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ORIGINAL PAPER

Synthesis of Structured Phosphatidylcholine Containing n-3 PUFA Residues via Acidolysis Mediated by Immobilized Phospholipase A_1

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Abstract Structured phosphatidylcholine (PC) was successfully synthesized by acidolysis of PC derived from soybean with n-3 polyunsaturated fatty acids (PUFA) obtained from fish oil using an immobilized phospholipase-A_1 from *Thermomyces lanuginosus*/*Fusarium oxysporum* as the biocatalyst. The effect of water activity in the range from 0.43 to 0.93 on n-3 PUFA residue content was investigated. The immobilized phospholipase was more active at water activities greater than 0.53. The yield of PC decreased as the water activity increased from 0.43 to 0.93. The yield of lysophosphatidylcholine (LPC) increased significantly during the first 4 h of reaction as a consequence of hydrolysis of the PC. As the enzyme loading increased, the proportion of n-3 PUFA residues in the PC and LPC present at various times in the mixture of these species increased to as much as 15% of the total weight of substrate. For all enzyme loadings tested, both the proportion of n-3 PUFA residues in the total PC (unreacted and modified) and the production of LPC increased significantly when the reaction time was increased, although differences in n-3 PUFA content were observed. When both the n-3 PUFA content of the PC and the total yield of PC are considered, the optimum water activity and enzyme loading for production of structured PC and LPC are 0.65 and 15%, respectively.

Keywords Fish oil · Lysophosphatidylcholine · Phospholipase A_1 · Phosphatidylcholine · Water activity

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Introduction

Phospholipids (PL) are major constituents of cell membranes and play essential roles in the biochemistry and physiology of cells [1]. Some oilseeds and egg yolks are particularly rich in PL. These substances are widely used in the food, pharmaceutical, and cosmetic products industries in whose products these materials function as emulsifiers, stabilizers, and antioxidants [2, 3]. Fatty acids could be more easily absorbed in the body as PL than as the corresponding triglycerides or ethyl esters [4]. Interest in the production of structured PL containing specific fatty acid residues at particular positions on the glycerol backbone of the PL has increased significantly in recent years. Replacement of fatty acid residues present in a native PL by fatty acids with beneficial physiological effects leads to tailored PL which represent an intriguing marketing opportunity for manufacturers of nutraceuticals. In particular, incorporation of n-3 PUFA residues into PL is of great interest because of the possibilities for use of these products for medical applications [5, 6]. Lemaitre-Delaunay et al. [7] have provided evidence for higher bioavailability of DHA for incorporation into erythrocytes in human adults when the DHA was provided in PL rather than as triacylglycerol (TAG) residues. Wijendran et al. [8] also reported that PL were about 2.1-fold more effective than TAG as substrates for accretion of brain arachidonic acid in the development of the brains of neonatal primates.

n-3 Polyunsaturated fatty acid (PUFA) residues such as those from eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, are present at high levels in fish oils. In recent decades these substances have received significant attention from the nutrition community because of the health benefits they provide. These health benefits include improved immune function and prevention of heart disease and certain cancers [9–11]. Consumption of n-3 PUFA has also been reported to provide important benefits with respect to functioning of the brain [12] and retina [13], as well as to acceleration of the growth of preterm infants [14, 15].

Lipases and phospholipases are widely used as biocatalysts for processes designed to modify PL. Both lipases and phospholipases can mediate enzyme-catalyzed transesterification reactions in organic media, reactions in which the water activity (a_w) of the reaction medium plays a crucial role. A minimal amount of water must be present if the enzyme is to be present in the configuration that is quasi optimum from the standpoint of catalytic activity and selectivity [16]. However, at higher levels of a_w, the additional moisture may be detrimental from two perspectives: it may either decrease the catalytic activity of the enzyme or it may lead to undesired hydrolysis reactions [17–19]. The physical properties of enzymes and other proteins may vary depending on the degree of hydration thereof. Hydration influences the molecular configuration of enzymes in organic media with concomitant effects on enzyme activity and selectivity, even though the details of the manner in which this occurs are not known [20–23]. It is clear that the optimum efficacies of different enzymes in organic media are characterized by quite different requirements for water activity.

