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Enzymatic Synthesis of Medium-Chain Triglycerides in a Solvent-Free System

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Abstract

The synthesis of tricaprylin, tricaprin, trilaurin, and trimyristin in a solvent-free system was conducted by mixing a commercial immobilized lipase (Lipozyme IM 20, Novo Nordisk, Bagsvaerd, Denmark) with the organic reactants (glycerol and fatty acids) in a 20-mL batch reactor with constant stirring. In a first set of experiments, the effect of water concentration (0–6%) on the reaction conversion was shown to be negligible. In a second set of experiments, the effects of temperature (70–90°C), fatty acid/glycerol molar ratio (1–5), and enzyme concentration (1–9% [w/w]) on the reaction conversion were determined by the application of a 3×3 experimental design. The reactions were carried out for 26 h and the nonpolar phase was analyzed by gas chromatography (GC). Appreciable levels of medium-chain triglycerides were achieved, except for tricaprylin. For the triglyceride production, higher selectivity was attained under the following conditions: molar ratio of 5, enzyme concentration of 5 or 9% (w/w) and temperatures of 70°C (tricaprin), 80°C (trilaurin), and 90°C (trimyristin). Statistical analysis indicated that the fatty acid/glycerol molar ratio was the most significant variable affecting the synthesis of triglycerides.

Index Entries: Medium-chain triglycerides; immobilized lipase; esterification.

Introduction

There is a growing interest in medium-chain triglycerides (MCTs), which are specialty fats composed primarily of medium carbon-chain fatty acids *(1)*. MCTs' chemical structure affords them unique properties over regular vegetable oils and other food-grade solvents in many food applications. Because MCTs are not metabolized like conventional fats, they are

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excellent energy sources for individuals who have various fat malabsorption conditions and for patients requiring energy-dense diets such as preterm infants and severely malnourished patients *(2,3)*. Medium-chain triglycerides are also used as carriers for flavors, colors, vitamins, and pharmaceuticals.

The commercial manufacturing process of medium-chain glycerides involves the direct esterification of medium-chain fatty acids and glycerol at high temperature and high pressure, followed by alkali washing, steam refining, molecular distillation, ultrafiltration, and activated carbon treatment for the purification of the product *(4,5)*. However, in recent years, the employment of triacylglycerol lipases (EC 3.1.1.3) as biocatalysts for esterification reactions has emerged as a potential route to replace the conventional chemical procedures, which are expensive owing to the extreme conditions required. The milder operating conditions and the possibility of a more efficient process owing to the lipase's selectivity *(6,7)* make the use of these biocatalysts a promising alternative to the conventional chemical procedures.

Recently, some authors *(4,8–12)* have also shown that such reactions could be achieved in a medium solely composed of substrates in the presence of immobilized lipase without any solvent or surfactant. Such a system avoids the problems of separation, toxicity, and flammability of organic solvents, lowering the cost of the final product and permitting recovery of product without further purification or evaporation steps.

Although the use of lipase for the synthesis of medium-chain triglycerides is an area of increasing interest and research, in general there is little information about the selectivity of lipases towards the size of carbon-chain fatty acids and the best conditions under which to perform such reactions.

The aim of this work was to study the effects of the reaction temperature as well as the effect of fatty acid/glycerol molar ratio and enzyme concentration on the synthesis of tricaprylin, tricaprin, trilaurin, and trimyristin in solvent-free media.

Materials and Methods

Materials

Lipozyme IM-20 (*Mucor miehei* lipase immobilized on a weak anion-exchange resin) was a donation from Novo Nordisk A/S (Bagsvaerd, Denmark). All fatty materials (substrates and gas chromatography [GC] standards) were obtained from Sigma (St. Louis, MO). Analytical-grade glycerol, *n*-hexane, ethyl acetate, ethanol, and acetone were purchased from Merck (Darmstadt, Germany).

