Enzymatic Synthesis of Low-Calorie Structured Lipids in a Solvent-Free System

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ABSTRACT: For the synthesis of low-calorie structured lipids (LCSL), transesterification between triacetin and stearic acid using immobilized lipase in a solvent-free system was investigated. Stearic acid, a long-chain saturated fatty acid, was incorporated mainly into the *sn*-1 and/or *sn*-3 positions of triacetin by lipase-catalyzed reaction. Three types of reactor systems (open, closed, and vacuum) were studied for the production of LCSL. The effects of various reaction variables such as water activity of substrates and lipase, molar ratio of substrates, stirring speed and reaction temperature were investigated. In the vacuum reactor system, a certain amount of water was added periodically to maintain the optimal water content of the reaction system. When a suitable amount of water (0.65 wt% of substrates) was added at every 1 h into the vacuum reactor system, more than 88% LCSL was obtained within 4 h using Chirazyme® L-2.

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KEY WORDS: Lipase-catalyzed transesterification, low-calorie structured lipids, solvent free system, vacuum application.

SALATRIM (short and long acyl triglyceride molecule) fats, developed by Nabisco Inc. (East Hanover, NJ), are defined as mixtures of triacylglycerols (TAG) formed by alkali metal-catalyzed interesterification of hydrogenated vegetable oils with short-chain TAG (1–3). An advantage of this class of structured lipids (SL) is to provide the physical properties of fat with approximately half of the calories of typical edible oils (4). The properties of SALATRIM depend on the chain length and positional distribution of acyl moieties on the TAG backbone (1,2). For example, absorption of long-chain fatty acid (stearic acid) by the human body is determined by its stereo-position on TAG and the presence of calcium and magnesium in the diet (4,5). When stearic acid is located at the *sn*-2 position on TAG, the resultant *sn*-2 monostearin after hydrolysis by pancreatic lipase is well absorbed. However, free fatty acids released from the *sn*-1 or *sn*-3 position on TAG tend to be poorly absorbed, suggesting that the stereo-position of long-chain saturated fatty acids is important in calorie intake (4–7). At present, most familiar low-calorie structured lipids (LCSL), so-called SALATRIM, are produced by chemical interesterification, which is conducted at high temperature in the presence of a toxic catalyst (e.g., sodium methoxide) (8). Therefore, removal of the toxic materials after reaction and subsequent purification steps are needed to obtain LCSL. Furthermore, long-chain acyl moieties are esterified randomly on TAG molecules in the chemical reaction (8–10).

Lipase-catalyzed esterifications offer the advantages of milder reaction conditions with negligible amount of by-products and regioselective specificity toward the acyl moieties on TAG molecules (3,11,12). Moreover, the products from enzymatic synthesis may satisfy consumers' demand for medical and nutritional products (13). Therefore, the enzymatic method may be an alternative to conventional chemical synthesis for producing SL with certain fatty acids incorporated at specific positions, and several studies of this type have been performed (14–18).

In this study, enzymatic synthesis of LCSL was investigated using Chirazyme® L-2 (Roche Molecular Biochemicals, Mannheim, Germany) as the catalyst. Since the products were intended to be used as food ingredients, organic solvent was avoided. Chirazyme® L-2 shows nonspecificity or *sn*-1, 3 specificity, depending on reactants and reaction conditions (19). By transesterification between long-chain saturated fatty acid and short-chain TAG using Chirazyme® L-2 in a solvent free system, long-chain saturated fatty acid was placed mainly at the *sn*-1 and/or *sn*-3 positions of the glycerol backbone during the initial reaction period. For monitoring the synthesis of LCSL, the transesterification reactions were performed in open-, closed-, and vacuum-reactor systems. The comparison of transesterification reactions in open- and closed-reactors and the effects of various parameters such as water activity of substrates and lipase, molar ratio of substrates, stirring speed, and reaction temperature were examined. Finally, we explored the use of a vacuum reactor system for effective removal of a by-product (acetic acid) and periodically added a certain amount of water to maintain a constant water level in the reaction medium during the vacuum application.

