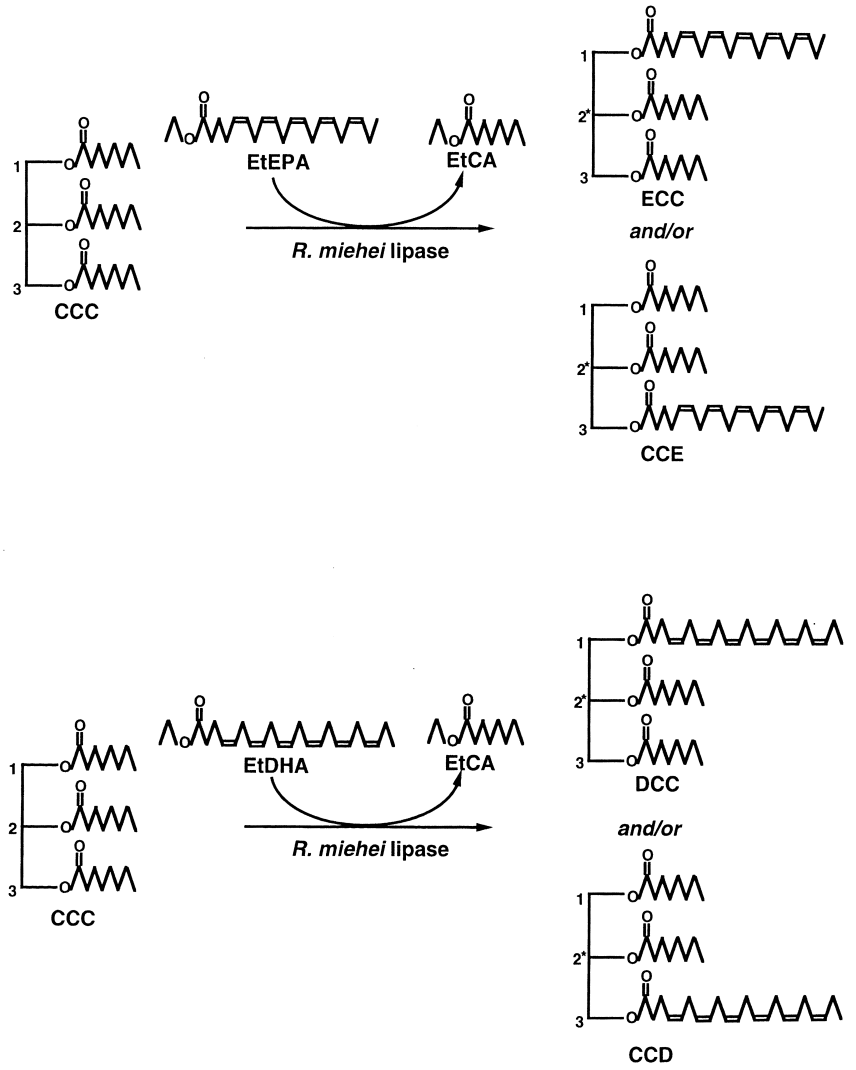

Fig. 1. Lipase-catalyzed interesterification for syntheses of asymmetric TGs. 1,2,3-Tricapryloylglycerol (CCC) is reacted with ethyleicosapentaenoate (EtEPA) or ethyldocosahexanoate (EtDHA) to afford 1-eicosapentaenoy-2,3-dicapryloyl-*sn*-glycerol (ECC) plus 1,2-dicapryloyl-3-eicosapentaenoyl-*sn*-glycerol (CCE) or 1-docosahexaenoy-2,3-dicapryloyl-*sn*-glycerol (DCC) plus 1,2-dicapryloyl-3-docosahexaenoyl-*sn*-glycerol (CCD), respectively, with liberation of ethylcaprylate (EtCA). The carbons in the glycerol moiety were numbered stereospecifically. The chiral centers at the *sn*-2 positions are indicated by asterisks.

