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Suppression of acyl migration in enzymatic production of structured lipids through temperature programming

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Abstract

Acyl migration in the glycerol backbone often leads to the increase of by-products in the enzymatic production of specific structured lipids. Acyl migration is a thermodynamic process and is very difficult to stop fully in actual reactions. The objective of this study was to investigate the feasibility of suppressing acyl migration by a programmed change of reaction temperature without loss of reaction yield. The model reactions were the acidolysis of tripalmitin with conjugated linoleic acid (CLA) or with caprylic acid (CA) targeted for human milk fat substitutes. Acyl migration was considerably inhibited in the temperature-programmed acidolysis of PPP with CLA or CA, with only slight reduction of acyl incorporation, the reaction leading to the required products. Acyl migration was reduced by 29% (35 h) and 45% (48 h), respectively, in the acidolysis of PPP with CLA under solvent and solvent-free systems, in comparison with 37% (35 h) and 61% (48 h), respectively, for the acidolysis of PPP and CA. Acyl migration in the acidolysis of PPP with CA was, in general, lower than the acidolysis of PPP with CLA in both systems. Temperature programming was more prominent in solvent-free systems for the reduction of acyl migration. Acyl incorporation was not significantly affected by temperature programming. The study suggests that it is feasible to reduce acyl migration by programmed change of acidolysis temperature without significant loss of reaction yield.

Keywords: Acidolysis; Acyl migration; Conjugated linoleic acid (CLA); Structured lipids; Temperature programming; Tripalmitin

1. Introduction

Structured lipids (SLs) are triacylglycerols (TGs) containing specific fatty acids in designated positions (Gunstone, 1999) and have become of academic and industrial interest because beneficial effects of TGs are closely associated with their structures. Molecular structures of TGs influence their metabolic fate in organisms (digestion and absorption) (Iwasaki & Yamane, 2000). For the synthesis and production of SLs using enzymatic catalysis, wide research work has been conducted in the past 10 years (Akoh & Yee, 1997; Hamam & Shahidi, 2004; Iwasaki, Han, Narita, Rosu, & Yamane, 1999; Quinlan & Chandler, 1992; Schmid, Bornscheuer, Soumanou, McNeill, & Schmid, 1998, 1999; Sharma, Arora, & Wadhwa, 2001; Shimada et al., 1996; Senanayake & Shahidi, 2002; Soumanou, Bornscheuer, & Schmid, 1998a, 1998b; Willis & Marangoni, 1999; Xu, 2000a; Yang & Xu, 2002). Based on structure and fatty acid composition, SLs are often referred to as human milkfat substitutes (HMFS), cocoa butter equivalents (CBE), and MLM-type TGs (M-medium chain and L-long chain fatty acids). MLM-type TGs, containing medium-chain fatty acids at *sn*-1 and *sn*-3 positions and long chain fatty acids at the *sn*-2 position, provide rapid delivery of energy via oxidation of the more hydrophilic medium-chain fatty acids. HMFS are supposed to contain 20–25% palmitic acid predominantly

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esterified at the *sn*-2 position, which lead to considerable significance and consequence for the absorption of fat and minerals in infants.

SLs are extremely difficult to prepare by chemical synthesis due to the many purification steps required to remove intermediate products (Soumanou et al., 1998a, 1998b). Another approach is enzyme-catalyzed interesterification, especially *sn*-1,3 specific lipase-catalyzed acidolysis. The advantages of enzymatic reaction lie in its selectivity, mild reaction conditions, little or no unwanted side-reactions or by-products, ease of product recovery, easy control over the process and less waste disposal (Akoh, 1997). The one-step acidolysis reaction route is depicted in Fig. 1. Regiospecificity is an essential requirement for the acidolysis to produce pure SLs. This process is to replace fatty acids (A) specifically at the *sn*-1 and *sn*-3 positions of TG (AAA) with the desired one (B) by a 1,3-specific lipase, leaving the fatty acid (A) at the *sn*-2 position unchanged. Nevertheless, side-reactions (acyl migration) could not be avoided due to the existence of diacylglycerols (DGs), which leads to the formation of undesirable products (*sn*-ABA, *sn*-BBA and *sn*-ABB). Acyl migration generally involves migration from *sn*-1,3 to *sn*-2 positions but also occurs with migration of acyls from the *sn*-2 into the *sn*-1,3 positions. The possible mechanisms of acyl migration have previously been discussed (Xu, 2000b). It is difficult to efficiently separate the desirable product from by-products on an industrial scale. The effect of reaction parameters on acyl migration has been elucidated and temperature is one of the most important parameters that have a profound influence on the migration rate, since acyl migration is a thermodynamic process (Xu, 2000b). There have been no possible methods to fully stop the acyl migration, but minimization of acyl migration can be expected under optimized conditions.

