Chapter 5

Interesterification Reaction between Medium and Long Chain Fatty Acid Triglycerides Using Modified Lipase

Introduction

MCT composed from caprylic acid (C8:0) and capric acid (C10:0) are known to have a nutritional function. MCT are more rapidly absorbed and utilized for energy compared to LCT, and MCT are used in the diets of patients with pancreatic deficiency, premature infants and surgery patients to provide the necessary calories. On the other hand, pure MCT alone cannot provide essential fatty acids. Simple physical mixtures of MCT and LCT may be thought to meet nutritional requirements, however, the absorption efficiency has been reported to be low[17,50]. The production of structured triglycerides (ST), in which each triglyceride contains both medium chain fatty acids (MCFA) and long chain fatty acids (LCFA), has been investigated as an alternative to a physical mixture of MCT and LCT. The ST of MCFA and LCFA have several nutritional benefits compared to a physical mixture of MCT and LCT[51]. Enzymatic interesterification in a non-solvent system is very practical for the commercial production of ST, since it is safe, cost effective and environmental friendly.

In Chapter 2, the interesterification of tripalmitin and stearic acid using the modified lipase which is a complex of lipase, *Rhizopus japonicus* and surfactant, sorbitan monostearate in *n*-hexane was studied. The modified lipase was also used for the interesterification in a non-solvent system, and the modified lipase had an activity at 75°C was found. In this section, in order to produce ST the interesterification of MCT and LCT using modified lipase was investigated in a non-solvent system. The interesterification kinetics in terms of the total MCT and LCT content changes are discussed. The effects of water activity (Aw) of the modified lipase, water content of the reaction system and the reaction temperature on the activity were also studied.

Materials and Methods

Materials

All chemicals and lipase used in this section except MCT and LCT were described in Chapter 2-1. MCT and LCT were a gift from Unilever Research

Colworth Laboratory, U.K. Table 5-1 shows the fatty acid composition of MCT and LCT.

Lipase modification

Lipase Saiken 100 (*R. japonicus*) and Emazol S-10 (F) (sorbitan monostearate) were used for the modification. Lipase modification was carried out according to the method described in Chapter 2-1.

Water activity (Aw) control of modified lipase

To adjust the Aw to the desired level, the modified lipase was kept in sealed jar with water saturated salt before the reaction. Several water saturated salts were used and their Aw values were 0.12, 0.55, 0.76, 0.86 and 0.97 for LiCl, $MgN_2O_6 \cdot 6H_2O$, NaCl, KCl and K_2SO_4, respectively. The equilibrium of the modified lipase was monitored using a water activity sensor Model AM3 with KGC humidity probe (Rotronic Instruments, Bassersdorf, Switzerland) and the water content was measured using a Karl Fischer volumetric titrator attached to a Radiometer Titralab (Radiometer A/S, Copenhagen, Denmark).

Adjustment of water content in the substrates

The water content of the reaction system was adjusted by the addition of water to the substrate. A certain amount of water was added to the substrate and dissolved or dispersed by stirring at 500 rpm and then stored at 50°C in a crimp sealed vial. The exact water content was analyzed using a Karl Fischer Coulometer Model 737 (Metrom Ltd., Herisau, Switzerland) before the reaction.

Interesterification of TGs

The substrates of MCT (3.67 g) and LCT (6.33 g), MCT: LCT = 1: 1 mol ratio, were used and heated in a heat block at 50°C in a crimp sealed vial. The modified

Table 5-1 Fatty acid composition of medium chain fatty acid triglycerides (MCT) and long chain fatty acid triglycerides (LCT)

MCT (mol%)	
C8:0	C10:0
62.47	37.53

LCT (mol%)						
C16:0	C18:0	C18:1	C18:2	C20:0	C22:0	C24:0
3.96	3.76	80.71	10.05	0.54	0.74	0.23

C8:0, Caprylic acid; C10:0, Capric acid; C16:0, Palmitic acid;
C18:0, Stearic acid; C18:1, Oleic acid; C18:2, Linoleic acid;
C20:0, Arachidic acid; C22:0, Behenic acid; C24:0, Lignoceric acid

lipase (9-20 mg/g TG) was added to the vial which was recapped and immediately replaced into the heat block. The interesterification was carried out while stirring at 500 rpm with a magnetic stirrer for 48 h. Samples were taken out through the septum by syringe and were filtered (LCR13-LH, pore size 0.5 μm, Millipore Co., Milford, MA) and then analyzed by GC and HPLC.

Analysis method

TG groups which had identical numbers of carbon atoms were analyzed using a slight modification of the AOCS Official Method Ce 5-86 (reapproved in 1997) as follows; Samples of using 10-15 mg were weighed and dissolved in 5 mL dichloroethane, then analyzed by Perkin Elmer 8500 series GC (Perkin Elmer, Norwalk, CO, USA) with FID. On-column injection was used and the sample volume was 0.1 to 0.2 μL. The column used was DB-5, i.d. 0.53 mm, 10 m length and 0.1μm methyl-5% phenyl film thickness (Quadrex Corporation, New Haven, CT, USA). The carrier gas used was helium at 55 kPa pressure. The injector temperature was controlled as 50°C at 0 min, 370°C at 0.5 min and 50°C at 3.5 min. The column temperature was controlled to increase from 120°C to 325°C at 25°C/min and 325°C to 355°C at 5°C/min. The detector temperature was 370°C.