activity to 0.12. Intermittently, 5 μl of the sample was withdrawn, and dissolved in 200 μl of *n*-hexane. The resultant lipid solution was filtered using a syringe equipped with a disposable polytetrafluoroethylene membrane filter unit (type DISMIC-13 HP, 0.2 μm pore size, Advantec, Japan) to remove the solid enzyme. A 1.5 μl aliquot of the filtered solution was injected into the HTGC, and the glyceride composition was calculated. Lipid solution (6 μl) was subjected to silver-ion HPLC, and the content of the positional isomer of the targeted TG was estimated. The ECC–CCE peak was collected manually from the outlet of the UV detector, dried, and redissolved in 10 μl of *n*-hexane. The resultant lipid solution (5 μl) was analyzed by chiral-phase HPLC with the CHIRALCEL OF column. Depending on

the progress of the enzyme reaction, the amounts of ECC and/or CCE injected onto the chiral-phase column varied (in the range 5–30 μg for each injection). The enantiomeric purity of the asymmetric TGs was evaluated from the enantiomeric excess (%ee) value, which was calculated from the peak areas:

$$\%ee = ([ECC] - [CCE])/([ECC] + [CCE]) \times 100$$

Lipase-catalyzed interesterification of CCC with EtDHA was performed similarly. The reaction was carried out at atmospheric pressure for 48 h and then at 3 mmHg for 24 h. The CHIRALCEL OD column was used for the optical resolution of DCC and CCD. The %ee value was defined as:

$$\%ee = ([DCC] - [CCD])/([DCC] + [CCD]) \times 100$$

3. Results and discussion

3.1. Separation of the authentic standards by chiral-phase HPLC

To our knowledge, the present paper is the first report concerned with the direct optical resolution of asymmetric TGs. Among the several commercial chiral columns tested, CHIRALCEL OF and CHIRALCEL OD were suitable for such resolution. Fig. 2 shows the resolution of the authentic ECC and CCE on the CHIRALCEL OF column. The authentic ECC and CCE were eluted at 45 and 41 min, respectively, with a resolution factor (R_s) of 1.07 (Fig. 2A). The peaks were identified by injecting each of the authentic enantiomers separately, as shown in Fig. 2B and C. The chromatograms of each of the authentic enantiomers (Fig. 2B and C) showed small peaks corresponding to the other enantiomeric counterparts, indicating that isomerization (i.e. acylmigration) occurred to some extent during the preparation of the authentic standards (especially in the deprotection step of the isopropylidene group). These isomerized impurities did not interfere with the identification of the peaks as above, because each of the enantiomers clearly gave one predominant peak and because the acid-mediated deprotection of the isopropylydene group proceeds with retention of the configuration at the *sn*-2 carbon [22].

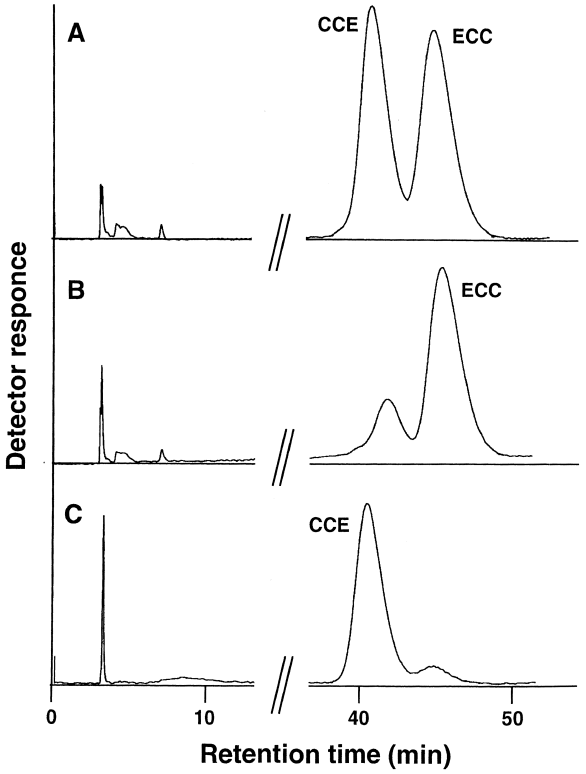

Fig. 2. Optical resolution of ECC and CCE on a CHIRALCEL OF column. Chromatograms of a mixture of ECC and CCE (A), ECC (B) and CCE (C) are shown. The mobile phase was *n*-hexane–2-propanol, 200:1.