In this study, an immobilized enzyme prepared from a commercially available phospholipase A_1 from *Thermomyces lanuginosa/Fusarium oxysporum* was used to synthesize structured phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) significantly enriched in residues of n-3 PUFA. The effects of a_w, and enzyme loading on the rate and extent of incorporation of n-3 PUFA into PC and LPC were ascertained by monitoring the time courses of these reactions.

Experimental Procedures

Materials

Granulated phosphatidylcholine (PC, purity >95%) was obtained from Avanti Polar-Lipids, Inc. (Alabasta, AL). Phospholipase A_1 (Lecitase® Ultra) from *T. lanuginosus/F. oxysporum* was provided by Novo Nordisk Biochem North America, Inc. (Franklinton, NC). Duolite® A568 was a gift from Rohm and Haas (Philadelphia, PA). Fatty acid standards were purchased from Sigma Chemical Company (St. Louis, MO). Free fatty acids were prepared from a fish oil concentrate (Ocean Nutrition, Dartmouth, Nova Scotia, Canada) by saponification according to the method of Kim et al. [24]. This mixture of fatty acids was used as the acyl donor for phospholipase A_1-catalyzed synthesis of structured PC. Other chemicals used in this study were of analytical grade unless otherwise noted.

Immobilization of Phospholipase A_1

The immobilized enzyme was prepared using the optimal conditions determined in a previous study [25]. The commercially available undiluted phospholipase A1 solution (Lecitase® Ultra) containing ca. 1.5% protein was mixed with an equal volume of 0.1 N Tris–HCl buffer set at pH 7. In a typical immobilization experiment, 10 mL of enzyme suspension was mixed with 1 g of support (Duolite A568) and placed in an orbital shaker operating at 300 rpm and 50 °C. After being shaken for 24 h, the suspension was filtered through a Büchner funnel to recover the resulting immobilized enzyme preparation from the Whatman #1 filter paper. The solids were rinsed with 50 mL of 0.1 N Tris–HCl buffer set at pH 7 and then dried overnight at 30 °C in a vacuum oven. The dried immobilized enzyme was subsequently used to catalyze the acidolysis reactions involved in modifying PC.

Equilibration of Water Activity

The premixed substrates (PC and fatty acids from fish oil) and the immobilized enzyme were equilibrated separately in sealed containers with saturated salt solutions of known water activity. The salts used were LiCl (a_w, 0.11), $MgCl_2{\cdot}6H_2O$ (a_w, 0.33), K_2CO_3 (a_w, 0.43), $Mg(NO_3)_2{\cdot}6H_2O$ (a_w, 0.53), $NaNO_3$ (a_w, 0.65), NaCl (a_w, 0.75), KCl (a_w, 0.84), and KNO_3 (a_w, 0.95). The equilibration process was carried out at 25 °C for 24 h.

Phospholipase A_1-Catalyzed Acidolysis of PC

The phospholipase A_1-catalyzed acidolysis of PC by the mixture of fatty acids from fish oil was accomplished in a 25-mL screw-capped Erlenmeyer flask. The fatty acid mixture (2.31 g, 7.2 mmol), PC (0.69 g, 0.90 mmol), and immobilized enzyme (0.3 g, 10% of the total weight of substrates) were loaded in the flask. The reaction mixture was agitated in an orbital shaker water bath (Model G76, New Brunswick Scientific Co. Inc., New Brunswick, NJ, USA) operating at 300 rpm and 50 °C. Individual samples were removed at selected times and analyzed. All trials were conducted in duplicate.

Analysis of Products

Once the reaction was completed, product mixtures were filtered first through a column packed with anhydrous sodium sulfate and then through a 0.45-μm nylon microfilter to remove water and enzyme, respectively. Samples (50 mg) from the reaction mixture were applied to TLC plates. The solvent system used to separate the PC and LPC consisted of chloroform/methanol/acetic acid/water (75:40:8:3, by volume). Spots on the TLC plates were detected under UV light with a 0.2% (w/v) solution of 2,7-dichlorofluorescein in methanol. The bands corresponding to the PC and LPC were scraped off the TLC plate and methylated using 14% BF_3 in methanol as the catalyst. Heptadecanoic acid (0.2 mg) was used as an internal standard. The fatty acid methyl esters were analyzed by GC. The total amounts of LPC and PC were calculated from the total amount of fatty acid methyl esters in each fraction.