Diglycerides (DG) and monoglycerides (MG) were analyzed using a modified version of the AOCS Official Method Cd 11d-96 (reapproved in 1997) as follows. The sample was dissolved in the solvent (1,2 dichloroethane 75 vol%: ethanol 25 vol%, HPLC grade) to a concentration of 0.1 g/mL, then 20 μL was injected with the internal standard (12-hydroxy octadecanol, Nu Chek Prep, Elysian, MN, USA). DG and MG concentrations were determined from the proportional relationships with the internal standard. The HPLC used was Varian 5560 (Varian Associates, Palo Alto, CA, USA) with evaporative light scattering detector (Altech 500 ELSD, heater set to 90°C, gas flow set to 30 psi, time constant 0.1 s, Altec UK, Lancashire, UK). The column used was Spherisorb silica (i.d. 4.6 mm, 100 mm length and 3 μm particle size, Sigma

Chemical Company, St. Louis, MO, USA). The solvent gradient was a multilinear combination of the three solvent mixtures of A: toluene 1: hexane 1, B: toluene 600: ethyl acetate 200: formic acid (98%) 16 and C: toluene 500: ethyl acetate 200: isopropyl alcohol 100: formic acid (98%) 16. Solvent flow rate was set at 1.0 mL/min.

The protein content of the modified lipase was analyzed according to the method described by Hartree[19].

Results and Discussion

Figure 5-1 shows the typical time history for the interesterification of total MCT (C24 to C30) and total LCT (C50 to C54) with the modified lipase. 103 mg of the modified lipase was used. The substrates of 3.67 g MCT and 6.33 g LCT (1:1 mole ratio) were used. The initial water content in the reaction mixture was below 0.09 wt%. The interesterification reaction was carried out at 500 rpm and 50°C. Carbon numbers denote the TG containing respective numbers of carbon atoms. The molar compositions of MCT and LCT decreased equally and were 13 mol% after a 48 h reaction period. ST (C32 to C46) were produced with time, and their composition was 74 mol% after 48 h. Each TG composition change is shown in Fig. 5-2. All of the MCT, C24, C26, C28 and C30 decreased significantly, and reduction ratios of those were 71 - 76% (Fig. 5-2-A). The LCT, C54 and C52 decreased significantly, and reduction ratios were 75% and 70%, respectively (Fig. 5-2-C). In the ST, the TG of C44, C36, C34 and C46 increased significantly (Fig. 5-2-B). The reaction system seems to reach equilibrium after 48 h. DG concentration increased from 1.5 to 4.1 wt% after 48 h. MG were not produced at all. When the interesterification reaction using non-modified lipase (Lipase Saiken 100, powdery) was also carried out for 24 h under the same conditions, neither interesterification nor hydrolysis reaction occurred. From these, the lipase modification process using surfactant was found to be useful for interesterification reaction between LCT and MCT in a non-solvent system with a low water content.

