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Parameters affecting incorporation and by-product formation during the production of structured phospholipids by lipase-catalyzed acidolysis in solvent-free system

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Abstract

By-product formation is a serious problem in the lipase-catalyzed acyl exchange of phospholipids (PL). By-products are formed due to parallel hydrolysis reactions and acyl migration in the reaction system. A clear elucidation of these side reactions is important for practical operation in order to minimize by-products during reaction. In the present study we examined the lipozyme RM IM-catalyzed acidolysis for the production of structured phospholipids between phosphatidylcholine (PC) and caprylic acid in the solvent-free system. A five-factor response surface design was used to evaluate the influence of major factors and their relationships on a number of responses reflecting the turnover of main reactions as well as side reactions. The five factors, including enzyme dosage, reaction time, reaction temperature, substrate ratio (mol/mol caprylic acid/PC) and water addition, were varied at three levels with two star points. All parameters besides water addition had an effect on the incorporation of caprylic acid into PC and lysophosphatidylcholine (LPC). Reaction time and enzyme dosage showed increased effect on incorporation into PC, while substrate ratio and reaction temperature showed opposite effect. The PC content decreased with increase of all parameters except for substrate ratio. Optimal conditions are recommended as enzyme dosage 40%, reaction temperature 55 °C, water addition 1%, reaction time 70 h, and substrate ratio 6 mol/mol caprylic acid/PC. Under these conditions an incorporation of 46% with PC accounting for 53% of the PL fraction can be obtained. Regiospecific analysis of the product revealed that the caprylic acid was mainly incorporated into the sn-1 position accounting for 80% of the fatty acids incorporated.

Keywords: Acidolysis; Solvent-free system; Lipase; Response surface methodology; Structured phospholipids

1. Introduction

Applications of structured phospholipids (PLs) in food, pharmaceuticals and cosmetics have increased interest in lipase-catalyzed interesterification for production of such compounds. Several attempts have been made over the last two decades for the enzymatic acyl exchange of phospholipids, however in general the yield for these reactions has been low [1–3]. Lipase-catalyzed interesterification (acidolysis) is a two-step reaction involving hydrolysis and esterification. Lysophosphatidylcholine (LPC) produced in the first step is reactant in the second step. The amount of LPC in the reaction mixture therefore affects the overall reaction rate. However, LPC also causes acyl migration or the formation of by-products, and as a consequence the formation of LPC decreases the yield and purity of the structured PLs. Acyl migration, a non-enzymatic reaction, is a problem often encountered in selective synthesis of region-specific glycerol-PLs, i.e. intramolecular transfer of one fatty acid moiety from one hydroxyl group to the adjacent one [4]. The intermediate 2-acyl-LPC during the lipase-catalyzed interesterification is less stable than 1-acyl-LPC, and is easily converted into the more stable 1-acyl-LPC by acyl migration, which can be further hydrolyzed by the lipase producing glycerophosphorylcholine (GPC) [5,6]. GPC can then be reacylated with the novel fatty acid in the sn-1 position. If the acyl group migrates from the sn-1 to the sn-2 position the

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lipase has the possibility to incorporate yet another new fatty acid into the sn-1 position, which would give rise to PC with novel fatty acids on both positions. In several studies the recovery of the phospholipid after the reaction have been measured without consideration of the distribution of by-products formed [7,8]. The formation of GPC and the LPC containing the novel fatty acids is a direct consequence of acyl migration and should be minimized in the reaction system in order to have high product yield and high product purity.

For the lipase-catalyzed production of structured PLs, the use of solvents would increase the capital investment when the process is scaled up. Preferably the reaction should be conducted in solvent-free systems. So far only few studies have looked into using solvent-free systems for the production of structured PLs [1,5,9]. Usually the reactions have been conducted with the assistance of organic solvents such as hexane or toluene [10–12]. However it has been reported that lipase-catalyzed acidolysis is faster in solvent-free systems compared to solvent systems [5].

Reaction conditions should be selected with care during lipase-catalyzed acyl exchange of PLs as demonstrated from previous studies [13]. Several parameters important for the main reaction also affect by-product formation resulting in lower recoveries. Usually there is a tendency of decrease in yields with increase in acyl incorporation for these types of reactions. With increasing lipase amount and reaction time the incorporation as well as the hydrolysis increases during lipozyme RM IM-catalyzed acidolysis reaction in solvent-free systems [1,5]. Water has been reported to have a complex role during acidolysis reaction. Varying the water content showed that 5% water addition to the enzyme resulted in the highest incorporation of novel fatty acids; however this water addition also resulted in the highest degree of hydrolysis [5]. Others have reported that water addition had no influence on the incorporation of novel fatty acids during lipase-catalyzed acidolysis reactions [9]. Substrate ratio and reaction temperature are other important parameters during the reactions [9,14]. It was claimed that too high temperature resulted in deactivation of the enzyme, and too high substrate ratios caused substrate inhibition in solvent-free systems [9].