Results and Discussion

The fatty acid compositions of the original PC and the mixture of fatty acids obtained by saponification of fish oil are presented in Table 1. The primary fatty acid residues in the starting PC were those of linoleic acid, 18:2n-6 (62.2 mol%), palmitic acid, 16:0 (14.7 mol%), and oleic acid, 18:1n-9 (10.4 mol%). The primary species in the mixture of fatty acids used as acyl donors were DHA, 22:6n-3 (60.7 mol%), EPA, 20:5n-3 (12.2 mol%), and docosapentaenoic acid (DPA, 22:5n-3; 10.1 mol%). For present purposes, we defined n-3 PUFA as the sum of EPA, DPA, and DHA.

Screening to Determine Effects of Water Activity

The effect of a_w on the n-3 PUFA content (mol%) of the total fatty acid residues in all the PC species present at 6 h into the phospholipase A_1-catalyzed acidolysis is shown in Fig. 1. For these trials the enzyme loading, reaction time, and temperature were held constant at 10% of the total weight of substrates, 6 h, and 50 °C, respectively. A marked increase in the proportion of n-3 PUFA residues present in the total PC (unreacted plus modified) was observed as the water activity increased from 0.43 to 0.65. However, when a_w was further increased from 0.65 to 0.95, the incremental increase in the percentage of n-3 PUFA residues was relatively small. Moreover, no incorporation of the n-3 PUFA in the PC was observed at an a_w of 0.11, and the n-3 PUFA proportion of the total residues was only 3% when a_w was 0.33. Hence in subsequent trials we employed an a_w of 0.43 or greater for synthesis of PC containing n-3 PUFA residues. The n-3 PUFA proportion

Table 1 Composition of the fatty acid residues (mol%) of the starting mixture of fatty acids from fish oil and the original unmodified PC and LPC

Fatty acid	Fatty acid from fish oil[a]	PC	LPC
C14:0	0.2	1.1	1.1
C16:0	0.5	14.7	13.1
C16:1(7)	1.2	0.2	0.1
C16:2(4)	0.4	–	–
C16:3(4)	0.3	–	–
C18:0	0.5	3.4	3.4
C18:1(9)	2.0	10.4	11.0
C18:1(7)	0.6	1.6	1.5
C18:2(6)	0.4	62.2	63.7
C18:3(3)	0.2	6.4	6.1
C18:4(3)	0.6	–	–
C20:1(9)	1.3	–	–
C20:2(6)	0.3	–	–
C20:4(6)	0.7	–	–
C20:4(3)	0.8	–	–
C20:5(3)	12.2	–	–
C22:1(11)	7.0	–	–
C22:5(3)	10.1	–	–
C22:6(3)	60.7	–	–

Tabular entries are the average of duplicate determinations from different experimental trials

[a] The mixture of fatty acids used as the acyl donor was obtained by saponification of fish oil. The original PC was composed of 97% PC and 3% LPC