MATERIALS AND METHODS

Materials. Chirazyme® L-2 (immobilized lipase B from *Candida antarctica*) was kindly donated by Roche Molecular Biochemicals. Triacetin, 1-monostearin, 1,3-distearin, tristearin, tridecane, and acetic anhydride were purchased from Sigma Chemical Co. (St. Louis, MO). Stearic acid was pur-

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chased from Junsei Chemical Co. Ltd. (Tokyo, Japan). Diacetylstearoylglycerol and distearoylacetylglycerol were prepared chemically in our laboratory as standards. Analytical-grade methanol and chloroform were obtained from Merck (Darmstadt, Germany). All other chemicals and solvents were of the highest purity available.

Synthesis of diacetylstearoylglycerol and distearoylacetylglycerol as standards. Diacetylstearoylglycerol was prepared as previously described (9). Added was 2.5 g 1-monostearin to 25 mL acetic anhydride in a 50-mL round-bottom flask equipped with a magnetic stirrer and a reflux condenser. The system was heated to 137°C for 12 h at atmospheric pressure. The crude TAG product was cooled with crushed ice water, and then dissolved in 70 mL chloroform followed by neutralization with 70 mL 3 M sodium bicarbonate. The solution was extracted with 70 mL chloroform three times, and the organic layers were combined, dried over anhydrous sodium sulfate, filtered, and evaporated under vacuum. The reaction mixture was then separated by thin-layer chromatography on silica gel 60 F_{254} (20 × 20 cm, 1-mm layer thickness, Merck). The plates were developed with a mixture of benzene/diethyl ether/ethyl acetate/acetic acid (80:10:10:0.2, by vol). The bands were visualized by spraying the chromatographs with 55% (w/w) sulfuric acid containing 0.6% $K_2Cr_2O_3$ (w/w) and heating at 100°C for 10 min. The fraction corresponding to diacetylstearoylglycerol was scraped off, extracted with chloroform, centrifuged at 1200 × *g* for 20 min, and concentrated under nitrogen. Distearoylacetylglycerol was synthesized by the same method as described above using 1,3-distearin and acetic anhydride. The reaction was performed at 145°C for 8 h at atmospheric pressure (9).

For the identification of the structure of diacetylstearoylglycerol and distearoylacetylglycerol prepared by the chemical method, nuclear magnetic resonance (NMR) analyses were performed using the methods discussed by Henderson *et al*. (20). ^{1}H NMR, ^{13}C NMR, and ^{1}H-^{13}C correlated spectroscopy (COSY) spectra were measured in $CDCl_3$ with tetramethylsilane as an internal standard. All NMR experiments were performed on a Bruker AMX FT 500 MHz spectrometer (Bruker, Rheinstetten, Germany) operating at 500 MHz for proton and 126 MHz for carbon. The results of NMR analyses were in good accord with the report of Henderson *et al*. (20) and used to determine the retention times of enzymatically synthesized LCSL in the gas chromatography (GC) chromatogram.

Adjustment of water activity (a_w) *of lipase and substrates.* To obtain a defined initial a_w of substrates and enzyme, triacetin and lipase were equilibrated separately in desiccators containing saturated salt solutions for 7 d at 25°C. For monitoring of the reaction at a defined a_w, a_w of enzyme and substrates should be adjusted to the same value. However, a_w of stearic acid was not controlled because it was barely influenced by water absorption. The salts used were LiCl (a_w = 0.13), K_2CO_3 (a_w = 0.44), $CuCl_2$ (a_w = 0.68), and KCl (a_w = 0.78). A desiccator containing pure water was used for a_w of 1.0. The a_w of substrate and enzyme were measured with a Novasina a_w-measurement instrument (Novasina Co., Pfaffikon, Switzerland).