In a "two-step" process for the production of pure SLs, alcoholysis was the first step and esterification was the second step. The "two-step" process efficiently suppressed acyl migration in both alcoholysis and esterification steps in a solvent system at low temperatures (Iwasaki & Yamane, 2000; Schmid, Bornscheuer, Soumanou, McNeill, & Schmid, 1998, 1999; Soumanou et al., 1998a, 1998b).

From our evaluation, we believe that temperature plays a very important role in the increase of acyl migration. On the other hand, temperature also affects the reaction activity of enzymes, in general, according to the Arrhenius law. For lipase-catalyzed acidolysis, we found that temperature had a stronger impact on migration than on incorporation, especially with the immobilized lipases tested (Xu, 1998). This provides a positive clue to apply a lower temperature for lipase-catalyzed acidolysis.

The reaction yield is very much based on the reaction equilibrium that can be reached. In the main reaction of Fig. 1, A is a released fatty acid from the reaction. If A can be removed from the reaction system, the reaction equilibrium will be broken and driven further to the product side. On consideration of low temperature for lipase-catalyzed acidolysis, we would expect A to be "frozen" and not actively participate in the reaction. This can be achieved by using a fatty acid with a higher melting point. This led to selection of the model system with tripalmitin as the substrate for production of HMFS. Palmitic acid has a melting point of 63 °C, much higher than caprylic acid or CLA as acyl donors. This would not, therefore, severely affect the reactivity of acyl donors at a lower temperature. Thus, lower temperature will reduce the enzyme activity and substrate mass transfer in the solvent-free system, but will push a higher reaction yield through the breaking of the reaction equilibrium by "inactivating" the released fatty acid. We expect that the former effect can be compensated by the latter, so that acyl migration can be eventually suppressed without the loss of reaction yield.

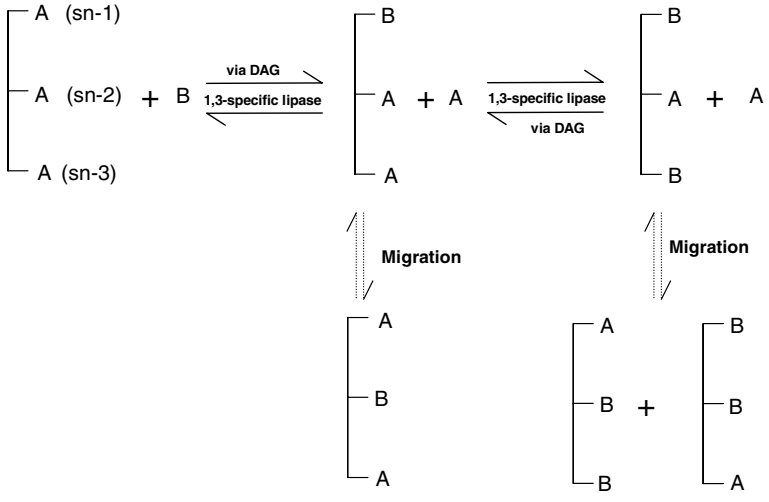

Fig. 1. Scheme of acidolysis catalyzed by *sn*-1,3 specific lipase. A and B stand for acyl groups. DAG is diacylglycerol.

