Article

Interesterification of Egg-Yolk Phosphatidylcholine with *p*-Methoxycinnamic Acid Catalyzed by Immobilized Lipase B from *Candida Antarctica*

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Abstract: The *p*-methoxycinnamic acid (*p*-MCA) is one of the most popular phenylpropanoids, the beneficial impact of which on the human health is well documented in the literature. This compound has shown many valuable activities including anticancer, antidiabetic, and neuro- and hepatoprotective. However, its practical application is limited by its low bioavailability resulting from rapid metabolism in the human body. The latest strategy, aimed at overcoming these limitations, is based on the production of more stability in systemic circulation bioconjugates with phospholipids. Therefore, the aim of this research was to develop the biotechnological method for the synthesis of phospholipid derivatives of *p*-methoxycinnamic acid, which can play a role of new nutraceuticals. We developed and optimized enzymatic interesterification of phosphatidylcholine (PC) with ethyl *p*-methoxycinnamate (*Ep*-MCA). Novozym 435 and a binary solvent system of toluene/chloroform 9:1 (*v/v*) were found to be the effective biocatalyst and reaction medium for the synthesis of structured *p*-MCA phospholipids, respectively. The effects of the other reaction parameters, such as substrate molar ratio, enzyme dosage, and reaction time, on the degree of incorporation of *p*-MCA into PC were evaluated by use of an experimental factorial design method. The results showed that substrate molar ratio and biocatalyst load have significant effects on the synthesis of *p*-methoxycinnamoylated phospholipids. The optimum conditions were: Reaction time of three days, 30% (*w/w*) of Novozym 435, and 1/10 substrate molar ratio PC/*Ep*-MCA. Under these parameters, *p*-methoxycinnamoylated lysophosphatidylcholine (*p*-MCA-LPC) and *p*-methoxycinnamoylated phosphatidylcholine (*p*-MCA-PC) were obtained in isolated yields of 32% and 3% (*w/w*), respectively.

Keywords: egg-yolk phosphatidylcholine; *p*-methoxycinnamic acid; lipase-catalyzed interesterification; Novozym 435; methoxy derivatives of phenolic acids

1. Introduction

Phenolic acids are gaining considerable interest to consumers and food manufacturers due to the literature reports indicating that their consumption has a great impact on the human body condition and health. It is generally well known that a diet rich in these compounds favors the prevention and reduces the risk of several degenerative diseases such as cardiovascular disease, atherosclerosis, and cancer. Among phenolic acids, especially useful properties exhibit hydroxy and methoxy derivatives of cinnamic acid. Their strong antioxidant properties are mostly determined by the presence of hydroxy substituents on the aromatic ring, while *p*-methoxy group in the structure of cinnamic acid is responsible for efficient insulin release [1–5] and enhancing cognition in an acute model of memory deficit [6–8]. Demonstrated in many published reports, less anticancer [9], antidiabetic [2],

and neuro- [7] and hepatoprotective [1] activity of hydroxylated aromatic acids than their methoxylated forms suggests that the health-promoting effects of the latter ones do not result from their antioxidant potential. Therefore, *O*-methoxylated derivatives of cinnamic acid are the subject of extensive studies aimed at evaluation of their mechanism of action and efficient delivery to the organism.

The *p*-methoxycinnamic acid (*p*-MCA, 4-methoxycinnamic acid, 4MCA) is a phenylpropanoid that commonly occurs in food products such as coffee [10], peanuts [11], sugarcane [12], brown rice [13], rice bran, and turmeric [14]. It is also one of the most biologically active ingredient of *Scrophularia buergeriana* and *Kaempferia galanga*, herbs that have been used for centuries in Oriental medicine as natural remedies for preventing and curing many diseases and ailments like cold, fever, coughs, asthma, dyspepsia, constipation, hypertension, swelling, rheumatism, inflammatory tumors, neuritis, and depression [6,15–17]. Most studies and reported data indicate a high therapeutic potential of *p*-MCA. In Table 1, presented below, the results of in vitro and in vivo tests, which confirm the significant hepato-, neuro-, antidiabetic and chemopreventive properties of *p*-MCA, are summarized. These reports also emphasize that α,β -unsaturated ester moiety and the presence of methoxy group in the para position of the benzene ring are crucial pharmacophores implying biological potential of this molecule. It is also worth noting that in some research this compound has shown higher activity than the reference compounds. Under the in vitro tests performed on rat hepatocytes with toxicity induced by carbon tetrachloride (CCl₄), it has been demonstrated that *p*-MCA shows comparable hepatoprotective activity to silybin even when it is used in several dozen times lower concentration [18]. Furthermore, this acid and its ethyl ester have been reported as effective inhibitors of α -glucosidase, exhibiting 100-fold higher activity than 1-deoxynojirimycin used as positive control [2]. In the aspect of chemopreventive properties, *p*-MCA exhibits comparable activity to that reported for doxorubicin, with the important difference that its action was more selective towards HCT-116 cells than against normal colonic epithelial cell line (NCM460) [19].

Table 1. Biological activity of *p*-MCA and its ester.