Nonpolar Phase Analysis

The fatty acid and mono-, di-, and triglycerides studied were analyzed by capillary GC. Each sample of 20 µL was diluted (1000 times) in a 1:1 hexane/ethylacetate mixture and 1 µL was injected into a Chrompack CP

9000 gas chromatograph with a flame ionization detector. The GC was fitted with a 10 m × 0.25 mm × 0.12 µm CP Sil 5CB Column. Helium was used as the carrier gas with a flow rate of 2 mL/min. The detector and injector temperatures were set at 350°C. The column temperature was set at 80°C for 1 min and was then programmed at 20°C/min to 320°C, which was maintained constant for 2 min. For the caprylic acid and caprylic glycerides, after injection of samples, the temperature of the column was kept constant for 1 min (65°C), then linearly increased to 300°C (25°C/min) and kept at this temperature for 2 min. All concentrations were calculated as mole fractions based on the actual number of moles.

Water Content Determination

The water concentration in the reaction media was measured by the titrimetric Fischer method using a Karl Fischer titrator (Metler DL 18).

Determination of Esterification Activity of Lipozyme

Fifteen mmoles of lauric acid and five mmoles of glycerol were mixed vigorously by a magnetic stirrer in a 20-mL batch reactor. The reaction was started by adding 0.17 g of Lipozyme to the reaction mixture, which had been thermally equilibrated to 60°C in a water bath. The moisture produced was allowed to evaporate spontaneously during the reaction. Aliquots (150 µL) were taken from the reaction medium after 40 min and diluted in 20 mL of a 1:1 ethanol/acetone mixture, in which the residual fatty acid was checked by titration against 0.04 *N* NaOH using a Metler DL 21 titrator. One esterification unit of Lipozyme was defined as 1 mmole of lauric acid consumed/min under the experimental conditions described herein.

Esterification Experiments

All experiments were carried out in a 20-mL batch reactor with constant stirring, using a magnetic stirrer. The reactor (jacketed beaker) was kept at the desired temperature by a thermostatic water bath. Given quantities of glycerol and fatty acid were mixed together and preincubated at the desired temperature, followed by the addition of the immobilized lipase. The progress of the reaction was followed by withdrawing 20-µL aliquots at various time intervals, and then analyzing them by the GC method described previously. The water produced by the reaction was spontaneously removed by free evaporation from the reaction medium.

A 3 × 3 experimental design was used to determine the effect of the reaction parameters on the triglyceride synthesis. The following conditions were investigated: Trilaurin and tricaprylin—temperatures of 60, 70, and 80°C; fatty acid/glycerol molar ratios of 1, 3, 5; and enzyme concentration of 1, 5, and 9% (w/w); trimyristin and tricaprin—temperatures of 70, 80, and 90°C. The molar ratios and enzyme concentrations were the same as aforementioned.

Further experiments were performed to study particular conditions arising from the results of the experimental design indicated in Tables 1–4.

Table 1
Experimental Results Obtained in Trilaurin Synthesis

Reactions conditions			Results after 26 h of reaction (molar fraction)				
T (°C)	Molar ratio of lauric acid/glycerol	Enzyme concentration (%)	Lauric acid (%)	Monolaurin (%)	Dilaurin (%)	Trilaurin (%)	Selectivity (%)
60	1	1	29	56	15	0	0
	1	9	11	65	21	3	4
	5	1	76	0	10	14	59
	5	9	76	0	3	21	88
70	3	5	12	0	20	68	77
	3	5	17	0	15	68	82
	3	5	17	0	16	67	81
	3	5	19	0	16	65	80
80	1	1	57	30	11	2	3
	1	9	28	55	15	2	2
	3	5	17	0	11	72	87
	3	9	13	0	12	75	87
	5	1	67	0	10	24	71
	5	5	60	0	0	40	100
	5	9	62	0	0	38	100
	7	9	64	0	0	36	100
90	5	5	59	0	0	41	100
	5	9	60	0	0	40	100
100	5	5	87	10	3	0	0
	5	9	64	0	6	30	83