Therefore, the objective of this study was to test theoretical speculation on the system design and to investigate the feasibility of decreasing acyl migration by a programmed change of reaction temperatures. The direct purpose for the system was to produce more desirable SLs and to reduce the formation of by-products. Model acidolysis systems of tripalmitin were selected with conjugated linoleic acid (CLA) or with caprylic acid (CA) as acyl donors. Both solvent and solvent-free systems were used.

2. Materials and methods

2.1. Materials

Tripalmitin (PPP > 85%) was purchased from Sigma Chemical Co. (St. Louis, MO). Conjugated linoleic acid (CLA) was obtained from Natural Lipids (Sandvika, Norway). Caprylic acid was obtained from Riedel-de Haen (Seelze, Germany). Lipozyme RM IM, a commercial *sn*-1,3 specific lipase, in which *Rhizomucor miehei* lipase was immobilized on a microporous ion exchange resin, was donated by Novozyme A/S, Bagsvaerd, Denmark. Fatty acid methyl ester and triacylglycerol standards were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were of analytical grade.

2.2. Enzymatic acidolysis

About 20 g of tripalmitin and conjugated linoleic acid or caprylic acid (1:6, mol/mol) in a 50-ml conical flask, in solvent (hexane) or solvent-free system, were used for Lipozyme RM IM-catalyzed acidolysis at different temperatures and magnetic stirrings. Lipozyme RM IM load (10% by weight, based on total substrates) without additional water was used. Samples of 500 μl were withdrawn for analysis at certain time intervals. The experiments for the study were conducted only once for each set-up. However, the repeatability of the enzymatic acidolysis was tested by conducting the experiments three times with a typical experimental set-up. The standard deviation for incorporation or migration was ±1.8% for a fatty acid content of more than 10% and ±0.7% for fatty acid contents below 10%, using the following methods for analysis.

2.3. Methylation and GC analysis

About 10 mg of samples containing free fatty acids (FFA) were dissolved in 1 ml of heptane. To this, 60 μl of 2 M KOH-methanol were added and the contents were mixed by shaking for 20 s. Anhydrous sodium sulfate was added to dry the mixture. Centrifugation was carried out at 4000 rpm for 15 min. The upper layer,

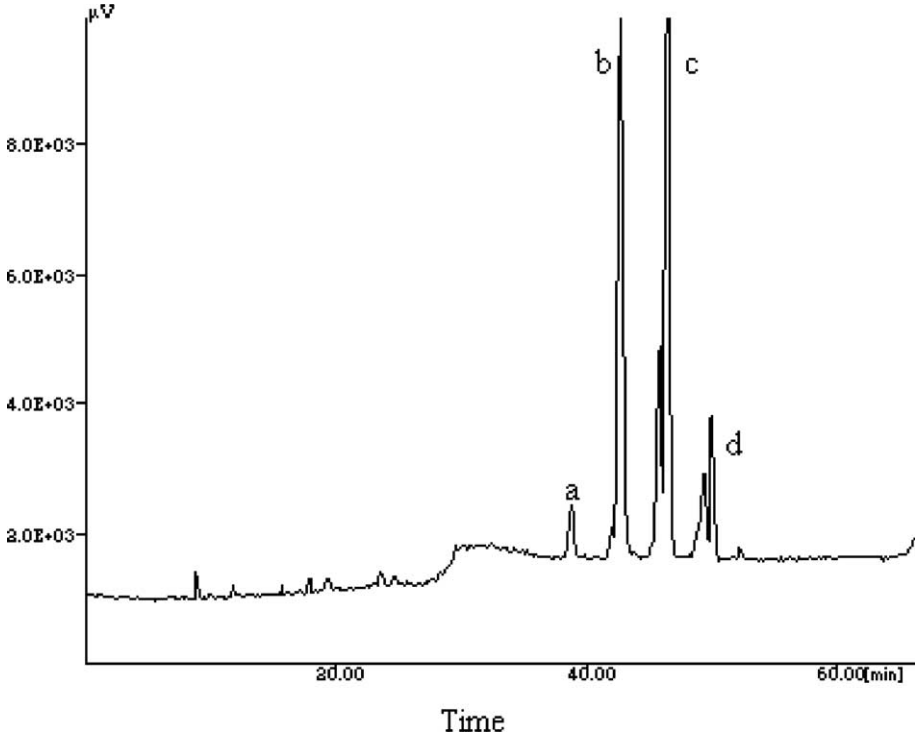

Fig. 2. HPLC chromatography of triacylglycerol composition of acidolysis products between tripalmitin (PPP) and conjugated linoleic acid (CLA, cL): (a) cLcLcL; (b) cLPcL; (c) cLPP; (d) PPP.