In this study, the incorporation of caprylic acid into PC and LPC together with the PL distribution was determined for the lipase-catalyzed acidolysis reaction between soybean PC and caprylic acid using a solvent-free system with lipozyme RM IM as catalyst. The parameters examined for their effects on incorporation and PL distribution were enzyme dosage, reaction time, reaction temperature, water addition, and substrate ratio (mol/mol caprylic acid/PC). Response surface methodology (RSM) was used to minimize the numbers of experiments. The objective of the study is to optimize a practical reaction system for lipase catalyzed acyl exchange of PC using the solvent-free system, and to have a clear elucidation of the by-product formation.

2. Materials and methods

2.1. Materials

Soybean PC (Epikuron 200, purity 93%) was obtained from Degussa Texturant Systems Deutchland GmbH & Co. KG (Hamburg, Germany). The fatty acid composition (mol%) of PC can be seen in Table 1. Caprylic acid (C8:0, purity 97%) was purchased form Riedel-de-Haen (Seelze, Germany). Lipozyme RM IM, an immobilized sn-1,3 specific lipase from *Rhizomucor miehei* and *Lecitase Novo* (phospholipase A_1), was donated by Novozymes A/S (Bagsvaerd, Denmark). *Crotalus adamenteus* snake venom (phospholipase A_2) was purchased from Sigma (St. Louis, MO). All solvents and chemicals used were of analytical grade.

2.2. Acidolysis reaction

Reactions between soybean PC and caprylic acid were carried out using a 10 g reaction mixture in a brown flask with tight screw cap. Reactions were conducted in a water bath with magnetic stirring at 300 rpm and reaction was started by the addition of lipase (wt.% based on total substrates). After reaction the samples were centrifuged at 4000 rpm for 5 min, and the supernatants were collected. All samples were stored at −20 °C prior to analysis.

Table 1
Fatty acid distribution in PC and structured PC (mol%)

Fatty acids	Soybean PC				Structured PC[c]			
	Direct analysis	sn-1 position (mol%)[a]	sn-2 position (mol%)[b]	Total calcd from sn-1 and sn-2	Direct analysis	sn-1 position (mol%)[a]	sn-2 position (mol%)[b]	Total calcd from sn-1 and sn-2
8:0	0.0	0.0	0.0	0	46.3	71.9	18.0	44.9
16:0	12.8	24.4	1.5	12.9	3.4	5.6	2.5	4.1
18:0	3.9	6.7	0.5	3.6	0.8	1.6	0.7	1.2
18:1	9.4	8.6	13.1	10.8	6.3	3.1	10.3	6.7
18:2	65.8	53.0	77.8	65.4	39.0	15.7	62.1	38.9
18:3	8.1	7.3	7.1	7.2	4.2	2.0	6.4	4.2

[a] The fatty acid composition (mol%) at the sn-1 position after enzymatic hydrolysis with snake venom.
[b] The fatty acid composition (mol%) at the sn-2 position after enzymatic hydrolysis with Lecitase Novo.
[c] Structured PC produced under optimal conditions (for details see exp 12 in Table 2).

2.3. Plate thin-layer chromatography (TLC)

Analytical separations were performed on Silica Gel 60 thin-layer plates (20 cm × 20 cm, Merck, Darmstadt, Germany). After development in chloroform–methanol–water (65:35:5, v/v), the plate was sprayed with 0.2% of 2,7-dichloroflourescein in ethanol (96%), making the lipid bands visible under UV-light. The lipid bands were scraped off, and methylated for analysis by GC.

2.4. Methylation of phospholipid species

The scrapings from TLC were transferred to test tubes with tight screw caps. One milliliter 0.5 M NaOH in methanol were added to each tube and placed at 80 °C for 5 min. Then 1 ml 20% BF_3 in methanol and 0.5 ml 0.5% hydroquinone in methanol were added and placed at 80 °C for 2 min. Two milliliters of 0.73% NaCl solution was added and subsequently 1 ml heptane. The upper phase was transferred to a new tube. One milliliter of saturated salt solutions was added to the new tube. After mixing and phase separation the upper phase was taken for GC analysis.

2.5. GC analysis

The methyl esters were analyzed on a HP6890 series gas–liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a flame-ionization detector (FID), as described elsewhere [9].