The resolution of ECC and CCE was also possible on the CHIRALCEL OD column with *n*-hexane–2-propanol (400:1) as the mobile phase. On this column, the order of elution of the enantiomers was reversed to the case of the OF column, giving an R_s value of 1.08 (Fig. 3).

Attempts to resolve the authentic DCC and CCD on the CHIRALCEL OF column were unsuccessful (data not shown), but the CHIRALCEL OD column enabled the complete separation of DCC from CCD. As shown in Fig. 4, DCC eluted at 21 min, whereas CCD eluted at 46 min, with an R_s value of >11.

3.2. Monitoring of lipase-catalyzed interesterification

In lipase-catalyzed interesterification, the replacement of one CA residue at either the *sn*-1 or 3

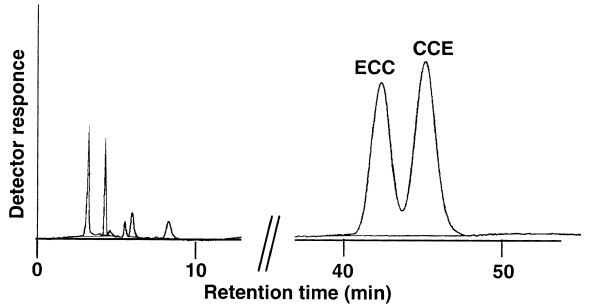

Fig. 3. Resolution of ECC and CCE on a CHIRALCEL OD column. A mixture of ECC and CCE were separated with *n*-hexane–2-propanol, 400:1.

position of the achiral (prochiral) CCC with one EPA or DHA residue generates a chiral center in the products (Fig. 1). Elucidation of the optical purity of the products is one of our main objectives in this analysis. The reaction course was monitored using HTGC, silver-ion HPLC and chiral-phase HPLC. None of these analytical methods required any derivatization steps, thereby enabling rapid analyses with minimal amounts of samples.

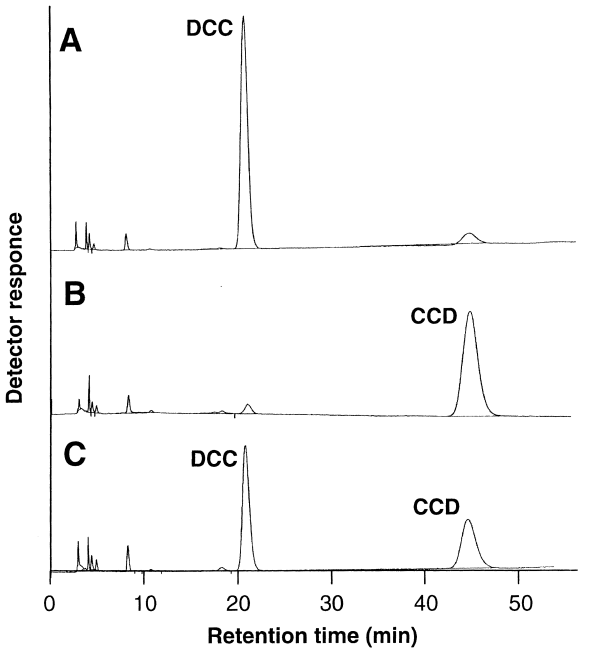

Fig. 4. Separation of DCC and CCD. The authentic DCC (A), CCD (B) and a mixture of DCC and CCD (C) were chromatographed on a CHIRALCEL OD column with *n*-hexane–2-propanol, 200:1.

Fig. 5A shows the time course of the reaction between CCC and EtEPA. Approximately 60% of the ethyl ester was consumed rapidly in 5 h and reached a plateau. Reducing the reaction pressure was effective for the further conversion of EtEPA, resulting in 71.1% consumption in 24 h. Consequently, the targeted TG amounted to 22.3 mol% of the glycerides in 24 h. Other components in the glycerides were CCC (75.8 mol%), diacylglycerol with two CA residues (1.1 mol%), diacylglycerol with one CA and one EPA residue (0.2 mol%) and ECE (0.6 mol%). Silver-ion HPLC analysis confirmed that CEC, the positional isomer of the targeted TG, was less than 2% throughout the reaction, as observed previously [18,21].