containing the methyl esters, was decanted to GC-vials for further GC analysis.

The FA methyl esters were analyzed by gas-liquid chromatography with an HP 6890 series gas chromatograph (Hewlett–Packard, Waldbronn, Germany) and a fused-silica capillary column (Supelco Wax-10, 60 m × 0.25 mm, i.d., 0.25 mm film thickness; Supelco Inc., Bellefonte, PA). Carrier gas was helium with a flow rate of 40 ml/min. The injector was used in split mode with a ratio 1:20. Oven temperature was programmed from 70 to 160 °C at a rate of 15 °C/min, increased to 180 °C at a rate of 1 °C/min, further to 185 °C at a rate of 0.5 °C/min, and finally to 200 °C at a rate of 20 °C/min and held there for 10 min. The injector and detector temperatures were 250 and 260 °C, respectively. The fatty acid methyl esters were identified by comparing their retention times with authentic standards obtained from Sigma Chemical (St. Louis, MO). Triple determinations were conducted and the average was used. The absolute standard deviations were less than 1.0% for all the fatty acids.

2.4. Grignard degradation

Fatty acid composition at the *sn*-2 position was determined by Grignard degradation with allyl magnesium bromide, followed by isolation and methylation (Becker, Rosenquist, & Hølmer, 1993).

2.5. Triacylglycerol composition by HPLC

Triacylglycerol compositions in the interesterification samples were determined by HPLC. The HPLC system was a JASCO high-performance liquid chromatograph (Jasco Corporation, Tokyo, Japan) equipped with two PU-980 pumps, an HG-980-30 solvent mixing module, an AS-950 autosampler, a UV-970 UV–VIS detector, and a Sedex 55 evaporative light-scattering detector (ELSD, Sedere, Alfortville, France). The ELSD was operated at a temperature of 40 °C with air as the nebulizing gas at 2.2 bar. The column used was Supelcosil LC-18 (250 × 4.6 mm, Supelcosil Inc., Bellefonte,PA), packed with a particle size of 5 μm. The mobile phase was a binary solvent system of acetonitrile and isopropanol/hexane (2:1 v/v) and flow rate was 1 ml/min at