2.6. Phospholipase hydrolysis of phospholipids

Caprylic acid-enriched PC was isolated on the TLC plates as described above, extracted with Chloroform-Methanol-water (20:10:0.5, v/v), and dried in a rotary evaporator. Water was removed by adding acetone during evaporation. Isolated PC was hydrolyzed to LPC with Lecitase Novo to remove the fatty acids at the sn-1 or with *Crotalus adamenteus* snake venom for the sn-2 position. A 2.5 mg portion of PC was dissolved in diethyl ether (2 ml) and incubated with 10 μl Lecitase Novo dissolved in 0.1 ml of water or 2.5 mg snake venom dissolved in 0.1 ml 10 mM Tris buffer (pH 8.0) containing 10 mM $CaCl_2$. After shaking vigorously for 5 min, the mixtures were washed into conical flasks with methanol (10 ml) and chloroform (20 ml), and the solution was dried over anhydrous sodium sulfate. The mixture was filtrated, dried and applied to TLC plates. The solvent system used to separate LPC from the other constituents was the same as described above.

2.7. Analysis of phospholipid profile by TLC–FID

One microliter of diluted sample were spotted to Chromarod SIII (Iatron Laboratories Inc.; Tokyo, Japan) and developed in a mixture of chloroform–methanol–water (45:20:2, v/v/v). After the development, chromarods were dried at 120 °C for 5 min, and PL species (PC, LPC and GPC) were analyzed by TLC coupled to a flame-ionization detector (TLC–FID) (Iatroscan MK6s, Iatron Laboratories; Tokyo, Japan). Flow rates of 200 ml/min for air and 160 ml/min for hydrogen were used during analysis. Peaks were identified by external standards. From GC-analysis the average molecular weight of PC and LPC were calculated in order to recalculate the TLC–FID data into molar distribution.

2.8. Viscosity measurements

Viscosity was carried out using a concentric cylinder bob cup CC25 measuring system by Stresstech rheometer (Version 3.8, Reologica Instruments AB, Sweden). A constant temperature of 50 °C was maintained during the measurements with a circulatory water bath. Shear stress was increased progressively from 0.5 up to 300 Pa in 20 logarithmic steps with continuous upward sweep direction. The viscosity was determined as the slope of shear stress versus shear rate curve.

2.9. Experimental design and statistical analysis

Experiments were conducted using a central composite design to investigate the linear, quadratic, and cross-product effects of five factors, each varied at five levels and also includes three center points for replication. The five factors chosen were enzyme dosage (E_d, wt.% based on substrate), reaction temperature (T_e, °C), water addition (W_a, wt.% based on total substrate), reaction time (T_i, h) and substrate ratio (S_r, mol/mol caprylic acid/PC). The design of the experiments employed is presented in Table 2. A software package (Modde 6.0, Umetri, Umeå, Sweden) was used to fit the second-order model to the independent variables. Where it was possible, the model was simplified by dropping terms which were not statistically significant ($P > 0.05$) by analysis of variance. The coefficient of determination (R^2) and the lack-of-fit test were used to determine whether the constructed model was adequate to describe the observed data. For process factors the main effect plot displays the predicted changes in the responses when factor varies from low to its high level, all other factors in the design being on their average.

3. Results and discussions

3.1. Model fitting

It has previously been demonstrated that LPC containing the fatty acids to be incorporated into PC was observed in the products during lipase catalyzed acidolysis reactions [5,16]. This is related to the acyl migration in the system. Therefore the amount of such LPC will indirectly indicate the extent of acyl migration. In the present study RSM was used to evaluate the effects of enzyme dosage, reaction temperature, water