	Molecule	Research Model	Active Dose	Mechanism of Action	Ref.
Antidiabetic and hepatoprotective activity	<i>p</i> -MCA	enzymes	0.044 ± 0.006 (IC ₅₀)	α -glucosidase inhibition	[2]
	<i>p</i> -MCA	* STPZ-induced rats	40 mg/kg	insulin secretion, gluconeogenesis inhibition	[3]
	<i>p</i> -MCA	INS-1 cell line	100 μ M	insulin secretion	[5]
	<i>p</i> -MCA	Wistar rats	5 mg/kg	insulin secretion	[5]
	<i>p</i> -MCA	* STPZ-induced rats	40–100 mg/kg	insulin secretion	[4]
	<i>p</i> -MCA	INS-1 cell line	100 μ M	Ca ²⁺ influx, insulin secretion	[20]
	<i>p</i> -MCA	* STPZ-induced rats	10–40 mg/kg	insulin secretion	[21]
	<i>p</i> -MCA	CCl ₄ intoxicated rat hepatocytes	1–5 μ M	-	[18]
	<i>p</i> -MCA	CCl ₄ -intoxicated rat	50 mg/kg	-	[1]
Neuroprotective activity	<i>p</i> -MCA	glutamate-insulted rat cortical cell	1 μ M	-	[6]
	<i>Ep</i> -MCA	glutamate-insulted rat cortical cell	0.01–1 μ M	Ca ²⁺ influx, glutamatergic antagonism	[7]
	<i>Ep</i> -MCA	ICR-mices	0.01–2 mg/kg	-	[8]
	<i>p</i> -MCA, <i>Ep</i> -MCA	Wistar rats with cognitive dysfunction	50–100 mg/kg	-	[22]

Table 1. Cont.

	Molecule	Research Model	Active Dose	Mechanism of Action	Ref.
Chemopreventive activity	<i>p</i> -MCA	HepG2 cell line	27.1 µg/mL (IC ₅₀)	induction of apoptosis	[17]
	<i>p</i> -MCA	Wistar rats	40 mg/kg	-	[14]
	<i>Ep</i> -MCA	** DMAB-induced mice	23.4 mg/kg (oil fraction)	induction of apoptosis	[23]
	<i>p</i> -MCA	HCT-116 cell line	10 µM (IC ₅₀)	induction of apoptosis	[19]
	<i>Ep</i> -MCA	MCF-7 cell line	360 µg/mL (IC ₅₀)	-	[24]
	<i>Ep</i> -MCA	HCT-116 cell line	42 µg/mL (IC ₅₀)	induction of apoptosis	[25]
	<i>Ep</i> -MCA	*** DMH-induced rats	40 mg/kg	-	[9]

* Streptozotocin (STPZ)-induced diabetic rats; ** Mice with colon cancer induced by dimethylbenz(a)anthracene (DMAB) *** Rats with colon cancer induced by dimethylhydrazine (DMH); INS-1, rats pancreatic β -cells; HepG2, human hepatocyte carcinoma cell line; HCT-116, human colon carcinoma cell line; MCF-7, human breast cancer cell lines.

The most important obstacles in practical application of phytochemicals are difficulties in achieving their, previously established in the in vitro tests, active blood concentration. They undergo extensive biotransformation in the human body by enzymes of phase I and phase II of xenobiotics' metabolism. The metabolic pathway of *p*-methoxycinnamic acid was determined on a rabbit model, following both intravenous and oral administration. It was observed that *p*-MCA disappeared from the serum very rapidly when it was injected into the rabbit's blood. Maximum serum concentration 41 mg/100 mL was observed three minutes after injection of 100 mg/kg of *p*-MCA and its half-life was only 0.4 h. The oral administration of the same dose resulted in very fast absorption from the digestive tract and slightly slower elimination from the serum. Peak concentration at the level of 7.38 mg/100 mL was obtained within 30–60 min after consumption but half-life was still lower than 1 h [16]. It was also reported that the main metabolite of *p*-methoxycinnamic acid identified in the serum is *p*-methoxybenzoic acid, which subsequently undergoes conjugation with glycine or glucuronic acid and in this form was excreted via urine [16]. In other studies, it has been reported that the radiolabeled [¹⁴C] 2-ethylhexyl-*p*-methoxycinnamate (EHMC) orally administered to rodents underwent rapid absorption and was excreted via urine after 72 h from consumption (65–80% in rats and 63–72% in mice). In addition, it has been determined that EHMC is mainly distributed to adipose tissues, bladder, liver, kidney, and intestines [26].

In the recent years, several strategies for delivering and/or releasing therapeutic molecules have been proposed to improve their pharmacokinetic properties in the biological systems. In order to increase activity of ethyl *p*-methoxycinnamate (*Ep*-MCA) Kusumawati and Yusuf prepared its phospholipid complex [27]. Its oral administration to mice resulted in a two-fold increase in the analgesic activity of ester due to the fact that PC is highly absorbed from the digestive system and can circulate in the blood for a long time [28]. However, such complexes possess some limitations and are rather unstable. A better option is formation of more stable bioconjugates with direct covalent binding between biologically active molecule and lipid carrier. Clinical studies have proven that therapeutic molecules administrated in the form of lipid-drug conjugates (LDCs) are characterized by greater oral bioavailability and lower toxicity. Moreover, their release may be controlled in the body, and this form of application reduces their potential side effects [29]. Therefore, in recent years, several LDCs have been approved by Food and Drug Administration (FDA) and European Medicines Agency, especially in the field of the treatment of diabetes, schizophrenia, and depression [30].

Lipases are enzymes whose biological function is to catalyze synthesis and hydrolysis of ester bonds in triacylglycerides [31]. However, due to their high specificity and selectivity they have found wide application in the biotechnology field [31]. They are often applied to the lipophilization process, even of non-natural for them, substrates such as sugars [32], amino acids [33], and phenolic