*Transesterification reaction in open- and closed-reactor system*s. A typical transesterification reaction was performed with 25 mmol triacetin and 30 mmol stearic acid using 0.61 g lipase (4 wt% of substrates) to synthesize the LCSL in a glass vial after pre-equilibration of triacetin and lipase to a predetermined a_w. For an open-reactor system, the enzyme reaction was performed in a glass vial without cap, whereas in a closed-reactor system a glass vial with a Teflon-lined cap was employed. The above-mentioned amount of stearic acid was added to a vial that had been thermally equilibrated to a predetermined temperature controlled by water circulation. After melting of the fatty acid, the reaction was initiated by adding triacetin and lipase, and the reaction mixture was agitated by a magnetic stirrer. Unless otherwise stated, transesterification was performed at initial a_w of 0.78, 400 rpm, and 80°C in an open-reactor system.

To investigate the effect of initial a_w of substrates and enzyme on the synthesis of LCSL, the transesterification reaction was performed at various levels of a_w (0.13, 0.44, 0.68, 0.78, and 0.98) in a closed-reactor system. The other reaction variables (molar ratios of substrates, stirring speed, and reaction temperature) were studied in an open-reactor system.

Transesterification reaction in vacuum-reactor system. For transesterification under reduced pressure, the reaction mixture contained 50 mmol triacetin, 70 mmol stearic acid, and 1.23 g lipase (4 wt% of substrates). In the vacuum-reactor system, enzyme and substrates were not pre-equilibrated to a specific a_w. The reaction was initiated by addition of lipase to a thermally equilibrated reaction flask containing triacetin and stearic acid, and then the reaction flask was immediately connected to a vacuum pump to maintain vacuum at specified value. A typical transesterification reaction was carried out at 80°C and 400 rpm under 700 mm Hg vacuum.

To investigate the optimal water content level in the reaction medium, we added periodically (*ca*. 1 h) various amounts of distilled water (0.33, 0.65, 1.30, 1.95, and 3.25 wt% of substrates) to the reactants.

GC analysis. For time-course analysis, aliquots were taken from the 15- and 30-mL vials, respectively, at predetermined intervals and extracted with chloroform/methanol (2:1, vol/vol) solution. Solid materials were removed by filtration through polytetrafluoroethylene membrane filters. The eluates were analyzed by GC using a Hewlett-Packard Model 5890 Series II gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame-ionization detector and HP 3396 series II integrator (Hewlett-Packard Co.). The column used for the separation was a Quadrex (Quadrex Corp., New Haven, CT) fused-silica capillary column (25 m × 0.25 mm i.d.) with a 0.1-μm film of bonded methyl 65% phenyl silicone. The oven temperature was programmed with an initial temperature of 100°C for 3 min, followed by ramping from 100 to 360°C at 10°C/min and held at 360°C for 5 min and then heated to 365°C at 5°C/min. The detector was maintained at 360°C. On-column injection was used, with the in-

jector operated in the temperature-track mode, which keeps the injector temperature close to the oven temperature. Hydrogen was used as a carrier gas with a constant flow rate operating at 163 kPa. Tridecane was used as an internal standard.

RESULTS AND DISCCUSION

For the synthesis of LCSL (mixture of diacetylstearoylglycerol and distearoylacetylglycerol), 10 commercial lipases [Chirazyme® L-2, Lipozyme IM (*Rhizomucor miehei*), lipase OF (*Candida rugosa*), lipase CES (*Pseudomonas* sp.), lipase R (*Penicillium roqueforti*), lipase PS (*Pseudomonas cepacia*), lipase CE (*Humicola lanuginose*), lipase D (*Rhizopus delemar*), lipase M (*Rhizomucor javanicus*), and lipase AP (*Aspergillus niger*)] were screened for their ability to catalyze the transesterification between triacetin and stearic acid at 70°C in a solvent-free system. Among them, Chirazyme® L-2 showed the highest synthetic activity for the incorporation of stearic acid into triacetin (data not shown). Therefore, Chirazyme® L-2 was used for the subsequent experiments.