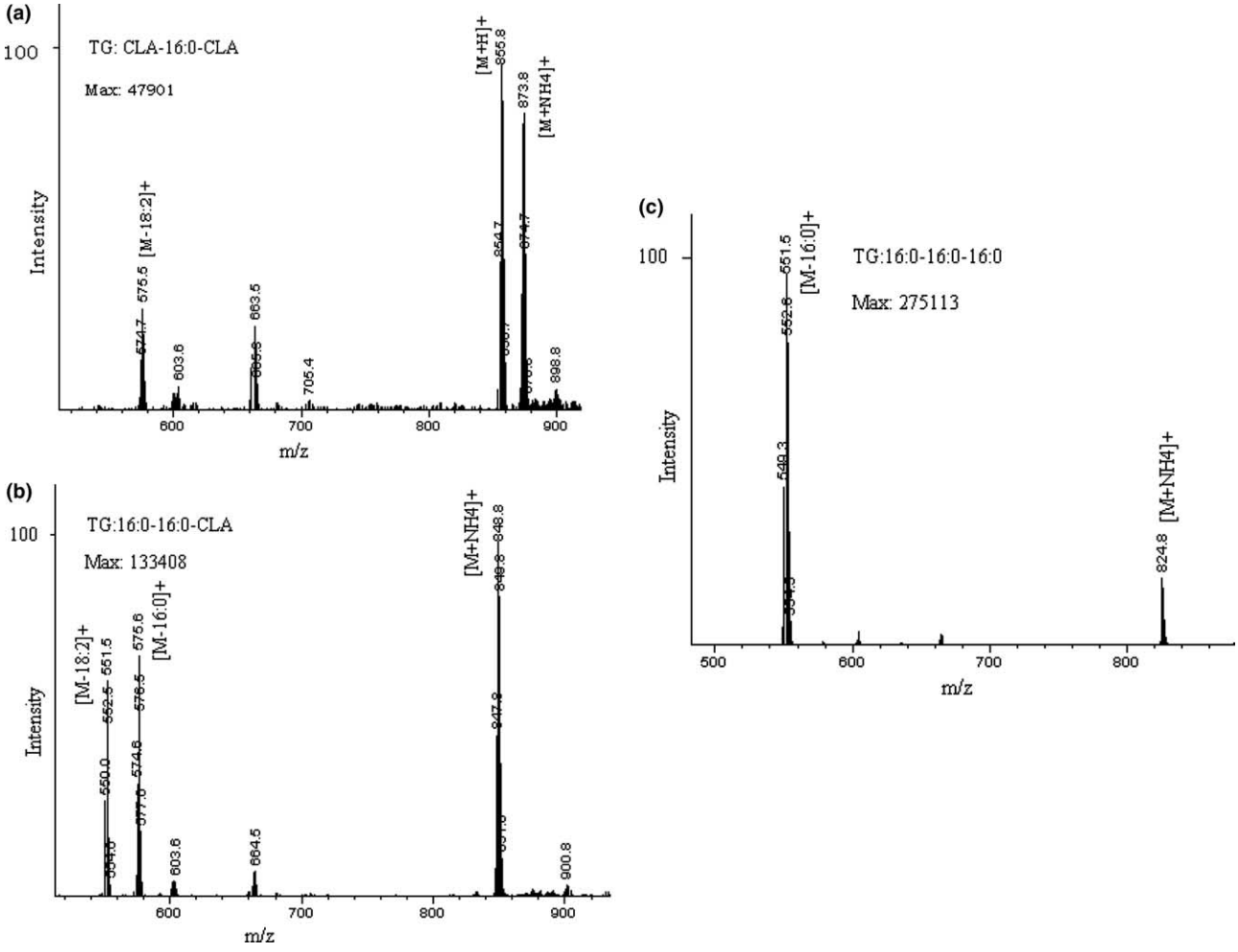

Fig. 3. LC–MS spectra of the triacylglycerols of the acidolysis mixture between tripalmitin (PPP) and conjugated linoleic acid (CLA).

ambient temperature. The samples were dissolved in chloroform (10 mg/ml) and 10 μl were injected. Triacylglycerol peaks were identified by LC–MS spectra, TG standards and theoretical carbon number (TCN).

2.6. Identification of triacylglycerol moiety by atmospheric pressure chemical ionization (APCI) LC–MS

The triacylglycerols were separated as described above. The column was fitted into an HP 1100 Series LC/MSD system, consisting of a quaternary pump, a vacuum degasser, an autosampler, a diode array detector, and an MS detector (Hewlett–Packard, Waldbronn, Germany). All of the column effluent was admitted to the mass spectrometer. The APCI was used in the positive mode, and the solvent vapour acted as the reagent gas. Ammonium acetate (50 mM in isopropanol/water = 1:1) was supplied after the separation column at a flow of 50 μl/min. The capillary voltage was 3000 V, the vapourizer temperature was 325 °C, and nebulizer gas (nitrogen) pressure was 60 psi. The heated nitrogen drying gas temperature and flow rate were 350 °C and 4.0 l/min, respectively. Full mass spectra were taken in the mass range of 65–950, and the step size was 0.1 *m/z*. System control and data evaluation were conducted using HP ChemStation (Mu, Sillen, & Høy, 2000).