compounds [34], carried out with different lipid carriers (fatty acids, fatty alcohols, acylglycerols, sterols, and phospholipids) delivering products with the desired physico-chemical properties, valuable for the pharmaceutical, food, and cosmetics industries. According to our best knowledge, little is known about enzymatic lyophilization of *p*-methoxycinnamic acid. Lee et al. synthesized octyl methoxycinnamate (OMC) by way of direct esterification reaction of *p*-MCA with 2-ethyl hexanol catalyzed by Novozym 435 at a temperature of 80 °C. The highest recorded level of conversion, 90%, was obtained after 24 h of the reaction. The authors indicated in their report that high conversion of *p*-MCA was favored by the use of nonpolar organic solvents as the reaction medium. The OMC ester obtained as a product of this reaction is a compound that effectively absorbs UV-B rays; therefore, it has found wide application in the cosmetics industry as a non-allergenic sunscreen [35]. The process of its enzymatic synthesis was later studied also by Kumar and co-workers, who used the lipase form *Rhizopus oryzae* as the biocatalyst [36]. In their studies, a slightly higher conversion of *p*-MCA, on the level of 91.3%, was observed in the reaction carried out in cyclo-octane at a temperature of 45 °C. Octyl methoxycinnamate (OMC) was obtained in high yield, 88.6%, after 96 h of reaction. Biological studies showed also that OMC exhibits better antioxidant activity than ascorbic acid and the free form of *p*-MCA as well as good antimicrobial activity against a series of pathogenic microorganisms. In 2006, Weber's research group presented the results of the enzymatic synthesis of long-chain alkyl *p*-methoxycinnamates, which are used in the food industry as lipophilic health-promoting additives. They performed the reaction of transesterification of methyl *p*-methoxycinnamate with oleyl alcohol (*cis*-9-octadecen-1-ol) at a temperature of 80 °C for 72 h using three commercially available immobilized preparations of lipases (Novozym 435, Lipozyme RM IM, and Lipozyme TL IM). The highest degree of conversion of *p*-MCA (92%) was obtained in the reaction catalyzed by lipase B from *C. antarctica* (Novozym 435). Lipase from *R. miehei* (Lipozyme RM IM) showed lower activity (60% of conversion), while lipase from *T. lanuginosus* (Lipozyme TL IM) was basically unable to catalyze this reaction [37].

Enzymatic lyophilization of phenolic acids with fatty alcohols and triacylglycerols was extensively studied during the last two decades [38–40], whereas application of phospholipids for production of phenolipids is a new area of research [41,42]. There are only a few reports in the literature about modifications of phospholipids with phenolic acids and their *O*-methylated derivatives. In our previous research we reported chemical and enzymatic synthesis of phospholipid derivatives of selected *O*-methylated phenolic acids [43–47] and proved, in the *in vitro* tests, their higher anticancer [43] and antidiabetic [48] activity in comparison to the none-conjugated forms. Here, we focused on the development and optimization of the biotechnological process of production of bioconjugates of *p*-MCA with phosphatidylcholine with potential application as food additives and nutraceuticals. The use of lipases for this purpose can be beneficial, due to their broad substrate specificity and absence of any cofactor dependence. Furthermore, the currently available preparations of immobilized lipases at hydrophobic support allowed us to carry out biotechnological processes in the presence of an organic solvent and a high concentration of substrates with rewarding reaction efficiency and selectivity [49]. Hence, we performed lipase-catalyzed transesterification of egg-yolk phosphatidylcholine (PC) with *p*-methoxycinnamic acid ethyl ester (*Ep*-MCA) and evaluated such reaction parameters as biocatalyst, organic solvent, substrate molar ratio, enzyme loading, and reaction time. To minimize the costs of the process and at the same time more thoroughly analyze the relationships between variables, we applied Box-Behnken design (BBD), one of the most efficient statistical models of experiment.

2. Results and Discussion

2.1. Screening of Lipase on the Interesterification Reaction

We started our research from evaluation of four preparations containing different lipases (Lipozyme[®], Lipozyme TL IM, CALB, and Novozym 435) for their ability to catalyze the interesterification process between egg-yolk phosphatidylcholine and ethyl *p*-methoxycinnamate (*Ep*-MCA). Selection of lipases was made on the basis of the literature data indicating them as

the most effective biocatalysts in the modifications of phenolic acids with acylglycerols [38,50,51] and phospholipids [32,41,45–47]. At the stage of lipases screening, reactions were incubated in the temperature of 50 °C in toluene using PC/Ep-MCA molar ratio 1/10 and 30% (*w/w*) of enzyme dosage. This combination of reaction parameters was taken as the starting point and was based on our previous experience and literature data. In several studies of lipase-catalyzed modification of phenolic acids, toluene was one of the most efficient reaction media [41,46]. An applied temperature of 50 °C was previously selected as the optimal during phospholipid modification with anisic acid [47], whereas the selected substrate molar ratio was the most efficient in the process of interesterification of PC with 3,4-dimethoxycinnamic acid ethyl ester [46]. The course of the reaction progress was monitored by evaluation of the degree of *p*-MCA incorporation into the phospholipid fraction phosphatidylcholine/lysophosphatidylcholine (PC/LPC) in selected time intervals (1, 2, 3, and 4 days) and the results are presented in Figure 1. For this purpose, samples of reaction mixtures were collected, purified by the solid phase extraction (SPE) (Section 3.2), and then analyzed by gas chromatography (GC) (Section 3.5.2). Two from four evaluated biocatalysts, Lipozyme RM IM and Lipozyme TL IM, were not active under the proposed reaction conditions, while lipases CALB and Novozym 435 showed the ability to catalyze the process of incorporation of *p*-MCA into phospholipid fraction. In the reaction catalyzed by Novozym 435, the incorporation of *p*-MCA reached a maximum 19 mol% within three days, whereas in CALB-catalyzed interesterification only 3 mol% incorporation was observed. These results are the next ones, which confirm that among commercially available lipases preparations, Novozym 435 seems to be the most effective biocatalyst in the field of lipid modification with heterogeneous molecules. Based on this, we selected this enzyme for our further study.

Figure 1. Time course of different lipase-catalyzed interesterification of PC with Ep-MCA. Reaction conditions: Toluene; PC/Ep-MCA, 1/10; lipase dosage 30% (*w/w*); 50 °C.