Table 2
Actual experimental settings of the factors and the responses

Exp number	Factors					Incorporation of caprylic acid (mol%)		PL distribution (mol%)		
	E_d	T_e	W_a	T_i	S_r	PC	LPC	PC	LPC	GPC
1	20	45	1	30	12	12.6	28.7	76.7	11.9	11.4
2	40	45	1	30	6	31.2	46.5	69.5	18.5	12.0
3	20	55	1	30	6	28.2	50.4	67.4	21.5	11.1
4	40	55	1	30	12	27.1	61.9	66.8	18.9	14.3
5	20	45	3	30	6	36.6	34.5	57.0	26.6	16.4
6	40	45	3	30	12	23.9	48.4	59.6	22.8	17.5
7	20	55	3	30	12	8.5	47.3	65.9	17.9	16.3
8	40	55	3	30	6	33.0	65.9	43.0	32.3	24.7
9	20	45	1	70	6	30.6	54.8	65.2	24.8	10.0
10	40	45	1	70	12	38.2	66.1	58.2	21.8	20.1
11	20	55	1	70	12	15.8	60.3	71.4	16.4	12.2
12	40	55	1	70	6	46.3	66.2	53.2	29.9	16.9
13	20	45	3	70	12	22.3	67.7	49.8	27.0	23.2
14	40	45	3	70	6	19.5	47.0	47.0	24.8	28.2
15	20	55	3	70	6	25.4	64.3	42.9	28.1	29.0
16	40	55	3	70	12	27.6	75.9	41.6	25.6	32.8
17	10	50	2	50	9	16.7	39.9	69.1	16.9	14.1
18	50	50	2	50	9	32.6	60.6	54.5	20.7	24.8
19	30	40	2	50	9	35.3	53.1	57.6	23.5	18.9
20	30	60	2	50	9	19.7	78.2	40.3	22.6	37.2
21	30	50	0	50	9	28.9	59.1	80.6	10.9	8.5
22	30	50	4	50	9	33.2	58.8	37.0	24.3	38.7
23	30	50	2	10	9	10.7	24.4	74.2	15.1	10.7
24	30	50	2	90	9	34.3	74.2	41.5	31.8	26.7
25	30	50	2	50	3	31.1	46.0	49.3	31.4	19.4
26	30	50	2	50	15	22.7	68.0	54.4	18.9	26.7
27	30	50	2	50	9	30.5	58.0	52.6	27.2	20.1
28	30	50	2	50	9	28.5	63.2	53.4	24.9	21.7
29	30	50	2	50	9	31.7	64.4	57.3	25.5	17.2

Abbreviations: E_d, enzyme dosage (wt.% based on substrate); T_e, reaction temperature (°C); W_a, water addition (wt.% based on total substrate); T_i, reaction time (h); S_r, substrate ratio (mol/mol caprylic acid/PC).

content, molar ratio of reactants, and reaction time on incorporation of caprylic acid into PC as well as the existence of caprylic acid in LPC. Additionally the PL species distribution was examined in order to understand how the parameters influence on the product recovery or by-product formation. The best-fitting quadric models by multiple regression and backward elimination were determined. The observed and predicted values were sufficiently correlated except for no.

Table 3
Regression coefficients and significance (P) values of the second-order polynomials after backward elimination

Term	Incorporation of caprylic acid (mol%)				PL distribution (mol%)					
	PC		LPC		PC		LPC		GPC	
	Regression coefficient	P	Regression coefficient	P	Regression coefficient	P	Regression coefficient	P	Regression coefficient	P
Constant	28.06	2.31×10^{-11}	61.2	2.45×10^{-14}	52.51	8.85×10^{-14}	25.07	6.72×10^{-14}	22.42	9.19×10^{-9}
E_d	5.51	9.47×10^{-6}	5.88	5.74×10^{-5}	−4.20	7.06×10^{-4}	1.72	1.94×10^{-3}	2.48	0.03
T_e	−2.84	3.2×10^{-3}	4.96	2.75×10^{-4}	−2.12	0.04	−0.11	0.80	2.24	0.05
W_a	0.37	0.64	1.89	0.08	−9.32	2.35×10^{-7}	3.40	3.67×10^{-6}	5.91	6.20×10^{-5}
T_i	4.39	8.99×10^{-5}	10.33	1.31×10^{-7}	−6.53	1.18×10^{-5}	3.11	9.45×10^{-6}	3.41	5.27×10^{-3}
S_r	−2.41	9.2×10^{-3}	4.19	1.11×10^{-3}	1.69	0.10	−2.32	1.66×10^{-4}	0.64	0.54
$E_d \times E_d$	−1.25	0.10	−3.03	5.19×10^{-3}	2.64	8.71×10^{-3}	−1.37	4.45×10^{-3}	−1.27	0.19
$W_a \times W_a$	0.35	0.63	−0.85	0.37	1.90	0.05	−1.67	1.12×10^{-3}	−0.23	0.81
$T_i \times T_i$	−1.80	0.03	−3.26	3.18×10^{-3}	1.65	0.08	−0.21	0.62	−1.45	0.14
$E_d \times T_e$	4.95	2.4×10^{-4}	3.45	0.02	−2.69	0.04	2.41	8.34×10^{-4}	0.28	0.83
$E_d \times W_a$	−4.87	2.8×10^{-4}	−3.32	0.02	1.44	0.25	−1.36	0.03	−0.08	0.95
$E_d \times T_i$	−1.59	0.13	−5.23	1.16×10^{-3}	0.82	0.50	−1.39	0.03	0.57	0.66
$T_e \times W_a$	1.32	0.21	2.68	0.05	−1.49	0.24	0.39	0.50	1.10	0.41
$T_e \times T_i$	2.86	0.01	−0.38	0.77	−0.37	0.76	0.27	0.64	0.10	0.94
$W_a \times T_i$	−4.55	4.9×10^{-4}	−1.94	0.15	0.16	0.90	−1.84	5.93×10^{-3}	1.67	0.21