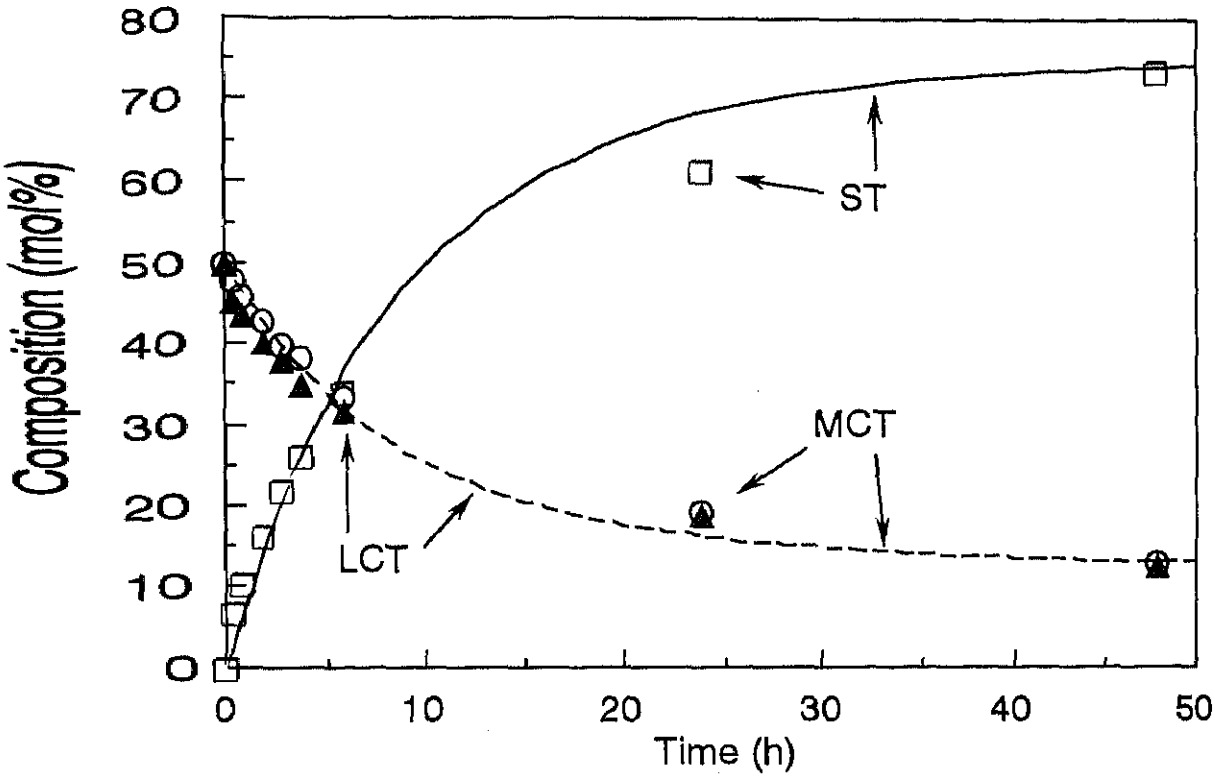

Fig. 5-1 Interesterification versus time for medium chain fatty acid triglycerides (MCT, ○), long chain fatty acid triglycerides (LCT, ▲) and structured triglycerides (ST, □) with the modified lipase [Lipase Saiken 100 (*R.japonicus*) and Emazol S-10 (S) (sorbitan monostearate)]. 103 mg of modified lipase was used. The substrates of 3.67 g MCT and 6.33 g LCT (1:1 mole ratio) were used. The initial water content in the reaction mixture was below 0.09 wt%. Interesterification reaction was carried out at 500 rpm and 50°C. The solid line represents simulation result of ST. The broken line represents simulation results of LCT and MCT. Carbon numbers of LCT C50, C52 and C54; those of MCT C24, C26, C28 and C30; and those of ST C32, C34, C36, C38, C42, C44 and C46.

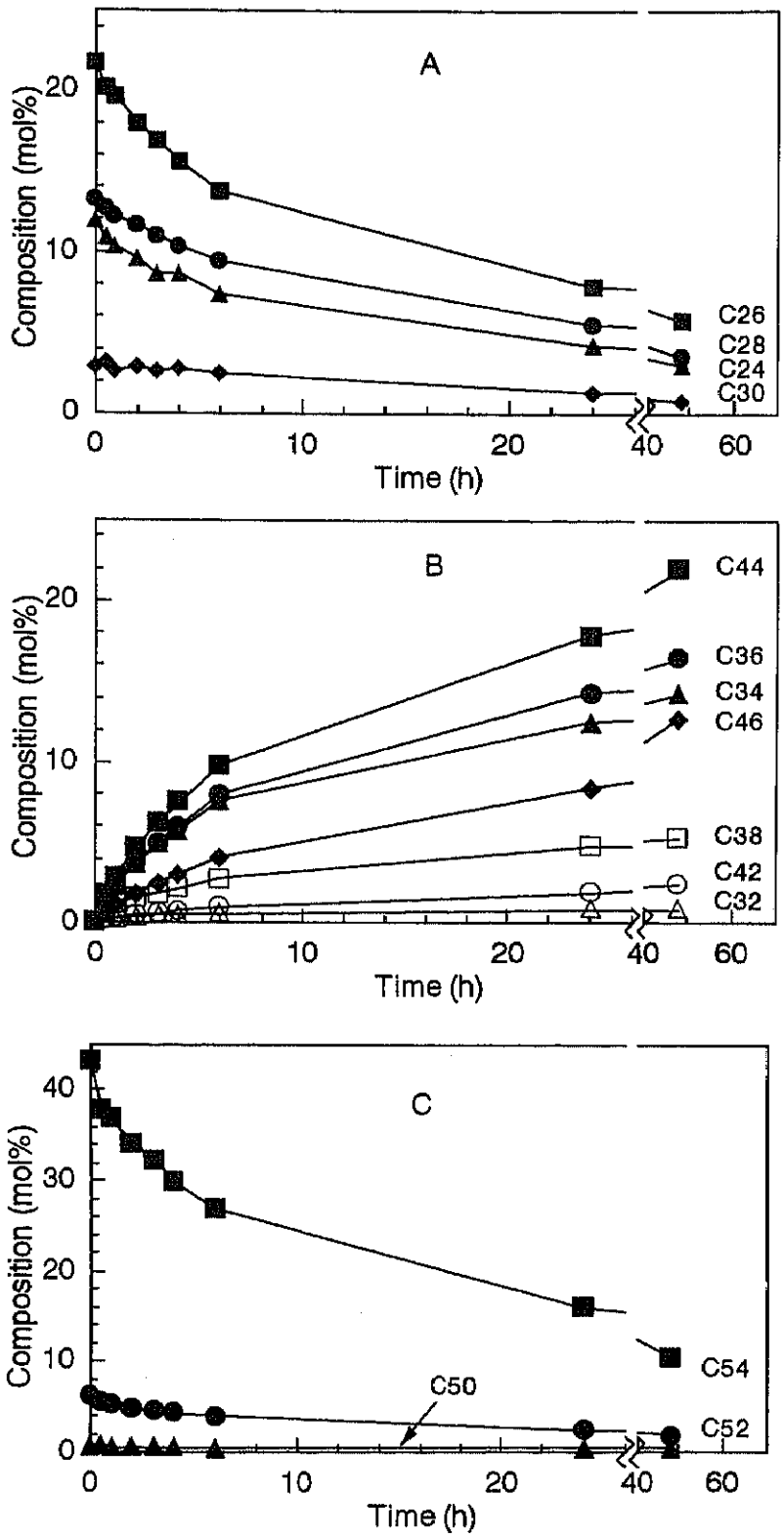

Fig. 5-2 Interesterification versus time for MCT (A), ST (B) and LCT (C) with the modified lipase. The substrates and reaction conditions as in Figure 5-1. Carbon (C) numbers represent TG containing respective number of carbon atoms.