2.2. Effect of Reaction Medium on the Interesterification Reaction

The presence of substrate–solvent interactions determines the availability of substrate to an enzyme. Therefore, the choice of solvent is very important for the productivity of the enzymatic reactions. During the selection of an appropriate reaction medium, four main factors should be taken under consideration: Solubilization of substrate, maintenance of enzyme activity and stability, the recovery of reactants, and the toxicity. The main problem during enzymatic lyophilization of phenolic acids is their poor solubility in hydrophobic solvents that are dedicated to lipase-catalyzed reactions. It is postulated that solvents with log *P* values < 2 are not suitable for enzyme-catalyzed systems because they can distort the essential water from the lipases, thereby inactivating them. For instance, Patil et al. performed lipase-catalyzed modifications of phenolic acids with glycerol

in polar solvents such as acetone, ethyl methyl ketone, acetonitrile, and 1,4-dioxane [52]. However, despite the increase in acid solubility in these reaction media, no increase in conversion was observed and use of a more hydrophobic medium was necessary to obtain a desirable product. The literature reports specify that the most proper solvents for lipase-catalyzed reactions are those with $\log P$ in the range of 2–4. In our study, three hydrophobic solvents, like toluene ($\log P = 2.5$), hexane ($\log P = 3.5$), and heptane ($\log P = 4$), were screened to optimize the reaction of interesterification of PC with *p*-MCA. In addition, since previous studies revealed that binary solvent systems are highly efficient in the PC modification with phenolic molecules, mixture of toluene/chloroform in the volume ratio 9:1 ($\log P = 2.5/2.01$) was also tested [41,47].

The second approach to increase solubility of substrates is to raise the reaction temperature. However, it has to be borne in mind that use of enzymes, even in immobilized form commonly considered as stable, in the presence of high temperature and at the same time with detergent-like molecules (fatty acids, phospholipids, etc.) can cause damage of the enzymes' carrier and lead to so-called enzyme "leakage". This phenomenon is consequently associated with partial loss of enzyme activity as well as loss of control over the course of the enzymatic process [53]. Thus, in our recently published studies, we used as an acyl donors' esters of phenolic acids [45,46], which has a lower melting point and at the same time higher $\log P$ value when compare to corresponding acid, which makes them dissolve better in hydrophobic media under mild reaction temperature. Driven by these observations, also in this research in order to get well-dissolved substrate in selected hydrophobic media during the modified phospholipids' synthesis, we applied ethyl *p*-methoxycinnamate (*Ep*-MCA) (melting point: 49–50 °C; $\log P = 3.07$) as an acyl donor.

From the results presented in Figure 2 it can be observed that reaction of interesterification of PC with *Ep*-MCA proceeded in all selected solvents. However, the reaction environment had a significant impact on the degree of incorporation. For solvents with higher values of $\log P$, the level of incorporation was relatively low, only about 9 and 6 mol% after three and four days of reaction for heptane and hexane, respectively. Toluene proved to be a better solvent for interesterification of PC with methoxylated derivative of cinnamic acid, giving 19 mol% of incorporation after two days of reaction. The best results were obtained for reaction carried out in a binary solvent system. Using as a reaction medium a mixture of toluene with 1/10 amount of chloroform, 29 mol% of incorporation was achieved. These results are in accordance with a previously published paper, and a relatively high degree of incorporation was obtained in the toluene/chloroform (9:1 (*v/v*)) reaction mixture during interesterification of PC with ethyl ferulate [45] and acidolysis of PC with anisic acid [47]. Therefore, the mixture of toluene/chloroform 9:1 (*v/v*) was selected as the medium for further evaluation of Novozym 435-catalyzed PC modifications.

Figure 2. Effect of organic medium on the incorporation of *p*-MCA into phospholipids (PLs). Reaction conditions: PC/*Ep*-MCA, 1/10; Novozym 435 30% (*w/w*); 50 °C.

2.3. Development of Response Surface Methodology (RSM) Model

The process of lipase-catalyzed interesterification of PC with *p*-MCA depends not only on the basal reaction parameters, such as biocatalyst and reaction medium, but its course varies during the time, depending also strongly on the reaction mixture composition and enzyme load. Due to that, in the next step of studies these reaction parameters, including PC/*Ep*-MCA molar ratio, enzyme load, and reaction time (independent variables), on the degree of incorporation of *p*-MCA into the phospholipid fraction (dependent variable) were investigated using 3-level-3-factor Box-Behnken model with three central replicates. Values of independent variables and degrees of incorporation, determined experimentally as well as calculated using model equation (Section 3.3), are presented in the Table 2.

Table 2. Box-Behnken design matrix for three independent variables on incorporation of *p*-methoxycinnamic acid (*p*-MCA) into phospholipid fraction (PC/LPC).

Run	Substrate Molar Ratio PC/ <i>Ep</i> -MCA	Enzyme Load [%]	Reaction Time [days]	Incorporation of <i>p</i> -MCA into PC/LPC [mol%] ^a (Experimental)	Incorporation of <i>p</i> -MCA to PC/LPC [mol%] (Predicted)
1	5	20	3	15 ± 0.2	16
2	15	20	3	7 ± 0.4	6
3	5	40	3	17 ± 0.3	18
4	15	40	3	9 ± 0.6	7
5	5	30	2	21 ± 0.8	20
6	15	30	2	8 ± 0.5	9
7	5	30	4	21 ± 0.7	20
8	15	30	4	9 ± 0.5	10
9	10	20	2	23 ± 0.3	23
10	10	40	2	24 ± 0.3	24
11	10	20	4	23 ± 0.1	23
12	10	40	4	24 ± 0.2	24
13	10	30	3	29 ± 0.5	29
14	10	30	3	29 ± 0.6	29
15	10	30	3	29 ± 0.4	29

^a Data are presented as mean ± standard deviation (SD) of two independent analyses.