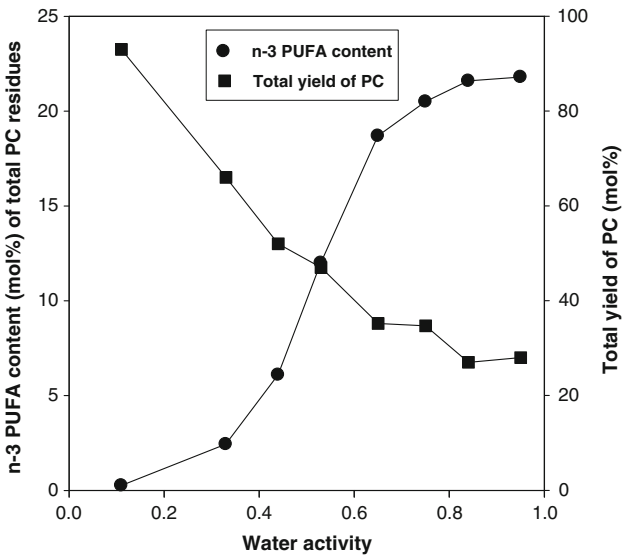

Fig. 1 Effects of water activity on the acidolysis of PC with a mixture of n-PUFA derived from fish oil. Reaction conditions: 2.31 g fatty acids, 0.69 g PC (mole ratio of substrates = 8 FA:1 PC), and 0.3 g immobilized enzyme; incubation for 6 h in an orbital shaker operating at 300 rpm and 50 °C; n-3 PUFA is defined as the sum of EPA (C20:5n-3), DPA (C22:5n-3), and DHA (C22:6n-3)

of the total residues was significantly less at a_w values of 0.33 or lower.

To quantify the efficacy of the reaction, we employed a measure that characterizes the yield of the reaction in terms of the total amount of PC (both the native unreacted PC and the modified PC produced via the phospholipase A1-catalyzed acidolysis reaction in which a native fatty acid residue has been replaced by a PUFA residue). The fractional total yield of PC is defined as the ratio of the total moles of PC (unreacted PC plus those PC containing n-3 PUFA residues) to the sum of the total moles of PC (unreacted and modified), the total LPC (unreacted LPC that was present in the original reaction mixture plus the LPC formed by hydrolysis of the modified PC formed by acidolysis), and glycerophosphatidylcholine (GPC) formed by hydrolysis of LPC. This approach assumes that the amounts of glycerol and phosphoric acid esters of glycerol that may be present as a consequence of other hydrolysis reactions are negligible. Percentage yields are defined as the fractional yield times 100. The yield of PC decreased significantly when a_w was increased from 0.11 to 0.65. For a_w above 0.65, the yield of PC continued to decline slowly with increasing a_w.

Water Activity

For the phospholipase A_1-catalyzed acidolysis trials involving a_w values of 0.43 and higher, the effects of a_w on the n-3 PUFA content (mol%) of the total fatty acid residues present in all PC species at incubation times between 2 and 48 h are shown in Fig. 2. For these trials the enzyme loading and temperature were held constant at 10% of the total weight of substrate and 50 °C, respectively. An earlier study in our laboratory [25] indicated that the optimum temperature for the immobilized enzyme used in this study was 50 °C. Hence, in subsequent trials we employed 50 °C as the reaction temperature.

During the first 4 h of reaction for trials in the a_w range from 0.65 to 0.93, the n-3 PUFA content of the total PC residues exceeded 10 mol%. However, during the same time frame for a_w values from 0.43 to 0.53, the n-3 content of the total PC residue was below 10 mol% even though the total yield of PC was greater than those for the a_w range from 0.65 to 0.93. For trials at a_w values from 0.43 to 0.53, the maximum n-3 content of the total PC residues was ca. 22 mol%., corresponding to a reaction time of 48 h. For a_w values from 0.65 to 0.93, the maximum n-3 content of the total PC residues was as high as ca. 35 mol% at a reaction time of 48 h. For the entire series of acidolysis reaction trials, lower levels of n-3 PUFA content were observed during the trials at a_w values of 0.43 and 0.53 than during the trials at higher water activities. These results demonstrate that the immobilized phospholipase employed in this

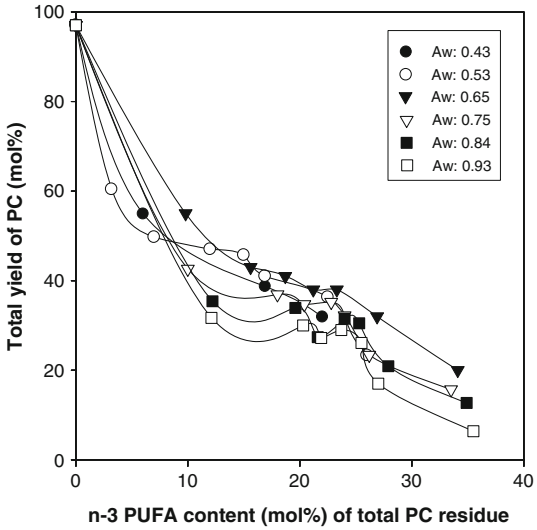

Fig. 2 Yield of PC as a function of the extent of incorporation of n-3 PUFA from fish oil at different water activities. Incubation times were 0, 2, 4, 6, 9, 12, 24, and 48 h. Other conditions were the same as those for the trials in Fig. 1