Simulation of final TG composition by stochastic model

Because the modified lipase used in this study has 1,3 positional specificity (Chapter 2-1 and 2-2), ST distribution at equilibrium can be theoretically described by the 1,3-random 2-random stochastic model[23]. The theory assumes that two different pools of fatty acids are separately and randomly esterified to the 2 and 1,3 positions. The compositions at positions 1 and 3 are identical. On the basis of this hypothesis, the amount of a given TG can be calculated as follows.

$$\%sn\text{-XYZ} = [\text{mol\% at 1,3}] \times [\text{mol\% at 2}] \times [\text{mol\% at 1,3}] \times 10^{-4} \quad (1)$$

Table 5-2 shows initial fatty acid distribution at *sn*-1,3 positions and *sn*-2 position in the substrates. Using Eq. 1, TG distribution at equilibrium was calculated. Figure 5-3 shows the experimental and calculated results of TG compositions, in which both results agree quite well. TG composition after interesterification reaction at equilibrium between MCT and LCT was estimated using the 1,3-random 2-random stochastic model.

Kinetic model

The modified lipase used in this study has 1,3 positional specificity, and showed little specificity toward the types of fatty acid moiety. The formation of DG was detected, but the content was less than 5wt%. No MG was produced. Based on these findings, the kinetic model of the interesterification reaction will follow the model described in Chapter 3-2. Assuming that DG and MG are not produced, there are six potential products of the interesterification reactions between MCT (MMM) and LCT (LLL) which can be modeled as follows.

$$\mathrm{MMM + LLL} \underset{k}{\overset{4k}{\rightleftharpoons}} \mathrm{MML + MLL} \quad (2)$$

Table 5-2 Initial fatty acid distribution of the substrates. Distribution ratio in *sn*-1,3 positions and *sn*-2 position

	sn-1,3 (mol%)	*sn*-2 (mol%)
C8:0	30.82	32.10
C10:0	18.51	19.30
C16:0	2.93	0.00
C18:0, C18:1, C18:2	46.63	48.60
C20:0	0.40	0.00
C22:0	0.55	0.00
C24:0	0.16	0.00
	100.00	100.00

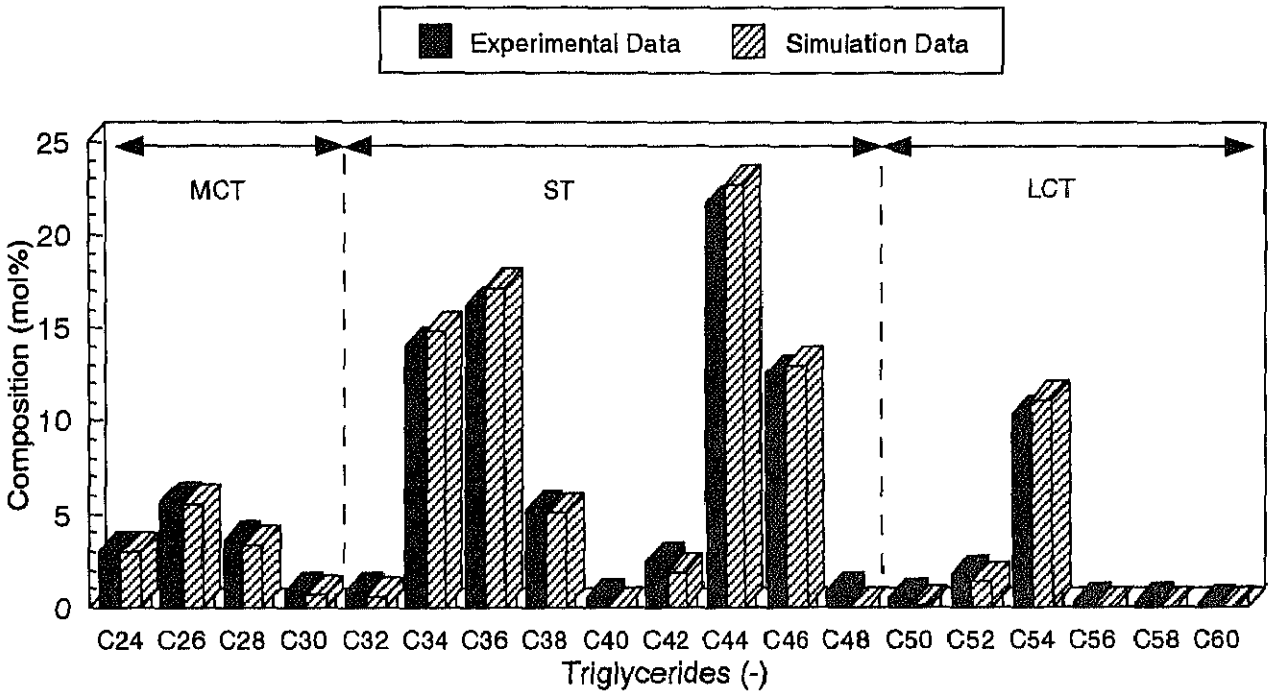

Fig. 5-3 Comparison of the TG composition between experimental and simulation results. The substrates and reaction condition as in Figure 5-1.