3. Results and discussion

3.1. General

Acyl migration is an unavoidable side reaction of lipase-catalyzed acidolysis. The existence of diacylglycerols (DGs) is the main reason leading to acyl migration in lipase-catalyzed interesterification. The mechanism of acyl migration is initiated by the nucleophilic attack of the lone pair of electrons of the free hydroxyl oxygen in DGs on the ester carbonyl carbon, which will form an unstable five-membered ring intermediate orthoester. The ring opens by cleavage of the original ester carbon oxygen single bond, which results in acyl migration (Yang, Xu, & Li, 2001). There are many factors that could possibly influence acyl migration, such as reaction temperature, time, acyl chain length, water activity, acid, base, reactor type, behaviour of lipase and others (Boswinkel, Derksen, van't Riet, & Cuperus, 1996; Heisler, Rabiller, & Hublin, 1991; Kodali, Tercyak, Fahey, & Small, 1990; Sjursnes & Anthonsen, 1994; Xu, 2000b). The primary hydroxyl oxygen is a more active nucleophile than the secondary hydroxyl oxygen and, therefore, acyl shift from a secondary hydroxyl to a primary hydroxyl is favoured.

Acyl migration has a good linear relationship with time course of the enzymatic acidolysis, which indicates that the reaction is a non-enzymatic process. Fatty acids with different chain lengths and numbers of double bonds have different migration rates (Xu, 2000b). The different positions and geometry of double bonds also produce different acyl migration rates (Yang et al., 2001). This means linoleic acid and CLA may have different migration rates. To investigate the effects of programmed temperatures on acyl incorporation and migration in enzymatic acidolysis, two reaction systems (PPP + CLA and PPP + CA) were used in this study.

3.2. Acyl migration and incorporation in the acidolysis of PPP with CLA

In the acidolysis of tripalmitin (PPP) with CLA (cL) (PPP + cL ⇌ cLPP + P + cL ⇌ cLPcL + cL + P), there are three main triacylglycerol components, namely

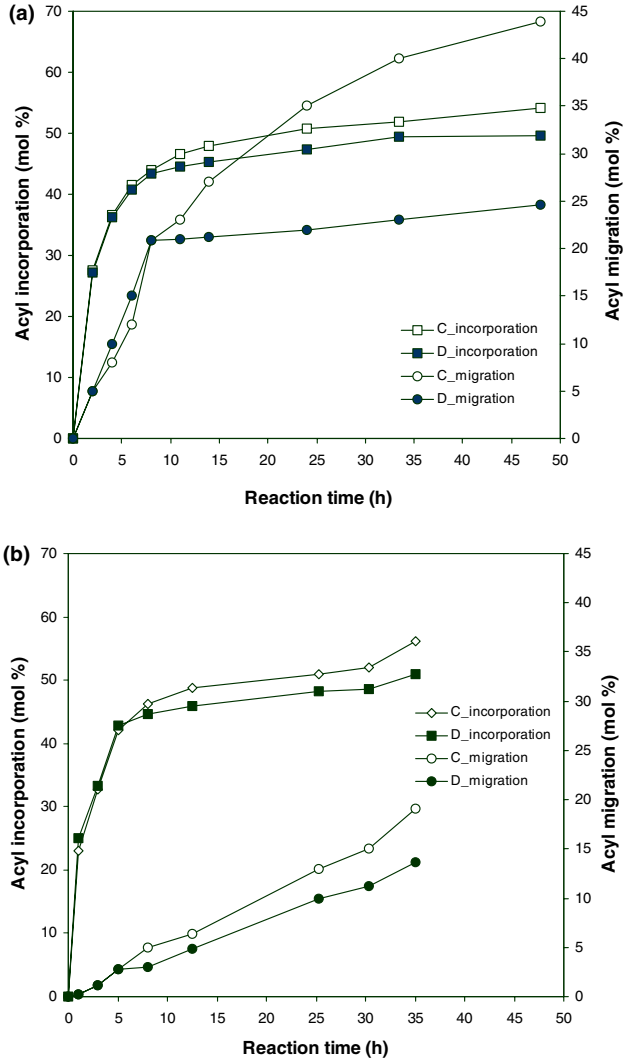

Fig. 4. Time course of conjugated linoleic acid (CLA) in the acidolysis of tripalmitin and CLA (1:6, mol/mol); 10 wt% Lipozyme RM IM (on total substrates), 250 rpm stirring: (a) solvent-free system, control (C) 65 °C and programmed (D) 65 °C (8 h), then 40 °C; (b) solvent (hexane) system, control (C) 55 °C and programmed (D) 55 °C (5 h), then 35 °C.