Transesterification in open- and closed-reactor system. To compare the synthetic activity of Chirazyme® L-2 in open- and closed-reactor systems, the transesterification reactions in both systems at different initial a_w levels were carried out. In the open-reactor system, spontaneous evaporation of by-product (acetic acid) and water might occur, and the system could be influenced by environmental conditions (e.g., relative humidity). However, in the closed-reactor system, there was no elimination of by-product or water in the reaction mixture, and thus, as reaction proceeded, accumulation of by-product and water could occur. Figure 1 shows the time course of LCSL production in the open- and the closed-reactor systems. The LCSL content after 24 h at an initial a_w of 0.78 was slightly higher than that at any other a_w condition in the closed-reactor system. At initial a_w higher than 0.78, the net reaction rate decreased, presumably due to a predominance of the hydrolysis of triacetin. On the other hand, at initial a_w lower than 0.78, the reaction rate became also slow probably due to the lack of essential water for the enzyme catalytic activity. Based on these results, a_w of 0.78 was determined to be optimal for the closed-reactor system.

In the open-reactor system, LCSL production reached maximum after 6 h reaction, and the content of product was higher than that at a_w of 0.78 in the closed-reactor system for the first 10-h reaction (Fig. 1). Furthermore, the initial reaction rate in the open-reactor system was also faster than that in the closed-reactor system. In our transesterification reaction, acetic acid as well as mono- and di-acetin is formed during the reaction as a by-product. As the acetic acid thus formed increases, the pH of the reaction medium shifts to lower side. Moreover, it may deactivate the enzyme catalytic activity and also affect the reaction equilibrium. In an open-reactor system, however, acetic acid produced from the reaction would be evaporated spontaneously because the reaction was carried out at a relatively high temperature (70– 80°C).

Characterization of positional isomers of LCSL. Time-course variations of positional isomers content and their ratio in the open-reactor system are shown in Table 1. Early in the reaction, the contents of 1,2-diacetyl-3-stearoylglycerol (AAS) and 1,3-distearoyl-2-acetylglycerol (SAS) were higher than that of 1,3-diacetyl-2-stearoylglycerol (ASA) and 2,3-distearoyl-1-acetylglycerol (ASS), respectively. As the reaction proceeded, however, an increase in the selectivity of Chirazyme® L-2 for the *sn*-2 position and acyl migration likely occurred, and consequently the content of *sn*-2 positional isomers (ASA and ASS) increased. The isomeric ratios, R_1 (= AAS/ASA) and R_2 (= SAS/ASS), for enzymatically synthe-

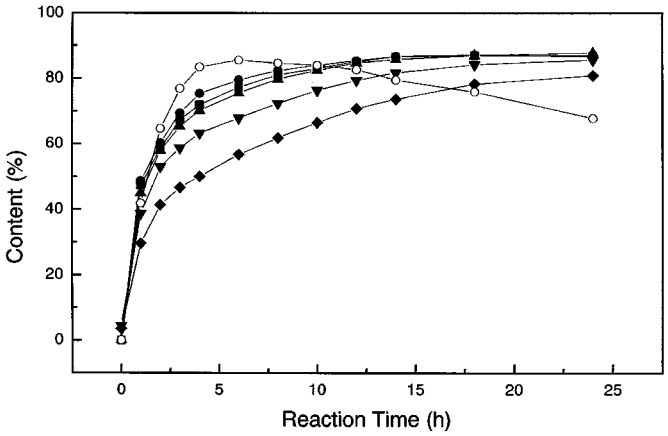

FIG. 1. Time course of low-calorie structured lipids synthesis at different initial water activities. In closed-reactor system; a_w = 0.98 (■), a_w = 0.78 (●), a_w = 0.68 (▲), a_w = 0.44 (▼), and a_w = 0.13 (◆). In open-reactor system; a_w = 0.78 (○). Reaction conditions: molar ratio of triacetin to stearic acid = 1:1.4 (25 mmol/35 mmol), 400 rpm, 80°C, and lipase content = 0.61 g of Chirazyme® L-2. Content = [product area/total triacylglycerol (TG) area] × 100 (%).