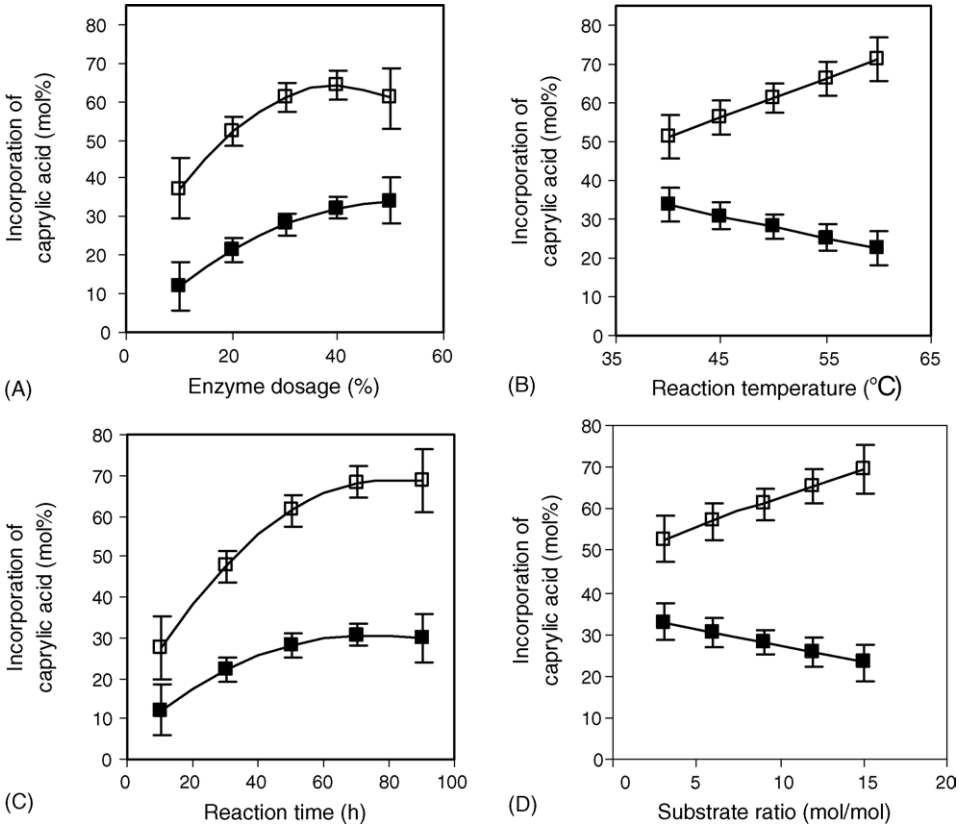

Fig. 1. Main effects of parameters on the incorporation of caprylic acid catalyzed by lipozyme RM IM in solvent-free system (■) PC and (□) LPC. (A) Enzyme dosage, (B) reaction temperature, (C) reaction time, and (D) substrate ratio.

3, which was treated as an outlier. The statistics for the model coefficients and probability (*P*) values for the response variables were calculated (Table 3). The coefficients of determination (R^2) of the models were 0.92, 0.94, 0.93, 0.94, and 0.83 for the five responses, i.e. caprylic acid incorporation into PC, caprylic acid existence in LPC, PC content, LPC content and GPC content, respectively. According to the analysis of variance there was no lack of fit for all the models.

3.2. Main effects of parameters on incorporation

Plots of main effects can be used to evaluate the major influence of parameters (Figs. 1 and 2). All parameters showed to have an effect on either the incorporation of caprylic acid or the PL distribution. In order to have a practical operation system, some compromises have to be made for the different parameters since some of them not only have a beneficial effect on the incorporation into PC, but also lead to lower yields.

3.2.1. Enzyme dosage

Enzyme dosage had the most significant effect on the incorporation into PC. Incorporation into PC increased for increasing enzyme dosage (Fig. 1A). It has been reported that high enzyme dosages are needed for effective incorporation of novel fatty acids into PLs by acidolysis in solvent-free system [1,5]. The use of high enzyme loads however gives problems with agitation and decrease the mass transfer. Even though the increased enzyme load has beneficial effect on the incorporation into PC it also results in increased existence of caprylic acid in LPC. A compromise has to be made since increased enzyme concentrations not only favour incorporation into PC, but LPC as well. With increasing enzyme dosage the content of PC decreased whereas the content of LPC and GPC increased (Fig. 2A). Only few lipases are commercially available in the immobilized form. Lipozyme RM IM is the most commonly used enzyme for the lipase-catalyzed production of structured PLs [1,5,7,10]. Lipozyme RM IM uses anion exchange resin as lipase carrier. This type of carrier can catalyze acyl migration in the reaction system [16]. With the lipase from *Rhizopus oryzae* immobilized on polypropylene support no incorporation of acyl donor into LPC was observed [16]. It seems that acyl migration could be affected by enzyme carriers under the issue of enzyme dosage.