Using Design of Experiments (DOE) methods for process optimization, it is important to verify the quality and statistical significance of the empirical model before proceeding to the results' evaluation. Therefore, at the beginning, we interpreted the F-value of the model as appropriate based on the *p*-value, which was significantly less than 0.05. Furthermore, a high value of coefficient of determination, $R^2 = 0.98771$, implies an almost perfect relationship between the model and the data. In addition, comparison of predicted and actual degrees of incorporation of *p*-MCA depicted in the Figure 3A reveals significant correspondence between them. All these observations can be concluded as a highly satisfactory fit of the empirical model, which can be used to predict response, depending on the value of the input factors.

The effect of three independent factors on the reaction course, as well as interaction between them, can be discussed from analysis of variance (ANOVA), shown in Table 3 and Pareto chart, depicted in Figure 3B. Based on these data, it can be determined that among all tested parameters, statistically important were substrate molar ratio in quadratic (Q) and linear (L) terms and enzyme loading (Q) ($p < 0.05$). It can also be observed that none of tested variables underwent linear interactions (X_1L by X_3L , X_1L by X_2L , X_2L by X_3L) and, therefore, did not affect the incorporation degree, according to their significantly higher-than-0.5 *p*-value. It is worth noting that the bars shown on the Pareto chart (Figure 3B) are mostly expressed by positive values, which means that as the parameter increased incorporation also increased. Only the substrate molar ratio (L) was characterized by a negative value, leading us to conclude that this parameter can exert a reverse effect on this process and, after exceeding

a certain value, it may cause a decrease in the degree of incorporation. These findings are in agreement with our previous observations from phospholipid modification with phenylpropanoid molecules, which clearly indicate that the most crucial parameter in this process is the molar ratio of substrates followed by the enzyme dose and time of the reaction [45,46]. Furthermore, Zheng et al. also concluded that, during dual-responses, surface-optimized, lipase-catalyzed synthesis of ferulated diacylglycerols, among all tested variables, the most affecting one was substrate molar ratio [54].

Figure 3. (A) Correlation of actual and predicted degrees of incorporation of *p*-MCA into phospholipids. (B) Pareto chart of the effect of the selected reaction parameters on the incorporation of *p*-MCA into phospholipids.

Table 3. Analysis of variance (ANOVA) for interesterification variables pertaining to the response of percent incorporation of *p*-MCA to PC/LPC.

Evaluated Factors	Sum of Squares	Degrees of Freedom	Medium Square	F-Value	<i>p</i> -Value
(X ₁) Substrate molar ratio (L)	210.1250	1	210.1250	97.7326	0.000181
Substrate molar ratio (Q)	612.0577	1	612.0577	284.6780	0.000013
(X ₂) Enzyme load (L)	4.5000	1	4.5000	2.0930	0.207617
Enzyme load (Q)	62.8269	1	62.8269	29.2218	0.002929
(X ₃) Time of reaction (L)	0.1250	1	0.1250	0.0581	0.819037
Time of reaction (Q)	6.9808	1	6.9808	3.2469	0.131434
X ₁ by X ₂	0.0000	1	0.0000	0.00000	1.000000
X ₁ by X ₃	0.2500	1	0.2500	0.1163	0.746968
X ₂ by X ₃	0.0000	1	0.0000	0.0000	1.000000
Error	10.7500	5	2.1500		
Total error	874.4000	14			
$R^2 = 0.98771$					

In accordance with the Box-Behnken model, it is also possible to generate three-dimensional response surface plots, which allow for a pragmatic, and at the same time, comprehensible analysis of the relationship between input parameters (substrate molar ratio, enzyme loading, and reaction time) and the obtained response (degree of incorporation). In general, there are four types of three-dimensional plots for RSM analysis: Type I to Type IV [55]. Type I, also called “dome shaped”, is one of the most characteristic for the lipases-catalyzed process [55]. In typical dome-shaped charts, there is a slight increase (almost linear) in one axis and a critical point in the other axis, after exceeding which no further increase of the dependent variable is observed. Figure 4 illustrates surface plots for effect of the two various reaction variables: (A) Reaction time and enzyme loading, (B) substrate molar and enzyme loading, and (C) reaction time and substrate molar ratio on the incorporation of *p*-MCA into phospholipids, while the third independent is fixed at the central value. Based on Figure 4B,C, it can be observed that an increase in the ratio of PC/Ep-MCA results in an increase in the degree of incorporation but only until a critical concentration of substrate ratio is reached. It suggests that up to molar ratio 1/10 of PC/Ep-MCA the interesterification reaction is favored, but further increase in the concentration of the acyl donor no longer contributes to an increase of incorporation degree. It is

possible that, beyond this value, competing lipid substrates (especially released free fatty acids) bind to the enzyme and limit acyl–enzyme complex formation, thus reducing the efficiency of the desirable interesterification reaction. This phenomenon was also observed in our earlier investigations with lipase-catalyzed modifications of phosphatidylcholine [45,46,48,56]. In addition, on Figure 4A,C it is also visible that in the studied range there is no significant relationship between the reaction time and the degree of incorporation, and this factor is of little importance. Maximum incorporation (29 mol%) was achieved on the second day of the trial and remained stable until the fourth day. Subsequently, depicted on Figure 4, correlation between the incorporation and the content of the enzyme and the reaction time (Figure 4A) or ratio of the substance (Figure 4C) indicates that increases of the enzyme dose in the range of 20 to 30% increases the incorporation and then, after exceeding this value, slightly decreases. It can be explained that, when a certain value of biocatalyst was reached (30% *w/w*), a further increase in its dose does not cause improvements of the reaction productivity.

Figure 4. Response surface plots presenting effect of (A) time of reaction and enzyme loading, (B) enzyme loading and substrate molar ratio, (C) time of reaction and substrate molar ratio on the incorporation of *p*-MCA into phospholipids.