TABLE 1
Time-Course Variations of Positional Isomers Content on the Synthesis of Low-Calorie Structured Lipids in Open-Reactor System[a]

	Content (%)				Isomeric ratio[c]	
Time (h)	AAS[b]	ASA	SAS	ASS	R_1	R_2
1	39.5	2.3	0.0	0.0	16.9	—
2	56.4	3.7	4.5	0.0	15.2	—
3	63.4	4.6	7.8	1.1	13.8	6.9
4	63.9	6.9	10.5	2.0	9.2	5.2
6	56.8	6.9	16.5	5.4	8.2	3.1
8	49.1	7.1	19.5	8.9	6.9	2.2
10	39.1	9.3	19.9	15.6	4.2	1.3
12	34.2	9.1	17.1	22.2	3.8	0.8
14	27.4	13.0	16.1	22.8	2.1	0.7
18	20.2	18.0	12.4	25.2	1.1	0.5
24	11.6	19.9	11.1	25.1	0.6	0.4

[a]Molar ratio of triacetin to stearic acid, 1:1.4 (25 mmol/35 mmol); lipase content, 0.61 g of Chirazyme® L-2; stirring speed, 400 rpm; temperature, 80°C; initial a_w of substrates and enzyme, 0.78.
[b]AAS, 1,2-diacetyl-3-stearoylglycerol; ASA, 1,3-diacetyl-2-stearoylglycerol; SAS, 1,3-distearoyl-2-acetylglycerol; ASS, 2,3-distearoyl-1-acetylglycerol.
[c]R_1 and R_2 designate the ratio of AAS/ASA and SAS/ASS, respectively.

sized LCSL were found to be much higher than those of the chemically synthesized LCSL. According to the report of Huang *et al*. (9), R_1 and R_2 for chemically synthesized LCSL were 1.38 and 0.48, respectively, whereas R_1 and R_2 from enzymatic transesterification after 6-h reaction in the open-reactor system were 8.2 and 3.1, respectively. Therefore, in terms of calorie intake, it was concluded that the enzymatically synthesized LCSL should be more effective than chemically synthesized ones.

Effect of molar ratio of substrates. As the amount of stearic acid increased from 15 to 45 mmol, the content of synthesized LCSL increased (Fig. 2). However, unreacted stearic acid in the reaction mixture also increased, which is not desirable in terms of the purity of the final product, and there was no difference in reaction rate between molar reactants ratios of 1:1.4 and 1:1.8. Therefore, we fixed the molar ratio of triacetin to stearic acid at 1:1.4 for the investigation of the effects of other factors on the LCSL synthesis.

Effect of stirring speed. To investigate the effect of stirring speed on the synthesis of LCSL, reactions were performed at 200, 400, and 600 rpm, respectively. As the stirring speed increased from 200 to 600 rpm, synthetic rate increased. After 4-h reaction at 70°C with 200, 400, and 600 rpm, about 60, 69, and 70% LCSL were obtained, respectively, indicating that there was little difference between 400 and 600 rpm. Therefore, 400 rpm was chosen to be optimal stirring speed for the sake of mechanical stability.

Effect of temperature. We carried out the transesterification reaction in a solvent-free system above 70°C, because stearic acid, the acyl donor, melted above 70°C. The time-course variations of the LCSL production were monitored at 70, 75, 80, and 85°C, respectively (Fig. 3). The LCSL content at 70°C increased to about 78% after 6 h and remained constant thereafter, whereas at reaction temperatures above 70°C, the content and synthetic reaction rate were further increased. Acetic acid formed during the reaction might be removed more effectively by spontaneous evaporation at the higher temperature in the open-reactor system. However, as the reaction temperature increased, the content of product tristearin also increased, which made for a decrease in the content of LCSL. Therefore, the LCSL level was increased to over 80% at temperatures above 70°C, and the highest yield (85.5%) was obtained at 80°C after 6 h, which was taken as the optimal temperature.