3.2.2. Reaction temperature

The effect of the temperature in solvent-free systems has received very little attention. Commonly the temperature has been kept at 60 °C in order to decrease viscosity of the reaction mixture [1,5]. Previous study performed at our lab has shown that the incorporation of caprylic acid into soybean lecithin using lipozyme TL IM, a silica granulated

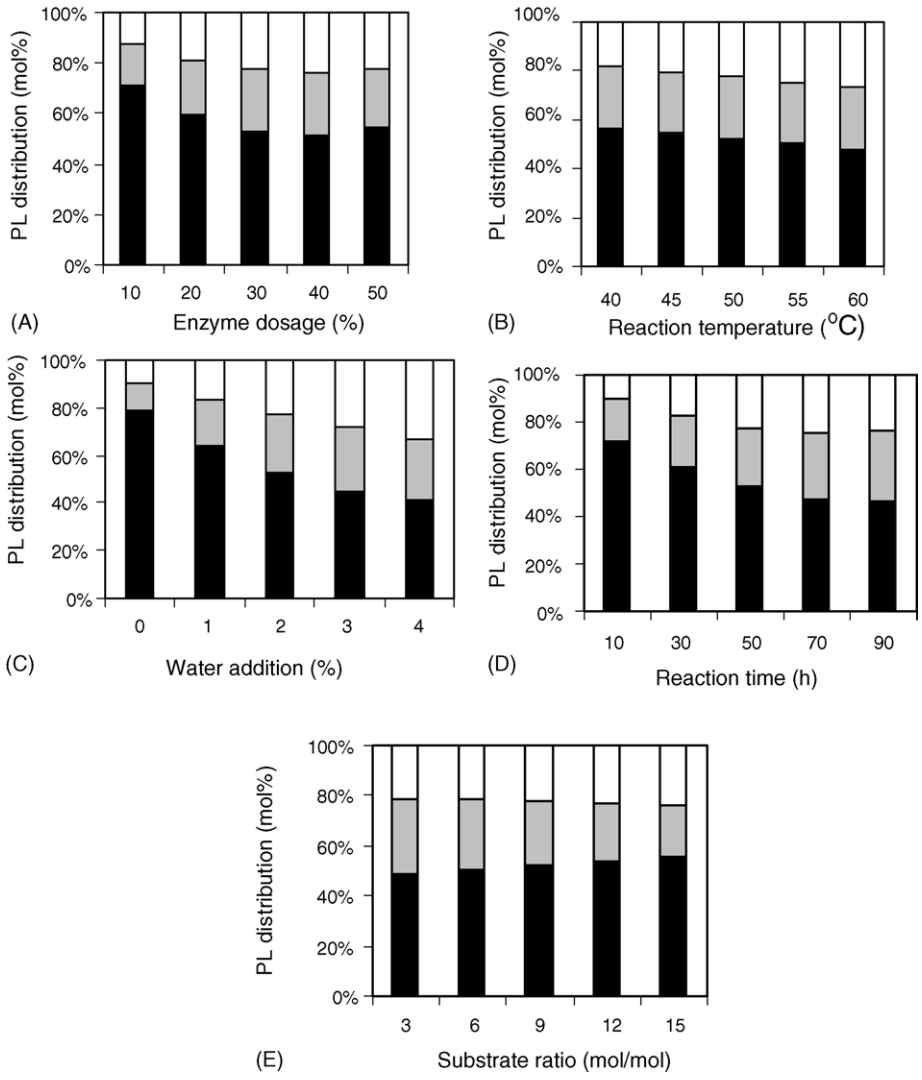

Fig. 2. Main effects of parameters on PL distribution during lipozyme RM IM catalyzed acidolysis reaction between PC and caprylic acid (■) PC, (▒) LPC, and (□) GPC. (A) Enzyme dosage, (B) reaction temperature, (C) water addition, (D) reaction time, and (E) substrate ratio.