study is more active at a_w values above 0.53. There is a critical threshold of a_w below which the biocatalyst does not function in an efficacious manner with respect to catalysis of the transesterification reactions of phospholipids and/or lipids. It also should be noted that increases in water activity also affected the yield of PC because of the negative impact of hydrolysis reactions that occur simultaneously within the reaction medium thereby producing LPC. Significant decreases in the total yield of PC were observed as the reaction proceeded. The decreased yield is attributed to the fact that the rates of the hydrolysis reactions increase as the water activity increases, thereby leading to additional release of free fatty acids and lower phospholipids with concomitant reductions in the yield of the PC product.

It is well recognized that hydrolysis is an unavoidable side reaction that accompanies enzyme-catalyzed acidolysis of phospholipids [26]. Hence LPC is expected to form during enzyme-catalyzed acidolysis as a consequence of hydrolysis reactions. Like PC, LPC is an effective emulsifying and solubilizing agent for many of the synthetic phospholipids used in the manufacture of foods, cosmetics, agrochemicals, and pharmaceuticals [27]. The diverse nature of these applications is a consequence of the unique structural properties of these substances that are reflected in their functional properties. The most widely used lysophospholipid is lysolecithin, a substance that is obtained by the hydrolysis of a single fatty acid residue from naturally abundant sources of lecithin [28–30].

The effects of a_w on the n-3 PUFA content (mol%) of the total fatty acid residues in all the LPC species

present at incubation times between 2 and 48 h during the phospholipase A_1-catalyzed acidolysis are shown in Fig. 3. During the initial stages of these reactions a significant increase was observed in the yield of LPC as a consequence of hydrolysis of both the modified and the unreacted PC fractions. However, during the same time frame no major differences were observed in the n-3 PUFA contents achieved in trials at different a_w. For a_w values of 0.43 and 0.53, no significant decreases in the total yield of LPC were observed for the range of n-3 PUFA contents between 10 and 35 mol%. On the other hand, in the a_w range from 0.65 to 0.93, the n-3 PUFA contents were as large as *ca.* 45 mol%. Furthermore, in this a_w range, the maximum yield of LPC (ca. 23%) was obtained at an a_w of 0.65. Consequently, for purposes of process optimization when both the n-3 PUFA proportion of all fatty acid residues and the yields of the modified forms of PC and LPC are considered, an a_w of 0.65 represents an appropriate compromise between these factors. By contrast, Svensson et al. [31] observed that for incorporation of heptadecanoic acid into PC via lipase-catalyzed transesterification the optimum a_w was as low as 0.064. There are multiple possible explanations for the discrepancy between the optimum reported by these researchers and our result. These explanations invoke differences in the substrate fatty acid employed, differences in the immobilized enzyme (source and support), and differences in reaction conditions.

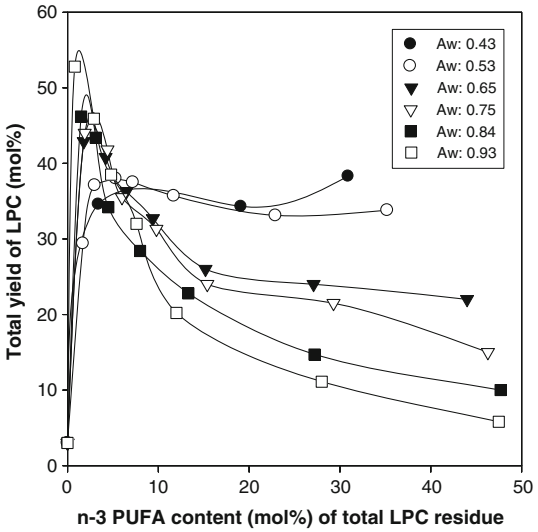

Fig. 3 Yield of LPC as a function of the extent of incorporation of n-3 PUFA from fish oil at different water activities. Incubation times were 0, 2, 4, 6, 9, 12, 24, and 48 h. Other conditions were the same as those for the trials in Fig. 1