$$MMM + MLL \underset{2k}{\overset{2k}{\rightleftharpoons}} MML + MLM \quad (3)$$

$$MML + LLL \underset{2k}{\overset{2k}{\rightleftharpoons}} LML + MLL \quad (4)$$

$$MML + MLL \underset{k}{\overset{2k}{\rightleftharpoons}} LML + MLM \quad (5)$$

$$MMM + LML \underset{k}{\overset{4k}{\rightleftharpoons}} 2MML \quad (6)$$

$$LLL + MLM \underset{k}{\overset{4k}{\rightleftharpoons}} 2MLL \quad (7)$$

where M is medium chain fatty acid, L is long chain fatty acid, k is the interesterification rate reaction constant, and MML, MLM, LML and MLL are the ST.

Different rate equations for MMM, LLL, MML, MLM, LML and MLL in the reaction system can be derived from Eqs. 2-7 as follows.

$$(1/k)\, d[MMM]/dt = [MLL][MML] + 2[MML][MLM] + [MML]^2 - 4[LLL][MMM] - 2[MLL][MMM] - 4[MMM][LML] \quad (8)$$

$$(1/k)\, d[LLL]/dt = [MLL][MML] + 2[MLL][LML] + [MLL]^2 - 4[LLL][MMM] - 2[LLL][MML] - 4[LLL][MLM] \quad (9)$$

$$(1/k)\, d[MLM]/dt = 2[MLL][MMM] + [MLL][MML] - [MLL]^2 - 2[MML][MLM] - 4[MLM][LML] + 4[MMM][LML] \quad (10)$$

$$(1/k)\, d[LML]/dt = [MLL][MML] + 2[LLL][MML] + [MML]^2 - 4[LML][MLM] - 2[MLL][LML] - 4[MMM][LML] \quad (11)$$

$$(1/k)\, d[MLL]/dt = -[MLL][MML] + 2[LLL][MML] + 4[LLL][MMM] - 2[MLL][LML] - [MLL][MML] - 2[MML][MLM] + 2[MML][MLM] + 4[LML][MLM] + 4[LLL][MLM] - [MLL]^2 \quad (12)$$

$$(1/k)\, d[MML]/dt = -[MLL][MML] - 2[LLL][MML] + 4[LLL][MMM] + 2[MLL][LML] - 2[MML][MLM] + 2[MLL][MMM] + 4[LML][MLM] - [MLL][MML] + 4[MMM][LML] - [MML]^2 \quad (13)$$

The above differential equations can be solved analytically, and the concentration changes of the components expressed as follows (Chapter 3-2).

$$[MMM]=[LLL]=(T/4)\{1+\exp(-4kTt)\}^2 \quad (14)$$

$$[MML]=[MLL]=(T/2)\{1-\exp(-8kTt)\} \quad (15)$$

$$[LML]=[MLM]=(T/4)\{1-\exp(-4kTt)\}^2 \quad (16)$$

$$[ST]=[MML]+[MLL]+[LML]+[MLM]$$

$$=T\{1-\exp(-8kTt)\}+(T/2)\{1-\exp(-4kTt)\}^2 \quad (17)$$

where T is the initial content of MCT and LCT and t is reaction time. The reaction rate constant can be determined as follows.

$$k=-\{1/(4Tt)\}\ \ln\{(4[MMM]/T)^{0.5}-1\}=-\{1/(4Tt)\}\ln\{(4[LLL]/T)^{0.5}-1\} \quad (18)$$

This model can therefore predict the kinetics of the interesterification by using only one parameter of the reaction rate constant k, and the initial concentration of MCT and LCT.

From Eq. 18, the rate constant (k) was determined as

$$k=0.030 \quad g/(mmol\cdot h) \quad (19)$$

From Eqs. 14, 17 and 19, the composition changes of MCT, LCT and ST were calculated. Simulation results were compared to the experimental results in Fig. 5-1, in which both agreed quite well. The agreement implies that the enzyme does not distinguish between fatty acids of MCT and LCT. The proposed model predicted the interesterification kinetics of the lipase-catalyzed reaction between MCT and LCT. The advantages of this model are that it requires only one parameter, which can be easily determined experimentally, and that an analytical solution is possible when the initial concentrations of the substrates are equal. When initial concentrations of the substrates are not equal, the model can easily be solved by simulation software.