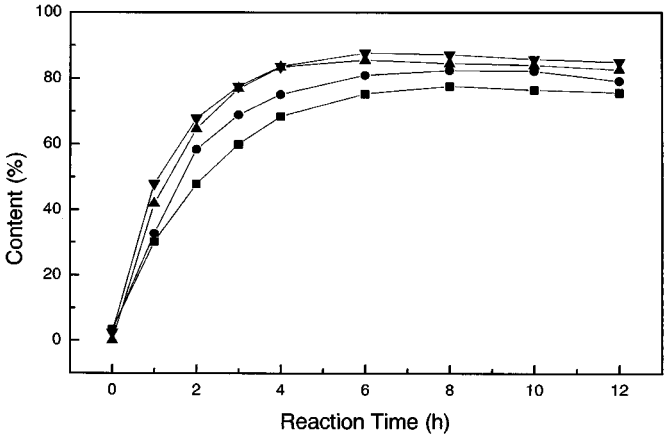

FIG. 2. Time course of low-calorie structured lipids synthesis at different molar ratios of substrates in an open-reactor system. Molar ratio of triacetin to stearic acid: ■, 25 to 15 mmol (1:0.6); ●, 25 to 25 mmol (1:1.0); ▲, 25 to 35 mmol (1:1.4); ▼, 25 to 45 mmol (1:1.8). Reaction conditions: 400 rpm, 80°C, initial a_w of substrates and enzyme = 0.78, and lipase content = 0.61 g of Chirazyme® L-2. Content = (product area/total TG area) × 100 (%). See Figure 1 for abbreviation.

Transesterification in vacuum reactor system. Transesterification for the synthesis of LCSL is a reversible reaction. Thus, the presence of acetic acid affects the reaction equilibrium and enzyme activity. In an open-reactor system, the spontaneous evaporation of acetic acid at higher temperature resulted in an equilibrium shift toward synthesis. However, this evaporation process might not be completely effective for removing acetic acid and we, therefore, applied high vacuum to remove the acetic acid more effectively. The effect of vacuum on the synthesis of LCSL is shown in Figure 4. As compared with open- and closed-reactor systems, the reaction rate was increased.

It is well known that nonaqueous enzyme reactions critically depend on the level of water in the reaction system, and several reports have shown that water has a profound influence on both the yield and rate of reactions (18,21–23). Therefore, maintaining an optimal water level in the reaction system is very important for maximal production of the targeted product. However, in this study, vacuum simultaneously removed water as well as acetic acid from the reaction medium and enzyme because the water has a lower boiling point (100°C) than acetic acid (118°C). Although it is often desired, so as to minimize unfavorable enzyme-catalyzed side reactions, i.e., hydrolysis, to reduce as much water as possible, the water bound to the enzyme plays an important role in determining catalytic properties (23). Therefore, a certain amount of water was periodically added to the reaction mixture during the vacuum application.

As shown in Figure 4, when 700 mm Hg of vacuum was applied and water (0.65 wt% of substrates) was added to reaction system at every hour, the content of LCSL and initial rate of production were higher and faster, respectively, than those with no added water or an open-reactor system. In the vacuum-reactor system, we could obtain 88.3% LCSL (70.2% diacetylstearoylglycerol and 18.1% distearoylacetylglycerol) after 4 h of reaction with 0.65 wt% water addition (Fig. 5), whereas 87.0% LCSL after 8-h reaction without water addition. When water was added at a level higher than 0.65 wt% of substrates, the LCSL production profiles were very similar (data not shown).