Table 2

Experimental Results Obtained in Trimyristin Synthesis

Reactions conditions			Results after 26 h of reaction (molar fraction)				
T (°C)	Molar ratio of myristic acid/glycerol	Enzyme concentration (%)	Myristic acid (%)	Monomyristin (%)	Dimyristin (%)	Trimyristin (%)	Selectivity (%)
70	1	1	44	32	22	2	4
	1	9	19	55	23	3	4
	5	1	83	1	0	16	91
	5	9	74	0	2	24	93
80	3	5	14	0	16	70	82
	3	5	14	0	16	70	82
	3	5	21	0	15	64	81
	5	9	73	0	0	27	100
90	1	1	91	4	3	2	23
	1	9	55	25	18	2	4
	5	1	94	3	3	0	0
	5	5	72	0	2	26	93
	5	9	65	0	0	35	100
	7	9	82	0	1	17	93
	7	5	58	0	4	38	91
100	5	5	95	0	4	1	23
	5	9	70	3	2	25	82

Table 3
Experimental results Obtained in Tricaprin Synthesis

Reactions conditions			Results after 26 h of reaction (molar fraction)				
T (°C)	Molar ratio of capric acid/glycerol	Enzyme concentration (%)	Capric acid (%)	Monocaprin (%)	Dicaprin (%)	Tricaprin (%)	Selectivity (%)
60	1	1	52	34	14	0	0
	1	9	23	51	24	2	2
	5	1	74	0	14	12	55
	5	9	72	0	6	22	77
70	1	1	94	5	2	0	0
	1	9	22	56	22	0	0
	3	5	26	0	21	53	71
	3	5	29	0	19	52	74
	5	1	75	0	11	14	56
	5	9	75	0	0	25	100
	1	9	96	4	0	0	0
80	3	5	22	0	23	55	70
	5	1	100	0	0	0	0
	5	9	71	0	2	27	94
90	1	1	87	13	0	0	0
	1	9	90	7	3	0	0
	5	1	92	6	2	0	0
	5	9	92	5	3	0	0

Table 4
Experimental Results Obtained in Tricaprylin Synthesis

Reactions conditions			Results after 26 h of reaction (molar fraction)				
T (°C)	Molar ratio of caprylic acid/glycerol	Enzyme concentration (%)	Caprylic acid (%)	Monocaprylin (%)	Dicaprylin (%)	Tricaprylin (%)	Selectivity (%)
50	3	5	46	8	39	7	14
	3	9	39	8	41	12	20
	5	5	72	0	21	7	26
	5	9	73	0	21	6	22
60	1	1	100	0	0	0	0
	1	9	19	61	20	0	0
	3	5	39	7	43	11	17
	5	1	100	0	0	0	0
	5	5	63	0	18	19	50
	5	9	65	0	15	20	57
70	3	5	100	0	0	0	0
	3	5	96	4	0	0	0
	5	5	100	0	0	0	0
	5	9	97	3	0	0	0
80	1	1	88	12	0	0	0
	1	9	90	10	0	0	0
	5	1	95	5	0	0	0
	5	9	95	5	0	0	0

