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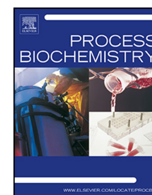

B. cereus phospholipase C engineering for efficient degumming of vegetable oil

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ABSTRACT

Enzymatic phospholipid removal (degumming) is a fast-growing and environmentally friendly process for vegetable oil refining. Type C phospholipases (PLC) are the preferred enzymes since they provide an extra yield in the oil recovery.

Bacillus cereus PLC can hydrolyze phosphatidylcholine (PC) but has a limited efficiency at removing phosphatidylethanolamine (PE), which together represent ∼70% of the phospholipids present in crude soybean oil. In the present work, we show that the *B. cereus* PLC mutant F66Y can remove up to 90% of PE while retaining its efficiency at hydrolyzing PC. Oil treatment with the engineered enzyme provides an extra yield of 1.84% making the *B. cereus* PLC F66Y mutant an attractive candidate for its industrial use.

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1. Introduction

Crude vegetable oil, obtained by press or solvent extraction, is a complex mix of triglycerides, phospholipids, sterols, tocopherols, free fatty acids, metallic traces and other minor compounds [1]. The process of oil refining includes several steps, being the first one the removal of phospholipids, or "oil degumming", which causes the major losses in the industrial process of oil refining [2].

Phospholipids can be partially or completely removed from oil using different methods including water degumming, acid degumming or enzymatic degumming [2,3]. Crude soybean oil may contain phospholipids in the range of 1.75–3%, depending on the extraction method used. The relative concentrations of different phospholipid species are not constant and large variations in the amounts of PC (35–47%), PE (20–30%), PI (20–24%) and PA (9–15%) are observed in different production facilities. The amount of each class of phospholipid in crude oil depends not only on the quality of the beans, but also on the seed preparation and oil extraction processes [1,2].

Enzymatic degumming has been employed using a wide variety of enzymes to hydrolyze phospholipids, generating products that are more easily removed by centrifugation [4–9]. In addition, as the phospholipids content is reduced, there is less oil trapped by the gums. Thus, upon enzymatic treatment the overall yield increases, which represents a significant economic benefit for the oil industry [3,10]. The use of phospholipase C enzymes in oil degumming has lately received considerable attention as they can produce an extra yield of refined oil not only by reducing the total mass of neutral oil retained in the phospholipid gums, but also by generating diacylglycerol, which is miscible with oil [3,11,12].

The phosphatidylcholine preferring phospholipase C from *Bacillus cereus* (PLC_{BC}) is a monomeric 28.5 kDa exoenzyme with three zinc ions in its active site [13–15]. Hydrolysis of phospholipids by PLC_{BC} produces diacylglycerol and a phosphate monoester. PLC_{BC} preferentially catalyzes the hydrolysis of PC, but it also hydrolyzes PE and phosphatidylserine (PS) with lower catalytic efficiencies [16]. The structures of native PLC_{BC} as well as PLC_{BC} complexes with different ligands have been solved by x-ray crystallography [14,17]. These structures have revealed that amino acids E4, Y56 and F66, are each within 5 Å of the trimethylammonium cation of the substrate head group, thus comprising the choline binding pocket. Site specific mutants for these amino acids have been studied to determine the contributions that these residues make to substrate

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recognition and specificity [16,18]. The substrate specificity of these mutant enzymes was studied in aqueous media with the water-soluble substrates 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (C6PC) and 1,2-dihexanoyl-*sn*-glycero-3-phosphoethanolamine (C6PE) [16,18]. In these studies, PLC_{BC} mutants F66Y and F66W displayed better kinetic parameters with the C6PE substrate than the *wild type* enzyme suggesting that this residue alters substrate specificity.

In the present study, we performed degumming experiments on crude soybean oil with *wild type* PLC_{BC} and mutants F66W and F66Y recombinantly expressed in *P. pastoris*. We show that the mutant F66Y greatly increases soybean oil degumming efficiency, making this engineered enzyme an attractive candidate for its industrial use.

2. Materials and methods

2.1. Site directed mutant plasmid construction

Plasmid pJ912 was purchased from DNA 2.0. A synthetic version of PLC gene derived from *Bacillus cereus* (PLC_{BC}) was codon optimized and synthesized by Genscript (GenBank accession no. KX881921). To obtain plasmid pJ912:$PLC_{BC,}$ the synthetic PLC gene was digested with *Xho*I and *Not*I and ligated into pJ912 vector for secretory expression.

pJ912:PLC_{BCY} pJ912:PLC_{BCW} were obtained by the site-directed mutagenesis protocol described by Horton et al. [19] using primers PLCfw (GACCTCGAGAAAAGATGGTCGGCGG), PLCrev (GACTCTAGATTAACGGTTGCCGTAAG) F66Yfw (GATGACTCTACCTACGCGTCACATTTC), F66Yrev (GAAATGTGACGCGTAGGTAGAGTCATC), F66Wfw (GATGACTCTACCTGGGCGTCACATTTC), and F66Wrev (GAAATGTGACGCCCAGGTAGAGTCATC). The resulting plasmids were all confirmed by DNA sequence analysis (Macrogen, Corea).