Thermomyces lanuginosa lipase, as catalyst had maximum performance at 57 °C [9]. From synthetic reaction using phospholipase A_2 (PLA_2) as catalyst it is known that elevated temperatures resulted in increased acyl migration and by-product formation [15]. It was reported that acyl migration was not observed at 25 °C. In this study it was observed that higher temperature individually decreased the PC content and incorporation of caprylic acid into PC (Figs. 1B and 2B). Reaction temperature did not influence the formation of LPC; however it had significant effect on the formation of GPC. In addition with the increase in temperature the incorporation of caprylic acid into LPC also increased. It is therefore best to apply temperatures at the low levels.

3.2.3. Water content

In this study the water content had no significant influence on the incorporation into PC and LPC. Of the parameters studied, water addition however had the most significant effect on formation of LPC and GPC (Fig. 2C). Increased water addition resulted in lower PC content and corresponding increase in LPC and GPC formation. It seems that excess water may act exclusively as a nucleophilic substrate for the hydrolysis rather than the esterification of desired fatty acids. Therefore water content is crucial for the optimization of the acidolysis reaction in terms of yield. Others have reported that the water content had significant influence on both incorporation and the yield. Haraldsson and Thorarensen reported that 5% water addition resulted in the highest incorporation into both PC and LPC; both also gave the highest degree of hydrolysis [5]. Aura et al. reported that the minimal water content of the reaction mixture for incorporation of novel fatty acids into soybean PL by lipozyme RM IM was below 0.5% (w/w) based on substrate [1]. The incorporation and degree of hydrolysis was not greatly influenced by the amount of water in the range 0.5–2.5%. Similar for the lipozyme TL IM-catalyzed acidolysis, the incorporation was not influenced by addition of

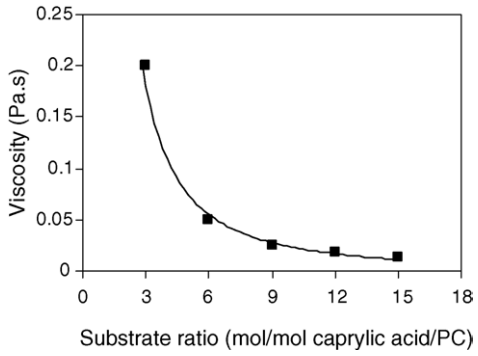

Fig. 3. Viscosity of the initial reaction mixture at different substrate ratios (mol/mol caprylic acid/PC). Two percent of water was added to substrate material. Measurements were conducted at 50 °C without enzyme addition.

1–5% water based on enzyme (0.2–1% based on total substrate) [9]. In reaction mixture with toluene as solvent it was observed that increased water activity increased hydrolysis reaction rate to a greater extent compared to the synthesis reaction rate. Water seems to have a complex role in terms of compromising the lipase activity, hydrolysis side reactions, reaction rate, and extent of incorporation. In order to have a high productivity it is however recommended that the water content should be low.

3.2.4. Reaction time

Usually there is an increase in incorporation of new fatty acids into both PC and LPC during progress in reaction time [5]. Increasing reaction time also results in higher degree of hydrolysis. Similar results were obtained in this study. Reaction time was the parameter having most significant effect on the incorporation of caprylic acid into LPC (Fig. 1C). The formation of LPC was higher compared to that of GPC with increasing reaction time (Fig. 2D). A compromise is also needed for the reaction time since it has positive effect on the incorporation of caprylic acid into PC, however it also results in higher by-product formation.

3.2.5. Substrate ratio

Increasing fatty acid concentration increased yield both for esterification and transesterification reactions [6]. Reaction rates for esterification reactions were independent of the fatty acid concentration. However, during transesterification, the reaction rates increased with increasing fatty acid concentration. Decreased reaction rates were thought to be caused by increased polarity of the reaction medium upon addition of fatty acids. Decreased reaction rates have also been reported during the PLA_2-catalyzed esterification reactions with increasing amounts of fatty acids, and were speculated to be caused by changes in polarity or viscosity [15].

In order to determine if the viscosity had any relationship with reaction rate the viscosity of the initial substrate materials at different substrate ratios were measured (Fig. 3). It was observed that the viscosity decreased with increasing substrate ratio. With higher substrate ratios the mass transfer would expect to increase due to the decrease in viscosity and thus resulting in higher reaction rates. However it was observed that the incorporation into PC decreased with increasing substrate ratio (Fig. 1D), and therefore mass transfer limitations do not seem to be the explanation for the decrease in reaction rate. The incorporation of caprylic acid increased for LPC with increasing substrate ratio, which illustrates that acyl migration probably increases with increasing substrate ratio.