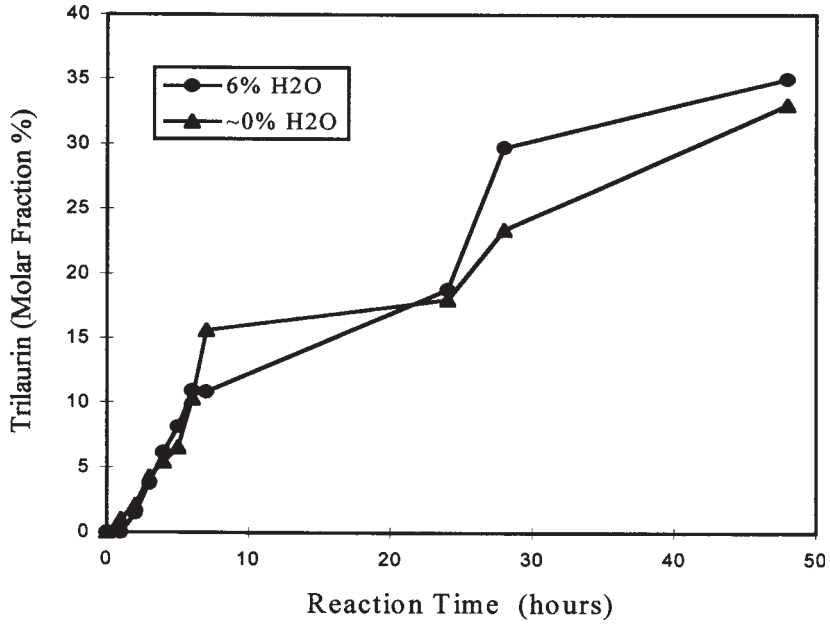

Fig. 1. Effect of initial water content on trilaurin synthesis carried out at 60°C, with lauric acid/glycerol molar ratio of 5 and 9% (w/w) of Lipozyme.

Results and Discussion

Esterification Enzyme Activity

As the amounts of reagents (fatty acid and glycerol) varied in our experiments, it was necessary to normalize the amount of enzyme used. Previous studies on triglycerides synthesis generally have not reported the esterification activity of the enzyme used in the reaction tests. Here, an estimate of esterification enzyme activity was made and it was observed that there is a linear relationship between activity and commercial enzyme weight. Thus, the commercial lipase showed an average esterification activity of 0.03 U/g.

Effect of Water Content

An initial set of experiments was performed at 60°C (the lower temperature investigated) for the synthesis of trilaurin. The results showed that the production of triglyceride was not significantly affected by initial water content in the reaction medium, as illustrated in Fig. 1, which presents the reaction progress curves for two extreme conditions (no water addition and initial water content of 6% [w/w]). Water was efficiently removed from the reaction medium by evaporation, which is favored by heating to 60°C in an open batch reactor. Similar results were reported by Ergan et al. *(8–10)* and Selmi et al. *(11,12)*.

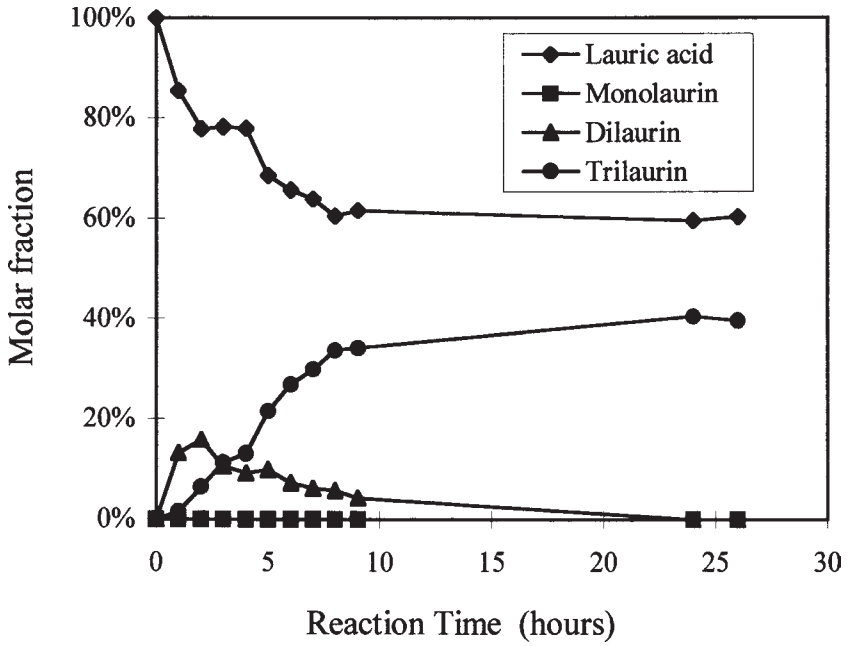

Fig. 2. Time course for trilaurin synthesis carried out at 80°C, with lauric acid/glycerol molar ratio of 5 and 5% (w/w) of Lipozyme.