Summarizing, based on RSM analysis, the highest degree (29 mol%) of incorporation of *p*-MCA into PC/LPC fraction was achieved when all evaluated independent variables were as follows: Substrate molar ratio PC/Ep-MCA 1/10, enzyme dosage 30% *w/w*, and two days of the reaction time. Furthermore, changes in reaction time and enzyme content did not significantly affect the process, while the reduction of substrate molar ratio (1/5) or its increase (1/15) resulted in a high reduction of degree of incorporation.

2.4. Identification of the Reaction Products

Qualitative analysis of products of the reaction mixture was made using thin-layer chromatography (TLC) (Section 3.5.1) and high-performance liquid chromatography (HPLC). Products of reaction mixtures and standards (PC-egg yolk, LPC-egg yolk, Ep-MCA) were spotted on TLC plate and their R_f values were compared. As a result, we identified two new spots with R_f values of 0.03 and 0.35

different than R_f values of standards (PC-egg (0.45), LPC-egg (0.1), and *Ep*-MCA (0.69)). Their higher polarity being different than PC and LPC of egg yolk suggested that these were new products of the enzymatic modification. This hypothesis was further confirmed by HPLC and NMR analysis.

Degree of incorporation of *p*-MCA into phospholipid fraction (PC/LPC) was analyzed by gas chromatography (GC) (Section 3.5.2). Fatty acid composition of native PC and *p*-MCA-enriched phospholipid fraction are presented below in Table 4. Comparing acids' composition of native phosphatidylcholine and modified phospholipid fraction (PC/LPC), it is visible that egg-yolk PC contains much more saturated fatty acids, such as palmitic (16:0) and oleic (18:0) acid, than their structured fraction. It can be explained by the fact that these acids usually are distributed in the *sn*-1 position of egg-yolk PC and that during Novozym 435-catalyzed interesterification they undergo replacement by the *p*-methoxycinnamoyl acyl residue.

Table 4. Fatty acid composition (% according to GC) of native egg-yolk PC and phospholipid fraction obtained in the interesterification reaction of PC with *p*-MCA.

Fatty and 4MCA Acids	Native PC	Modified Phospholipid Fraction PC/LPC
C16:0 (PA)	34 ± 0.2	8 ± 0.6
C16:1 (OPA)	1 ± 0.7	1 ± 0.1
C18:0 (SA)	15 ± 0.9	8 ± 0.9
C18:1 (OA)	26 ± 0.7	23 ± 0.3
C18:2 (LA)	20 ± 0.3	22 ± 0.7
C20:4 (AA)	4 ± 0.2	9 ± 0.4
<i>p</i> -MCA	-	29 ± 0.4

Modified phospholipid fraction was next analyzed by normal-phase, high-performance liquid chromatography (HPLC) equipped with a UV/CAD detector (at 300 nm) according to the procedure described in Section 3.5.3. As can be seen from Figure 5, retention time of both new products' modified PC and modified LP were as follow: $R_f = 11.067$ and $R_f = 15.320$, respectively, for *p*-MCA-PC and *p*-MCA-LPC.

Figure 5. Chromatogram of fraction of modified phosphatidylcholine obtained by HPLC analysis with UV/CAD detector.

Based on the optimized parameters (reaction medium toluene/chloroform 9:1 *v/v*, enzyme dosage 30% *w/w* of Novozym 435, 1/10 substrate molar ratio PC/*Ep*-MCA, reaction time of two days, and reaction temperature 50 °C), the interesterification reaction of natural phosphatidylcholine with *Ep*-MCA was next performed on a larger scale. Phospholipids were extracted from the reaction mixtures and purified by column chromatography (Section 3.4). The major product, *p*-methoxycinnamoylated lysophosphatidylcholine (*p*-MCA-LPC), was obtained in (high) 32% of isolated yield. The second identified product isolated in trace amount, only in 3% isolated yield, was *p*-methoxycinnamoylated phosphatidylcholine (*p*-MCA-PC). The chemical structure of *p*-MCA-LPC was fully characterized by NMR (^1H , ^{13}C , ^{31}P) spectral data (Section 3.5.4). On the ^1H NMR spectrum protons from *p*-methoxycinnamic acid, including signals from aromatic ring (6.70–7.48 ppm), olefinic protons (6.14 ppm and 7.47 ppm) and protons of methoxy group (3.63 ppm) were identified. Also, protons

from phospholipid backbone ($-N(CH_3)_3$ (3.06 ppm), $CH_2-\beta$ (3.54 ppm), CH_2-3' (3.70–3.78 ppm), $H-2'$ (3.81 ppm), $-OH$ (3.88 ppm), CH_2-1' , and $CH_2-\alpha$ (4.00–4.08 ppm), were clearly detected (Supplementary Materials).

Scheme of the Novozym 435-catalyzed synthesis of *p*-methoxycinnamoylated phospholipids in the reaction of interesterification between the egg-yolk PC and *Ep*-MCA is proposed below (Figure 6). The process begins by being catalyzed by Novozyme 435 hydrolysis of native PC and formation of 2-acyl-LPC-egg yolk. In the next step, free hydroxyl group, in the *sn*-1 position of formed lysophospholipid, is esterified with an *p*-methoxycinnamic acid ethyl ester, giving the first product of the reaction, 1-(4-methoxy) cinnamoyl-2-acyl-*sn*-glycero-3-phosphocholine (*p*-MCA-PC). A probable reason for such a low efficiency in the *p*-MCA-PC synthesis is that 2-acyl-LPC is subjected to 1-acyl-LPC as a result of fatty acid migration from *sn*-2 to *sn*-1 position, leading to glycerophosphocholine (GPC), which is an intermediate product next transformed into the main product of the enzymatic interesterification *p*-MCA-LPC. This pathway, based mostly on the formation of GPC as the intermediate product, confirmed its presence in the reaction mixtures identified during TLC and HPLC analyses. Obtained results are in accordance with previously published data about the synthesis of phenophospholipids [45–47].