The enantiomeric excess (%ee) of the targeted TG was more than 60% in the early stage of the reaction (0.5–1 h), and then gradually decreased to 25% as the reaction proceeded. The %ee value of more than zero throughout the reaction indicates that the *R. miehei* lipase has stereopreference towards the *sn*-1 position over the *sn*-3 position under the conditions tested. This observation is in agreement with the results of Chandler et al., who demonstrated the *sn*-1 stereopreference of the enzyme in acidolysis reactions [23].

Fig. 5B presents the reaction course of CCC with EtDHA. The reaction was very slow compared with the case of EtEPA due to the weak activity of the *R. miehei* lipase towards the DHA residue [24,25]. Forty-four percent of the EtDHA was consumed in 72 h, giving a glyceride mixture containing 13.8 mol% of DCC plus CCD, 81.6 mol% of CCC, 3.7 mol% of diacylglycerol with two CA residues and 0.9 mol% of diacylglycerol with one DHA and one CA residue. CDC, the positional isomer of the target, was not detected by silver-ion HPLC. The enantiomeric excess of the product was in the range 70–40, showing again the enzyme's *sn*-1 stereopreference over *sn*-3.

The present study provides a simple method for optical purity analyses of TGs. The method will

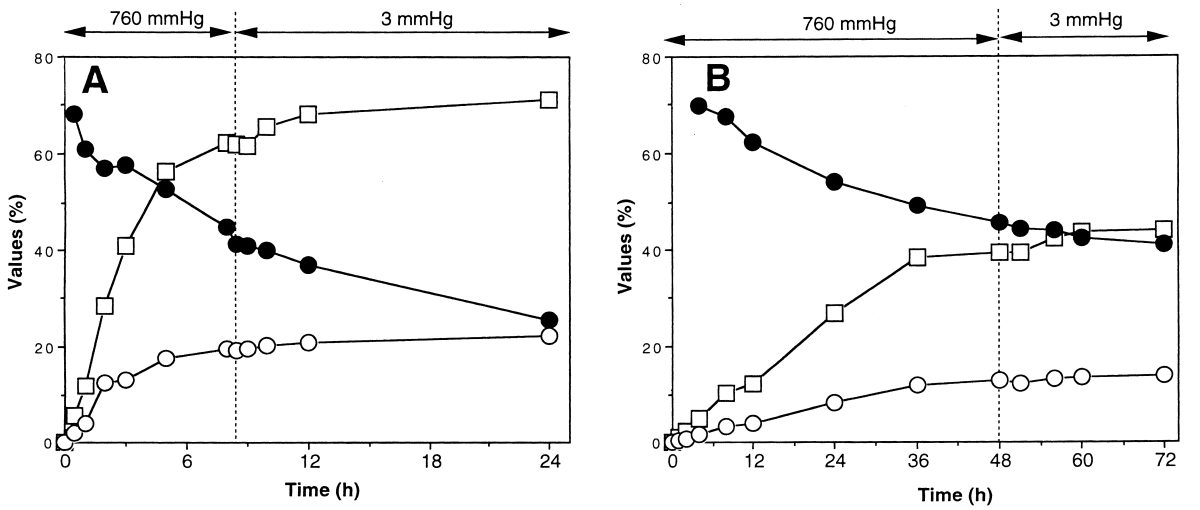

Fig. 5. Time course of lipase-mediated interesterification. For the reactions of CCC with EtEPA (A) or EtDHA (B), the consumption of the ethyl ester (squares), the molar content of the targeted TG in glycerides (○) and the enantiomeric purity (%ee) of the target (●) are shown. Vertical dashed lines indicate the time after which the pressure was reduced.

facilitate optimization of the reaction conditions for effective asymmetric synthesis of SLs. In addition, this resolution technique itself may be applied for preparative purposes, as it is a nondestructive method.

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