Enzyme Loading

The effects of enzyme loading on the n-3 PUFA content of the total PC (unreacted and modified) present at any time during the phospholipase A_1-catalyzed acidolysis at an a_w of 0.65 are shown in Fig. 4a. The proportion of n-3 PUFA residues in the total PC increased significantly as the amount of enzyme in the reaction mixture increased up to a loading level of 15% of the total weight of substrates. However, the differences between the n-3 PUFA contents achieved at loadings of 15 and 20% were minimal. If these trials are considered in their entirety, the yield of PC decreased significantly as the enzyme loading increased (Fig. 4b). In particular, for all enzyme loadings investigated, the total yield of PC decreased sharply during the first 4 h of reaction, even though there were differences in the amount of unreacted PC remaining that depended on the various enzyme loadings employed. However, at all enzyme loadings investigated, the overall yield of PC after 4 h decreased gradually as reaction time increased further. The total yield of PC depended not only on the enzyme loading but also on the initial a_w of the reaction mixture. For the trial involving an enzyme loading of 20% of the total weight of substrates, the yield at the conclusion of the trial (48 h) was only 2.2%. Previous researchers [32, 33] have also reported that the extent of hydrolysis increased with increasing incorporation of fatty acid residues in PC when higher enzyme loadings were employed.

For LPC, the n-3 PUFA contents achieved at enzyme loadings of 15 and 20% of the total weight of substrates increased significantly with time during the first 24 h but then approached a plateau at longer times (Fig. 5a). Moreover, throughout the course of these two trials (loadings of 15 and 20%) there was very little difference between the n-3 PUFA contents achieved at various times. At lower enzyme loadings (5 and 10%) the n-3 PUFA contents lagged significantly behind those achieved at the two highest loadings investigated. The maximum yield of LPC was ca. 50% (Fig. 5b). This yield was achieved at an enzyme loading of 15%. It should also be noted that for all loadings there were significant decreases in the yield of LPC as the reaction time increased from 2 to 12 h. Moreover, the magnitudes of the observed decreases in the yield of LPC were also a function of a_w (data not shown). Inspection of Fig. 3b indicates that the yield of LPC also depends on the water activity. For reaction times greater than 12 h, slow but steady decreases in the total yield of LPC were observed. At 48 h, the total yield of LPC in the trial involving an enzyme loading of 20% was only 4.5%.

The results reported here indicate that one can readily synthesize structurally modified forms of PC and LPC containing large proportions of n-3 PUFA residues using a route based on acidolysis of a PC derived from soybeans