Effect of modified lipase Aw on interesterification activity

The Aw of the modified lipase was 0.35. It took about 2 weeks for the Aw of the modified lipase to be adjusted to values of 0.55, 0.76, 0.86 and 0.97 using the salts

$MgN_2O_6 \cdot 6H_2O$, NaCl, KCl and K_2SO_4 respectively. The modified lipase placed in LiCl (Aw, 0.12) reached an Aw 0.22 after 3 weeks storage and the value was unchanged after a further week storage. Figure 5-4 shows the interesterification activity calculated as the ratio of reaction rate constant to enzyme protein content (from Eq. 18), TG composition and contents of DG and MG after 48 h reaction under the different modified lipase Aw conditions. The amount of modified lipase protein was 38 mg in each experiment. The activity increased with the increase in the modified lipase Aw from 0.22 to 0.35 with the highest activity reached at a modified lipase Aw of 0.35. The activity dropped down remarkably for modified lipase Aw higher than 0.55, however, the activity increased slightly at the modified lipase Aw of 0.97. For TG composition, at the modified lipase Aw of 0.22, ST composition was 60 mol% and both MCT and LCT compositions were 20 mol%. At the modified lipase Aw of 0.35, ST composition increased to 74 mol%. Both MCT and LCT compositions were 13 mol%. Both MCT and LCT reduction rates were almost same at the modified lipase Aw between 0.22 and 0.35. At modified lipase Aw higher than 0.55, ST composition decreased to less than 30 mol%. At Aw 0.76, ST composition was only 13 mol% which was the lowest, however, at Aw 0.96, ST composition increased to 29 mol%. For modified lipase Aw higher than 0.55, the MCT composition was lower than LCT, which is probably due to hydrolysis, i.e., hydrolysis against MCT may take place more than that of LCT. DG after 48 h increased from 3.1 wt% at Aw 0.22 to 12.7 wt% at Aw 0.96. MG were not produced up to Aw 0 .76, but increased to 1.3wt% at Aw 0.96.

Effect of the water content of the substrate on interesterification activity

Water content in the substrate at initial condition was 0.09 wt% of TG, and saturated water content in the substrate was about 0.25 wt%. The modified lipase with Aw 0.35 was used in the reaction experiment. Figure 5-5 shows the activity and produced ST composition, DG and MG contents after 48 h reaction under various initial water contents. At water content of 0.09 wt%, the activity reached the highest. And