LCSL could be very efficiently synthesized by lipase-catalyzed transesterification of triacetin with stearic acid in a solvent-free system. Although the vacuum-reactor system did not seem to be more effective than the open-reactor system with respect to overall production (88.3 vs. 85.5%, respectively), acetic acid, which was formed as a by-product, was collected in the condenser during vacuum application, thus

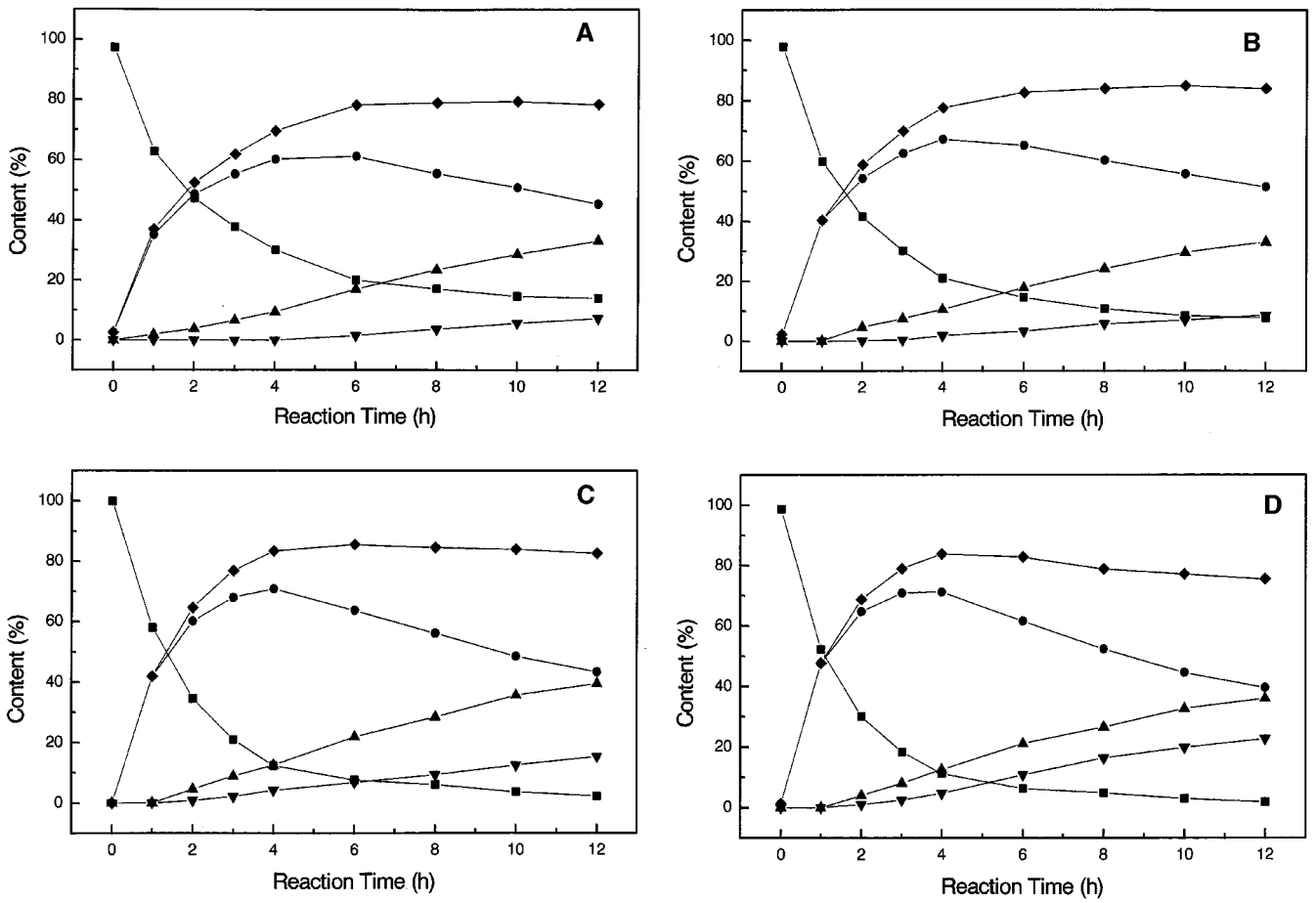

FIG. 3. Time course of low-calorie structured lipids synthesis at different reaction temperatures in an open-reactor system. The content of triacetin (■), diacetylstearoylglycerol (●), distearoylacetylglycerol (▲), tristearin (▼), and low-calorie structured lipids (◆). Reaction conditions: molar ratio of triacetin to stearic acid = 1:1.4 (25 mmol/35 mmol), 400 rpm, initial a_w of substrates and enzyme = 0.78, and lipase content = 0.61 g of Chirazyme® L-2. Content = (product area / total TG area) × 100 (%). See Figure 1 for abbreviation. A, 70°C; B, 75°C; C, 80°C; D, 85°C.

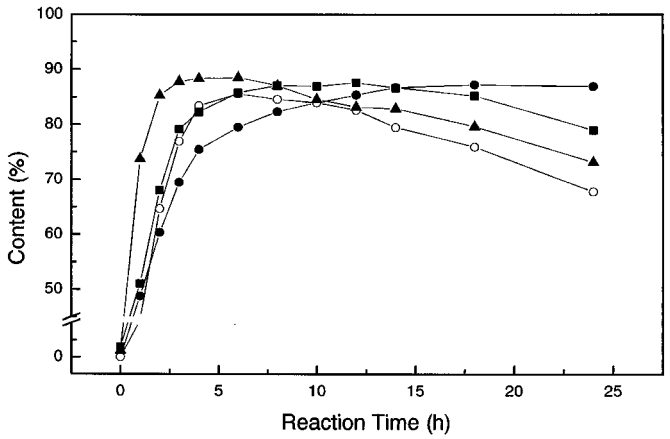