Figure 6. Possible changes occurring during the enzymatic interesterification of egg-yolk (PC) phosphatidylcholine with ethyl *p*-methoxycinnamate (*Ep*-MCA).

3. Materials and Methods

3.1. Substrates, Chemicals, and Enzymes

Ethyl *p*-methoxycinnamate (*Ep*-MCA) was synthesized (82% yield) according to the procedure described before [57] and its spectroscopic data were confirmed with the literature [58]. High, 98%, purity of *Ep*-MCA was verified by GC. Native egg-yolk phosphatidylcholine (PC), used as a substrate for interesterification reaction, was obtained and purified according to the previously presented protocol [56].

Immobilized lipases used in the research were purchased from Sigma-Aldrich from St. Louis, MO, USA (*Candida antarctica*: Novozym[®] 435 (>5000 U/g) and CALB (>1800 U/g)), Fluka from Buchs, Switzerland (*Rhizomucor miehei* Lipozyme[®] RM IM (>30 U/g)), and Novozymes A/S from Bagsvaerd, Denmark (*Thermomyces lanuginosus* Lipozyme[®] TL IM 250 U/g). The *trans* *p*-methoxycinnamic acid (4MCA), sodium methylate, and boron trifluoride methanol complex solution (13–15% $BF_3 \times MeOH$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All organic solvents used in the enzymatic reactions and chromatography, silica gel-coated aluminum plates (Kieselgel 6-F254, 0.2 mm), and the silica gel (Kieselgel 60, 230–400 mesh) used in the column chromatography were purchased from Merck (Darmstadt, Germany).

3.2. Enzymatic Interesterification of PC with Ep-MCA

At the beginning of the experiments, four lipases (Novozym 435, CALB, Lipozyme RM IM, and Lipozyme TL IM) were tested for interesterification reaction of egg-yolk phosphatidylcholine (PC) with ethyl *p*-methoxycinnamate (Ep-MCA). For this purpose, 20 mg of PC (0.026 mmol) were mixed on the magnetic stirrer (300 rpm) with Ep-MCA at 1/10 molar ratio in 2 mL of toluene and N₂ atmosphere. When the substrate solution reached reaction temperature of 50 °C, 30% of appropriate immobilized lipase (calculated by total weight of substrates) was added. In another set of experiments, the type of organic solvent (heptane, hexane, toluene, and toluene:chloroform 9:1 (*v/v*)) were also studied using Novozym 435 as a biocatalyst. All experiments were carried out in triplicate.

Intesterification reactions were stopped at the selected time intervals (1, 2, 3, and 4 days), by enzyme filtration on G4 Shott funnel with Celite layer. Then, phospholipids were separated from residue-unreacted ester and released fatty acids using silica gel columns (Discovery® DSC-Si SPE, 52654—U 500 mg) and SPE methodology [56]. Purified and concentrated phospholipid fractions were first qualitatively analyzed by thin-layer chromatography (TLC) (Section 3.5.1) and then their acid profile was quantitatively analyzed by gas chromatography (GC) procedure (Section 3.5.2).

3.3. Design of Experiment

In order to optimize reaction parameters, such as molar ratio of substrate, enzyme loading, and reaction time, 3-factor, 3-level Box-Behnken design was employed, which required 15 runs of experiments (Table 1). The independent variables in the selected Design of Experiment (DOE) of egg-yolk phosphatidylcholine interesterification with Ep-MCA were substrate molar ratio of PC/Ep-MCA (1/5, 1/10, 1/15), enzyme loading (20, 30, 40%), and reaction time (2, 3, 4 days), while the only dependent variable was incorporation [mol %] based on GC analysis. We used STATISTICA 13.3 (StatSoft, Inc.) software to generate the polynomial equation of the model (Equation (1)):

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{33} X_3^2 \quad (1)$$

where Y_i is predicted response, β_0 is model constant, X_1 – X_3 are independent variables, and β_1 – β_{33} are regression coefficients. All experiments were carried out in two independent analyses and the averages of incorporation were taken as response.

3.4. Large-Scale Interesterification of PC with Ep-MCA Catalyzed by Novozym 435

Intesterification of PC with ethyl *p*-methoxycinnamate was next successfully performed in the large scale. In this case, egg-yolk phosphatidylcholine (200 mg, 0.26 mmol) and Ep-MCA (at molar ratio PC/E4MCA, 1/10) were mixed in 20 mL of the solvent mixture of toluene/chloroform 9:1 (*v/v*) on a magnetic stirrer (300 rpm). Three-day reaction was carried out in N₂ atmosphere, at 50 °C, in a presence of 30% of Novozym 435. After this time, reaction mixture was filtered on G4 Shott funnel with Celite, and solvent was evaporated in vacuo. Reaction products were purified next by column chromatography, according to the procedure described before [45]. Purified products' fractions (PC-egg, LPC-egg, modified phosphatidylcholine (*p*-MCA-PC), and modified lysophosphatidylcholine (*p*-MCA-LPC)) were analyzed by TLC plates (Section 3.5.1), GC, and HPLC. The structure of major product, *p*-MCA-LPC, was confirmed NMR spectroscopy (¹H, ¹³C, ³¹P).

3.5. Analytical Methods

3.5.1. Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was employed to control interesterification reactions' progress and for qualitative analysis of the reaction products. Samples were dissolved in chloroform, spotted on TLC plates, eluted with the mixture of chloroform/methanol/water (65:25:4, *v/v/v*), and next identified

by spraying the TLC plates with the 0.05% primuline solution (acetone:water, 8:2, *v/v*) and then exposing them to UV light ($\lambda = 365$ nm).