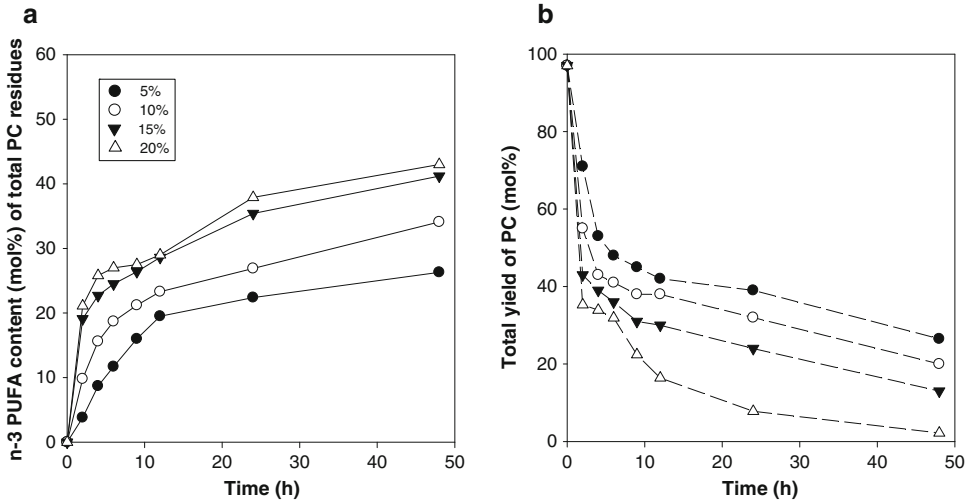

Fig. 4 Effects of enzyme loading on the acidolysis of PC with a mixture of n-3 PUFA from fish oil as a function of time at different water activities: **a** proportion of n-3 PUFA residues among all PC residues; **b** total yield of PC. Samples were analyzed at 0, 2, 4, 6, 9, 12, 24, and 48 h. Other conditions were the same as those for the trials in Fig. 1

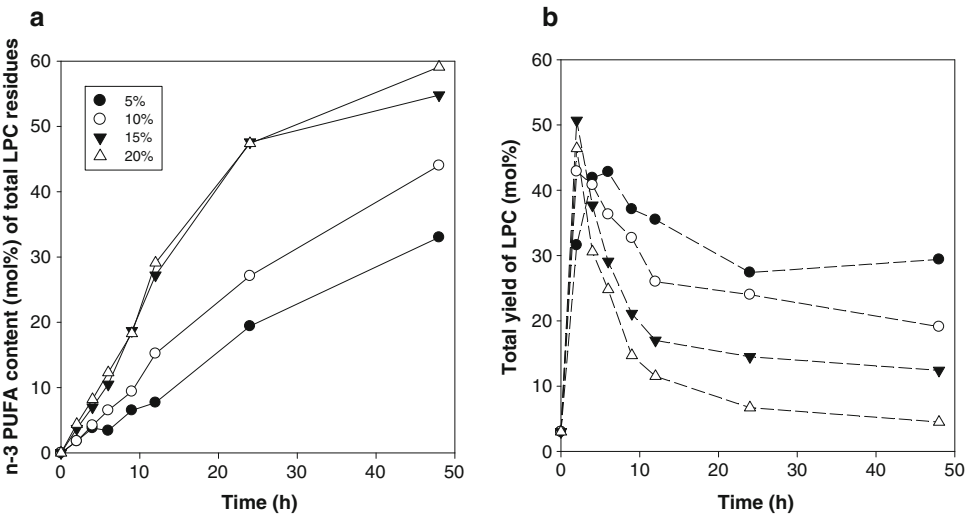

Fig. 5 Effects of enzyme loading on the acidolysis of the LPC formed by hydrolysis of PC with a mixture of n-3 PUFA from fish oil as a function of time at different water activities: **a** proportion of n-3 PUFA residues among all PC residues; **b** total yield of LPC. Samples were analyzed at 0, 2, 4, 6, 9, 12, 24, and 48 h. Other conditions are the same as those for Fig. 4

with a mixture of n-3 PUFA obtained by saponification of fish oil. The entire process can be regarded as solvent-free. Even though use of reaction media with very high water activities for acidolysis yields higher overall contents of n-3 PUFA residues in PC and LPC, the acidolysis reaction is inherently accompanied by parallel hydrolysis reactions that lead to a decrease in the yield of the structurally modified compounds.

Conclusions

The design of an optimum protocol for synthesis of structured PC and LPC containing significant levels of n-3 PUFA residues requires that one take into account both the n-3 PUFA residue contents of the various PC and LPC species and their corresponding yields. Analysis of the data presented in Figs. 1, 2, 3, 4, 5 indicates that the synthesis protocols leading to large proportions of n-3 PUFA residues in the total amounts of PC and LPC present at particular times in the reaction mixture suffer the disadvantage that they simultaneously lead to low yields of these compounds. Conversely, those reaction protocols that give high total yields of PC and LPC at particular times simultaneously produce much smaller contents of n-3 PUFA in the PC and LPC present at these same times. Selection of optimum synthesis conditions may well depend on the specific application of interest, but on the basis of the data reported here, the quasi optimum water activity is 0.65, while the corresponding quasi optimum enzyme loading is 15% of the total weight of substrates.

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