FIG. 4. Effect of water addition during vacuum application on the synthesis of low-calorie structured lipids. ●, closed reactor; ○, open reactor; ■, vacuum reactor without water addition; ▲, vacuum reactor with water addition (0.65 wt% water was added every 1 h to the reaction mixture.). Reaction conditions of vacuum-reactor system: molar ratio of triacetin to stearic acid = 1:1.4 (50 mmol/70 mmol), 400 rpm, 80°C, 700 mmHg vacuum, and lipase content = 1.23 g of Chirazyme® L-2. Content = (product area/total TG area) × 100 (%). See Figure 1 for abbreviation.

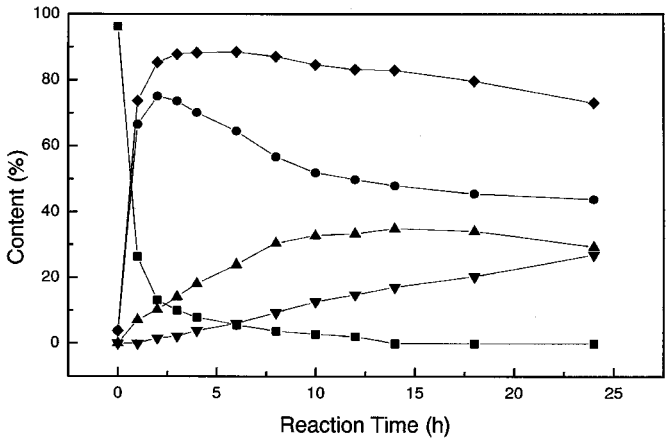

FIG. 5. Time course of low-calorie structured lipids synthesis in a vacuum-reactor system with water addition. The content of triacetin (■), diacetylstearoylglycerol (●), distearoylacetylglycerol (▲), tristearin (▼), and low-calorie structured lipids (◆). 0.65 wt% water was added every 1 h to the reaction mixture. See Figure 4 for reaction condition.

negating the air pollution. Besides, residual acetic acid in the reaction mixture might be negligible and consequently, the purification steps would be reduced. Vacuum application with the simultaneous periodic incorporation of a certain amount of water allowed efficient acetic acid removal and maintenance of the proper water content for maintenance of catalytic activity.

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