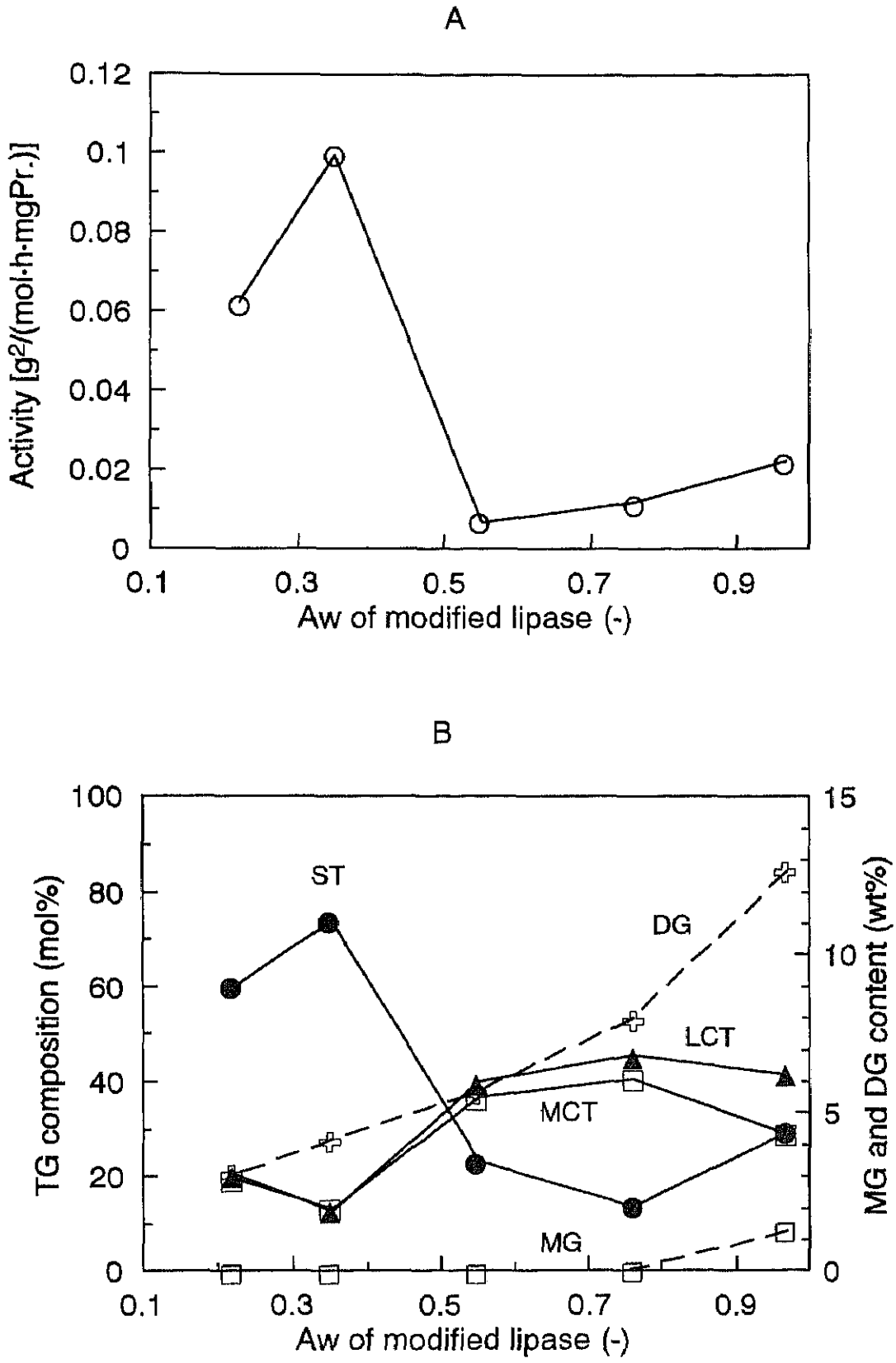

Fig. 5-4 Effect of Aw of the modified lipase on the initial activity (A), TG composition, and DG and MG contents (B). Each experiment used 38 mg of modified lipase protein. The substrates and reaction conditions as in Figure 5-1.

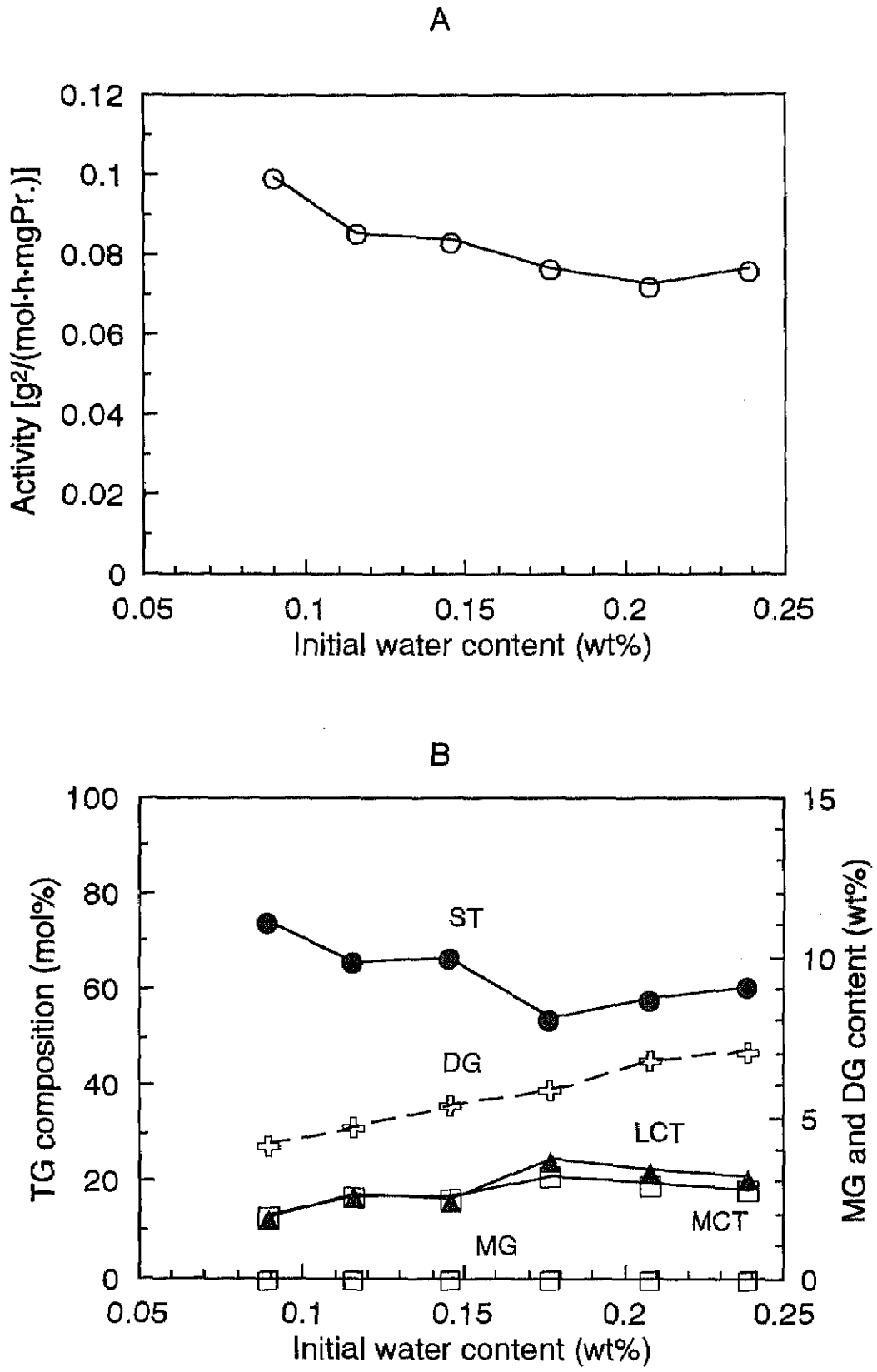

Fig. 5-5 Effect of the initial water content of the reaction system on the initial activity (A), TG composition, and DG and MG contents (B). 110 mg of modified lipase was used and its Aw was 0.35. The substrates and reaction conditions as in Figure 5-1.

the activity decreased gradually with increase in the water content. ST composition after 48 h reaction was the highest at the water content of 0.09 wt%, and decreased gradually with an increase in the water content up to 0.18 wt%. DG content after 48 h reaction increased with an increase in the water content. No MG was produced at all water contents of the substrates.