3.5.2. Gas Chromatography (GC)

Analysis of acid profile of interesterification products (PC-egg, LPC-egg, *p*-MCA-PC, *p*-MCA-LPC) and standards (PC-egg, *p*-MCA) was performed by gas chromatography (GC). All purified samples by SPE or column chromatography methods were derivatized into corresponding methyl esters, according to the procedure described before [45]. Then methyl esters were analyzed on an Agilent 6890N instrument equipped with HP-88 column (100 m $\times$ 0.25 mm $\times$ 0.2 μ m), manufactured by Agilent (Santa Clara, CA, USA). Initial oven temperature was set at 110 $^{\circ}$ C, then raised to 200 $^{\circ}$ C (rate of 5 $^{\circ}$ C/min), and next to 250 $^{\circ}$ C at 2 $^{\circ}$ C/min, and held there for 5 min. The total analysis time was 48 min. The injector temperature and the flame ionization detector temperatures were set at 250 $^{\circ}$ C. Retention times of the fatty acid methyl esters (FAME) were compared with retention time of a standard FAME mixture (Supelco 37 FAME Mix) obtained from Sigma Aldrich. GC Chemstation Version A.10.02 was used for the quantitative analysis of *p*-methoxycinnamic acid incorporation into phospholipid fraction, calculated as mol% based on the peak areas.

3.5.3. High-Performance Liquid Chromatography (HPLC)

Phospholipid fraction, obtained from the optimized interesterification reaction performed in the larger scale, was analyzed by HPLC. To determine PC-egg, LPC-egg, *p*-MCA-PC, and *p*-MCA-LPC in the purified product mixture, DIONEX UltiMate 3000 chromatograph from Thermo Fisher Scientific (Olten, Switzerland) equipped with UV/CAD detector (at 300 nm) and BetaSil DIOL column (Thermo Scientific, 150 $\times$ 4.6 mm, 5 μ m) were used. In this analysis, the injection volume was 15 μ L, whereas autosampler and column temperature were 20 and 30 $^{\circ}$ C, respectively. The elution was set at constant flow (1.5 mL/min) and performed in a gradient: solvent A (1% HCOOH, 0.1% triethylamine (TEA) in water), solvent B (hexane), and solvent C (2-propanol). The elution program was as follows: 3/40/57 (%A/%B/%C (*v/v/v*)), at 5 min = 10/40/50, at 9 min = 10/40/50, at 9.1 min = 3/40/57, and at 19 min = 43/40/57. Total analysis time was 19 min.

3.5.4. Spectroscopic Spectra (NMR)

In order to confirm the structure of obtained products, NMR analysis was conducted. However, due to the trace amount of the obtained modified PC (*p*-MCA-PC), NMR experiments were performed only for modified LPC (*p*-MCA-LPC). A sample of purified *p*-MCA-LPC was dissolved in 0.6 mL of CDCl₃/MeOH (2:1, *v/v*) in NMR tube and then analyzed on a Bruker Advance II 600 MHz spectrometer (Bruker, Billerica, MA, USA).

1-(4-methoxy) cinnamoyl-2-hydroxy-sn-glycero-3-phosphocholine (*p*-MCA-LPC)

Colorless, greasy solid (32% yield, R_f 0.03); ^1H NMR (600 MHz, CDCl₃/CD₃OD 2:1 (*v/v*)), δ : 3.06 (s, 9H, -N(CH₃)₃), 3.54 (s, 2H, CH₂- β), 3.63 (s, 3H, -OCH₃), 3.70-3.78 (m, 2H, CH₂-3'), 3.81 (s, 1H, H-2'), 3.88 (s, 1H, -OH), 4.00-4.08 (2m, 4H, CH₂-1', CH₂- α), 6.14 (d, 1H, $J = 15.9$ Hz, H-2), 6.71 (m, 2H, H-3'', H-5''), 7.30 (m, 2H, H-2'', H-6''), 7.48 (d, 1H, $J = 15.9$ Hz, H-3); ^{13}C NMR (150 MHz, CDCl₃/CD₃OD 2:1 (*v/v*)) δ : 54.11 ((-N(CH₃)₃), 55.22 (-OCH₃), 59.64 (C- α), 63.75 (C-1'), 64.95 (C- β), 66.24 (C-3'), 68.57 (C-2'), 113.38 (C-2), 114.36 (Ar), 126.76 (Ar), 129.87 (Ar), 145.61 (C-3), 161.73.21 (Ar), 167.80 (C-1); ^{31}P NMR (243 MHz, CDCl₃/CD₃OD 2:1 (*v/v*)) δ : -5.08.

4. Conclusions

In the present paper, we developed a novel biotechnological method for the synthesis of phospholipids structured with *p*-methoxycinnamic acid. During the studies, we proved that Novozym 435 is a lipase preparation that was the most effective among the tested biocatalysts and can be

successfully applied for the process of lyophilization of *p*-methoxycinnamic acid. In addition, we clearly demonstrated that course of incorporation of *p*-MCA into phospholipids varied during the time and strongly depended on the reaction mixture composition and enzyme load. In order to optimize crucial parameters, we conducted experiments according to Box-Behnken model and, based on these, we were able to observe that the most important factor was molar ratio of substrates. HPLC and NMR analyses confirmed that the products of modification were *p*-MCA-LPC, which was formed in 32% isolated yield and *p*-MCA-PC, which was isolated only in a trace amount (yield = 3%). Both of these products have not yet been described in the literature and are promising in the context of production of new nutraceuticals based on the natural products.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4344/10/10/1181/s1>, Figure S1: ¹H NMR spectrum of *p*-MCA-LPC, Figure S2: ¹³C NMR spectrum of *p*-MCA-LPC, Figure S3: ³¹P NMR spectrum of *p*-MCA-LPC, Figure S4: GC chromatogram of PC-egg (standard), Figure S5: GC chromatogram of *p*-MCA (standard), Figure S6: GC chromatogram of PLs

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