PPP, cLPP, and cLPcL. During the reaction, HPLC was used to monitor the change of TG composition (Fig. 2) and GC was used to determine the fatty acid composition. The identification of TGs in HPLC were made by LC–MS. A typical MS spectrum of the species in the acidolysis mixture is shown in Figs. 3(a)–(c).

An increase of reaction temperature usually results in an acceleration effect, according to the Arrhenius law, catalyzed by enzymes. High temperature favours higher yields for endothermic reactions due to the shift of thermodynamic equilibrium. At elevated temperatures, operation is also easy, since a higher temperature decreases the viscosity of solutions so as to reduce the mass transfer limitations (Martinek, 1993). Raising temperature can increase the rate of acyl incorporation, but simultaneously more migration occurs. Acyl migration was highly suppressed when temperature was reduced, after 8 h, during the acidolysis of PPP with CLA though acyl incorporation was slightly reduced as well (Fig. 4(a)). The acyl migration was defined as the mole percentages of CLA at the *sn*-2 position of produced TGs. There was almost no increase of acyl migration after temperature was reduced. On the other hand, incorporation increased in a pattern similar to the control experiment. Migration was reduced by 45% (48 h) and 29% (35 h), respectively, in the acidolysis of PPP and CLA in the solvent-free system and solvent system (Figs. 4(a) and (b), respectively).

Less acyl migration resulted in a lower content of the by-product, triconjugated-linolein (cLcLcL), which was also considered as one of the indications of acyl migration, in the acidolysis of PPP with CLA (Figs. 5(a) and (b)). Before 35 h, cLcLcL was not produced in the temperature-programmed (65–45 °C) acidolysis of tripalmitin and CLA, but amounted to 10% in the control (65 °C).

3.3. Acyl migration and incorporation in the acidolysis of PPP with CA

Migration was separately reduced by 61% (48 h) and 37% (35 h) by the programmed change of reaction tem-

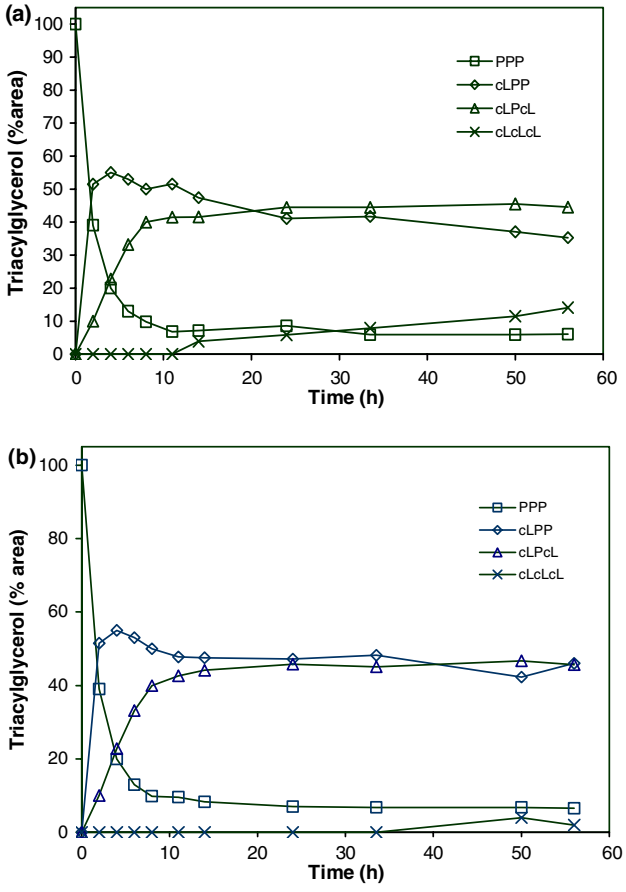

Fig. 5. Time course of triacylglycerol (TG) composition during the acidolysis of PPP and conjugated linoleic acid (CLA) (1:6, mol/mol). 250 rpm stirring; 10 wt% Lipozyme RM IM (on total substrates): (a) 65 °C (Control); (b) 65 °C (8 h), then 40 °C.