Effect of temperature on interesterification activity

The effect of the reaction temperature on the activity was investigated using the modified lipase of Aw 0.35 and the reaction system water content of 0.09 wt%. Figure 5-6 shows the interesterification activity and TG composition and DG content after 48 h reaction against temperature. The activities were almost same at 50°C, 60°C and 70°C, however the activity at 80°C decreased to 67% of that at 50°C. At 50°C and 60°C, ST compositions were almost same, however, ST composition decreased at 70°C and 80°C. DG contents of 3-4 wt% were not changed against the temperature. MG was not produced under all reaction conditions. These results showed that the modified lipase could react significantly the interesterification up to 60°C. Although the modified lipase was deactivated gradually at temperatures higher than 70°C, the modified lipase was found to keep its activity, even at 80°C.

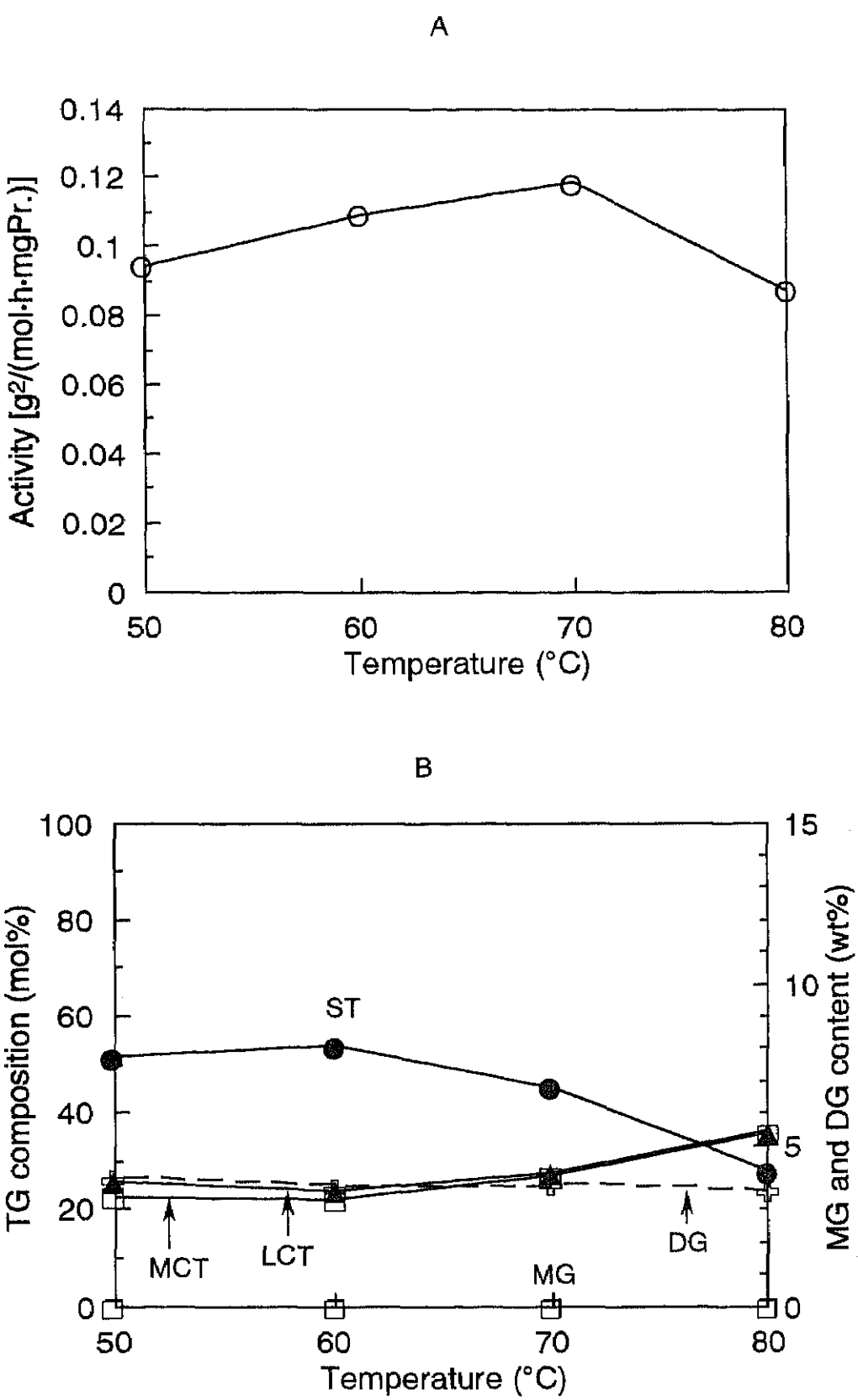

Fig. 5-6 Effect of reaction temperature on the initial activity (A), TG composition, and DG and MG contents (B). 65 mg of modified lipase was used and its Aw was 0.35. The substrates and reaction conditions as in Figure 5-1.