In theory the product yield under reaction equilibrium during acidolysis is determined by the substrate ratio. The maximum incorporation of acyl donors can be calculated at certain substrate ratios assuming no by-product formation and acyl migration. The equation is given below:

$$\ln c_{\max}\,(\text{mol\%}) = 50\frac{S_r}{S_r + 1} \quad (1)$$

Theoretical maximum of new fatty acids to be incorporated into PC is expected to reach 50% for the sn-1,3 specific lipase. Theoretically having substrate 3–15 mol/mol will result in conversion of 75–94% (incorporation of 38–47 mol% based on total PL). Higher substrate ratios will in theory result in higher incorporation of acyl donors. The LPC content in the reaction system generally decreased with increasing substrate ratio, whereas GPC was not affected (Fig. 2E). A compromise therefore has to be made concerning the substrate ratio, even though incorporation of novel fatty acids decreases with increasing substrate ratio, the yield increases.

3.3. Optimization of reaction system

The most important responses for the optimization of the process are the incorporation into PC and PC content. Optimization with these two related responses and five variables cannot be calculated mathematically as more than one optimum condition may exist. Possible optimum conditions, however, can be iteratively calculated in the set ranges and target responses of incorporation into PC and PC content (mol%). The best way to evaluate the relationships between responses and parameters and interactions that exist herein is to analyze the contour plots (Fig. 4). The tendency being the same for parameters as those discussed above. The optimal conditions were generated by the optimizer function of the software with interactive calculation within the low and high level of parameters studied (star points not included). The general conditions for optimal production were enzyme dosage 40%, reaction temperature 55 °C, water addition 1%, reaction time 70 h, and substrate ratio 6 mol/mol. Under these conditions, an incorporation of caprylic acid into PC up to 49% with PC accounting for 51% of the PL fraction can be obtained from the prediction. From Table 2 it can be observed that experiment no. 12 has the settings that are predicted to be the optimal conditions. Regiospecific analysis was performed on this sample (see Table 1). As could be observed most of the caprylic acid was found on the sn-1 position, accounting for 80% of the fatty acid incorporated. Due to acyl migration

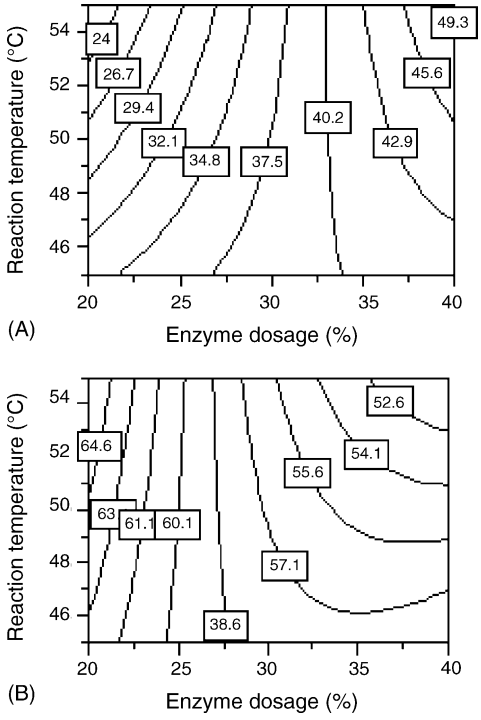

Fig. 4. Contour plots between enzyme dosage and reaction temperature for (A) incorporation of caprylic acid into PC and (B) PC content. Other conditions were as follows: water addition 1%, substrate ratio 6 mol/mol and reaction time 70 h.

caprylic acid could also be observed in the sn-2 position as well.

4. Conclusion

The quadric response models satisfactorily expressed the incorporation of caprylic acid and PL distribution in lipase-catalyzed acidolysis between PC and caprylic acid regarding enzyme dosage, reaction time, reaction temperature, substrate ratio and water content in the batch reactor. Several compromises have to be made in order to have high product yield since many of the parameters favouring acyl incorporation also results in increased hydrolysis and acyl migration in the reaction system. Increased temperature and substrate ratio decreased incorporation into PC, but increased the incorporation into LPC. Increasing all other parameters except for water, however, increased incorporation into both PC and LPC. With an increase of parameters there was seen a decrease in PC content, except for substrate ratio with no individual effect. According to the optimization, it is possible to obtain 49% incorporation of caprylic acid into PC with PC accounting for 51 mol% of the PL fraction by using 40% enzyme, 70 h reaction time, 55 °C temperature for reaction mixture with substrate ratio of 6 mol/mol caprylic acid/PC in the solvent-free system. Regiospecific analysis of structured PC produced under optimal conditions revealed that caprylic acid was not exclusively incorporated into the sn-1 position. Twenty percent of the caprylic acid incorporated could be found in the sn-2 position.

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