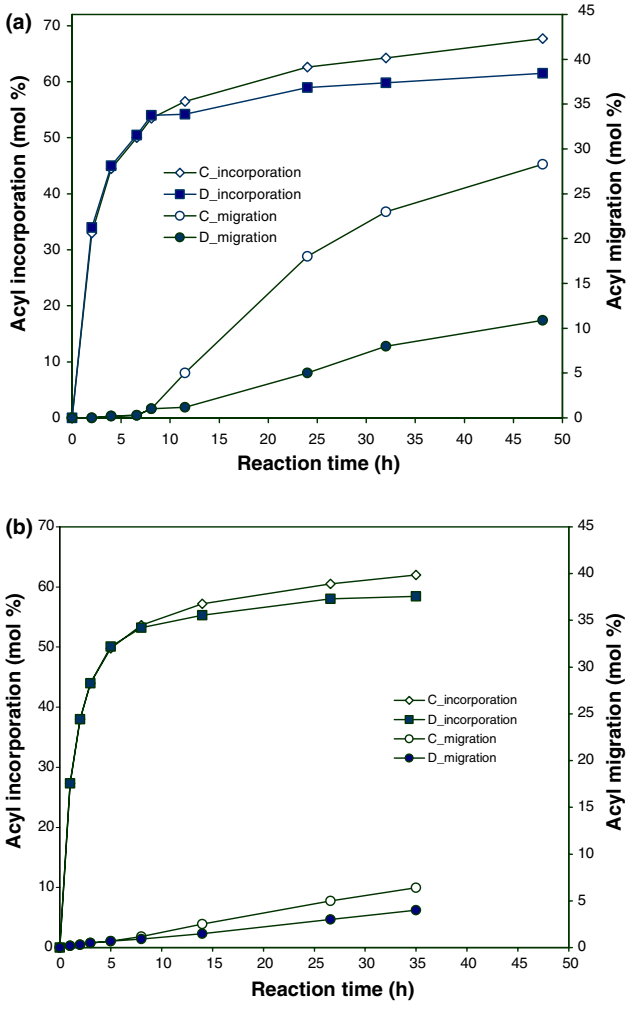

Fig. 6. Time course of caprylic acid (CA) during the acidolysis of tripalmitin and CA (1:6, mol/mol); 10 wt% Lipozyme RM IM (on total substrates), 250 rpm stirring: (a) solvent-free system, control (C) 65 °C and programmed (D) 65 °C (8 h), then 40 °C; (b) solvent (hexane) system, control (C) 55 °C and programmed (D) 55 °C (5 h), then 35 °C.

peratures in the acidolysis of PPP and CA in the solvent-free and solvent systems (Figs. 6(a) and (b)). CA was more incorporated in PPP than was CLA, probably due to the steric hindrance of CLA in the acidolysis (Yang et al., 2001). For the temperature-programmed acidolysis, the reduction of caprylic acid incorporation in the non-solvent was more significant than that in the solvent system, but solvent had no effect on CLA incorporation (Figs. 4(a) and (b)). Migration in the acidolysis of PPP with CA was significantly less than that in the acidolysis of PPP with CLA in solvent or solvent-free systems.

4. Conclusions

Acyl migration could be effectively reduced by temperature-programming with a lower formation of by-products in enzymatic acidolysis. TGs with different carbon-chain lengths had different acyl migrations. Solvent (hexane) in the reaction could inhibit more migration in the reactions and narrow the migration gap between control and temperature-programmed acidolysis.

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