Triglyceride Synthesis

Experimental results are summarized in Tables 1–4. The results are expressed as molar fraction of the component in the nonpolar phase. The selectivity parameter, chosen to define the best reaction conditions, was defined as the ratio between triglyceride and the total glyceride (mono-, di-, and triglyceride) content in a molar basis.

Trilaurin

Maximum selectivity values were only attained when molar ratios equal to or higher than 5 were used in the experiments conducted at 80°C (Table 1). As the use of a molar ratio of 7 does not promote an appreciable increase on trilaurin production rate, the ratio of 5 was taken as an optimal value. Temperature has also a marked effect on trilaurin synthesis. High selectivity values were only attained at temperatures of 80 and 90°C. At 100°C, enzyme denaturation is severe leading to lower selectivity values. Enzyme concentration affects final selectivity, which increases in the range of 1–5%. However, a further increase on enzyme concentration does not result in a significant selectivity improvement. Figure 2 illustrates the time course for the reaction of lauric acid and glycerol conducted at the selected conditions. Equilibrium is attained after 10 h of reaction. The residual lauric acid may be recovered from the reaction medium by acid-base extraction and reused. In an experiment made with both recovered

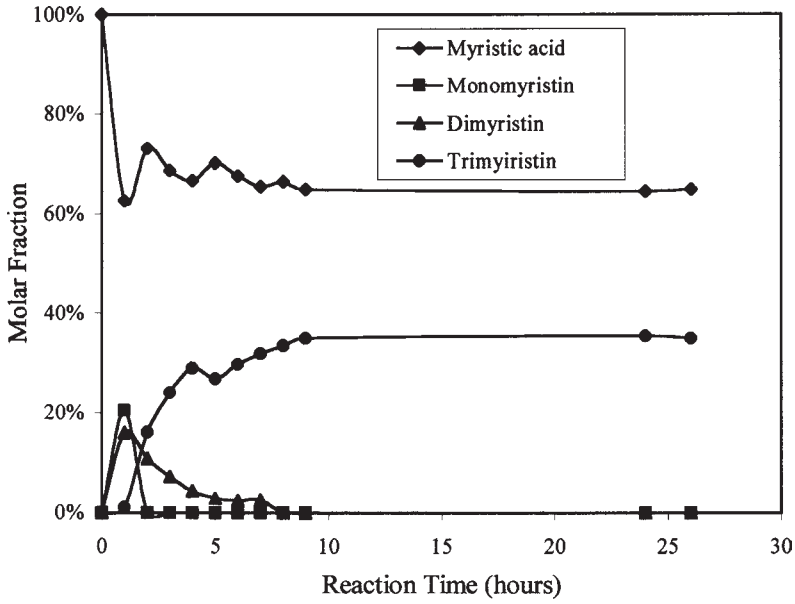

Fig. 3. Time course for trimyristin synthesis carried out at 90°C, with myristic acid/glycerol molar ratio of 5 and 9% (w/w) of Lipozyme.

enzyme and lauric acid, the selectivity reached 95% after 20 h of reaction. That is an important feature for process viability.

Trimyristin

Results shown in Table 2 are very close to those obtained for trilaurin synthesis. The best conditions for trimyristin synthesis were: 90°C, molar ratio of 5, and enzyme concentration of 9% w/w. Figure 3 shows the time course of the synthesis reaction. Reaction equilibrium is attained after 10 h. Utilization of recovered enzyme and myristic acid lead to a selectivity value of 92% after 20 h of reaction.

Tricaprin

Moderate levels of this triglyceride were reached in the set of experiments indicated in Table 3. Although in a few experiments a high selectivity was observed, a large amount of capric acid remained at the end of the reaction, indicating that the reaction conversion was low when compared with trilaurin and trimyristin synthesis reactions. Tricaprin was produced in small amounts by Kim and Rhee *(4)*, using Lipozyme in a similar reaction system. Figure 4 illustrates the results obtained in the experiment that lead to the highest selectivity value for tricaprin synthesis.

Tricaprylin

Table 4 shows data confirming that tricaprylin was not produced in most of the experiments. At 60°C, the molar fraction of tricaprylin in the nonpolar phase was 20%, with a selectivity value of 57%. Selmi et al. *(11,12)*,

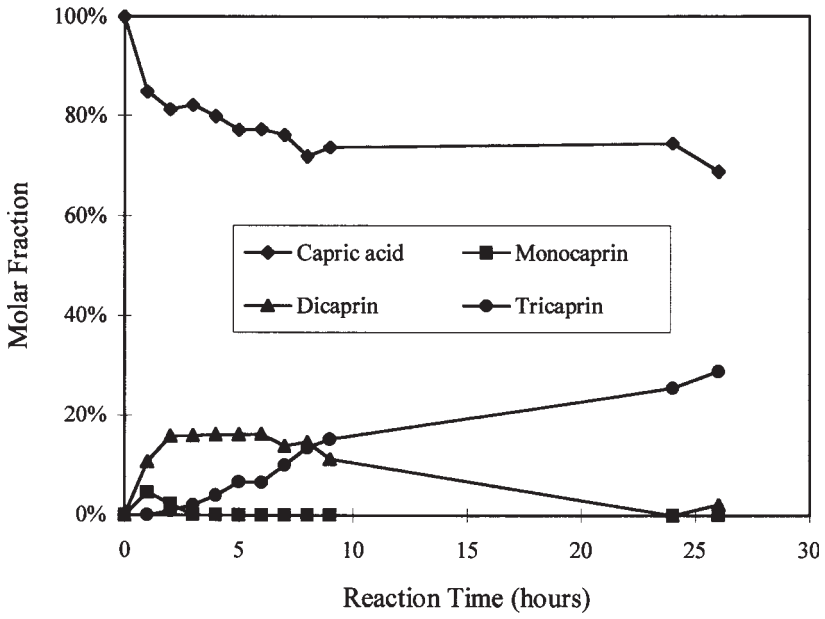

Fig. 4. Time course for tricaprin synthesis carried out at 70°C, with capric acid/glycerol molar ratio of 5 and 9% (w/w) of Lipozyme.

working with a similar reaction system, obtained higher contents of tricaprylin in their experiments.

Experimental Design Results

The results in the experimental design were further analyzed using the software Statistica. Piecewise linear regression (with breakpoint) was employed, and the correlations for trilaurin, trimyristin, and tricaprin are shown here. As the production of tricaprylin was very low, no statistical analysis was performed for that triglyceride.

Trilaurin production (S > 67.8%)

$$S = 0.52\ T + 2.14\ E + 2.32\ MR + 25.72 \quad (1)$$

(regression coefficient $R^2 = 0.993$)

Trimyristin production (S > 62.7%)

$$S = -0.05\ T + 0.21\ E + 2.93\ MR + 79.45 \quad (2)$$

(regression coefficient $R^2 = 0.993$)

Tricaprin production (S > 42.8%)

$$S = 1.22\ T + 4.25\ E + 4.21\ MR - 49.11 \quad (3)$$

(regression coefficient $R^2 = 0.997$)

where S, T, E, and MR represent selectivity, temperature, enzyme concentration, and molar ratio, respectively.

Table 5
Best Conditions for Medium-Chain Triglycerides Synthesis

Triglyceride	Temperature (°C)	Fatty acid/glycerol/ molar ratio	Enzyme concentration (% w/w)
Tricaprylin	60	5	9
Tricaprin	70	5	9
Trilaurin	80	5	5
Trimyristin	90	5	9

The statistical analysis also showed that the variable molar ratio has the most significant effect on selectivity for the three triglycerides produced.

The best conditions for medium-chain triglycerides synthesis are summarized in Table 5. The amounts of triglyceride produced and the optimum temperature increased with the fatty-acid chain length.

In conclusion, the results hereby obtained indicate that the synthesis of trialurin and trimyristin can be performed in a solvent-free medium, attaining high selectivity values. The possibility of enzyme reuse and recovery of residual fatty acid, as observed in this work, is a relevant result that contributes to increase process viability.

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