2.2. Protein expression and purification

PLC_{BC}, PLC_{BCY} and PLC_{BCW} were expressed and purified according to Elena et al. [20]. Briefly, plasmids pJ912:PLC_{BC}, pJ912:PLC_{BCY} pJ912:PLC_{BCW} were transformed by electroporation to *Pichia pastoris* strain GS115H according to Cregg et al. [21]. Highly expressing clones were selected in YPD plates containing 0.3% soybean phospholipids and further assayed for integrated plasmid copy number using real time-PCR [20,22]. Selected clones were grown in 1 l fermenter. The fermentation inoculum of *P. pastoris* was prepared by cultivating the cells at 30 °C for 20 h in a shaking flask containing YPD medium. This culture was diluted 1/10 in fermenter (Infors Labfors 4, Switzerland) with 900 ml of the BSM medium (Invitrogen), 40 g of glycerol and 4.35 ml of PTM1 trace salts solution per liter. PMT1 solution is composed of 6 g/l $CuSO_4{\cdot}5H_2O$, 0.08 g/l NaI, 3 g/l $MnSO_4{\cdot}H_2O$, 0.2 g/l $Na_2MoO_4{\cdot}2H2O$, 0.02 g/l H_3BO_3, 0.5 g/l $CoCl_2$, 20 g/l $ZnCl_2$, 65 g/l $FeSO_4{\cdot}7H_2O$, 0.2 g/l biotin and 0.5% v/v H_2SO_4. Throughout the entire cultivation process, temperature was maintained at 30 °C, pH was adjusted to 5 using NH_4OH 28%, dissolved oxygen was maintained over 20% of air saturation by controlling the agitation speed and the air flow rate (or pure oxygen when required) was kept at 0.5 VVM. The fermentation process comprised three different phases: the glycerol batch, the glycerol fed batch phase and the methanol fed batch phase. Briefly, once the initial glycerol from the batch culture was consumed and the OD600 reached approximately 150, a fed batch was started with a solution containing glycerol 50% w/v and 1.2% of PTM1. After 4 h of feeding with a constant flow rate of 18.1 ml/l h the induction phase was started with a solution containing methanol 100% and 1.2% of PTM1. The feed rate was started at 3.6 ml/l h during 4 h, then the flow rate was doubled to 7.2 ml/l h for the next 2 h (adaptation phase). Once the culture was fully adapted to methanol utilization, a constant flow rate of 11 ml/l h was maintained until the end of the process (35 h of induction). Samples were taken regularly to monitor biomass and protein production by phospholipase activity tests and quantified by SDS-PAGE analysis using BSA (Sigma Aldrich) as standard. Cultures were centrifuged and culture supernatant containing the secreted protein was ultrafiltrated and diafiltrated using Xampler Microfiltration cartridge (GE). The resulting protein preparation was concentrated up to 20 g/l and kept in 50 mM HEPES pH 7, 1 mM $ZnCl_2$ and 35% glycerol. Similar yields and purity were obtained with the three purified enzymes.

2.3. Kinetic comparison of PLC_{BC}, PLC_{BCW} and PLC_{BCY} with O-(4-Nitrophenyl-phosphoryl) choline as a substrate

PLC activity was measured in 96 well microplates using 1 mM *O*-(4-Nitrophenylphosphoryl)choline as a substrate and 2.2 μM of each PLC in buffer 250 mM HEPES pH 7, 60% sorbitol, 0.1 mM $ZnCl_2$ [23]. Absorbance at 405 nm was monitored for 1 h at 50 °C. To determine V_{max} and K_m, PLC activity was measured using 1.2 μM enzyme. Different substrate concentrations (50, 20, 10, 5, 2, 1, 0.5 and 0.1 mM) prepared in buffer 250 mM HEPES pH 7, 60% sorbitol, 0.1 mM $ZnCl_2$ were used and absorbance at 405 nm was monitored for 1 h at 50 °C. V_0 was determined for each substrate concentration and V_{max} and K_m were estimated from a V_0 vs [S] curve.

2.4. Kinetic comparison of PLC_{BC}, PLC_{BCW} and PLC_{BCY} in oil

In order to compare the activity of wild type and mutant enzymes in oil, 3 g of crude soybean oil containing 2.5% phospholipids (1000 ppm phosphate) was homogenized (1 min using Ultra-Turrax T8 Homogenizer, IKA) with 15 μg of PLC in 90 μl of buffer HEPES 15 mM pH7, 1 mM $ZnCl_2$, 10% glycerol. Each tube was incubated at 55 °C with constant agitation (VP 710 magnetic tumble stirrer, VP-Scientific). At different time points (5–10-20-40-60–120 min) the oil was homogenized and 200 μl of the homogenized oil were mixed with 200 μl of 2 M Tris-HCl pH 8 to stop the PLC reaction. Then, 800 μl of water was added, incubated for 1 h at 37 °C with constant agitation and centrifuged 5 min at 14,000*g* 45 μl of the aqueous phase were recovered and treated with 0.3 U of calf intestinal phosphatase (Promega, WI, USA) for 1 h at 37 °C following to the manufacturer instructions. Finally, inorganic phosphate was determined according to the method of Sumner [24]. Briefly, a 500 μl sample, containing 0.025–0.25 μmol of inorganic phosphate in 5% TCA was mixed with 500 μl of color reagent (4% $FeSO_4$, 1% $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 3.2% H_2SO_4). Spectrophotometric readings were made at 700 nm, and the micromoles of inorganic phosphate in the sample were calculated from a standard curve.

2.5. Enzymatic oil degumming

Oil degumming experiments were performed using 2 kg of crude soybean oil (1000 ppm phosphate), 3% H_2O and 10 mg of the different PLCs obtained. The oil was emulsified with water using an Ultra-Turrax T 50 Homogenizer (IKA) for 1 min, and the containers incubated with continuous stirring with a magnetic stirrer at 55 °C for 2 h. Finally, the enzyme was heat-inactivated at 85 °C for 10 min and the oil centrifuged at 3000*g* for 10 min to remove the remaining gums. Gums and recovered oil were weighed to determine oil yield. 1,2-diacylglicerol (1,2-DAG) content determination was performed as described [25].

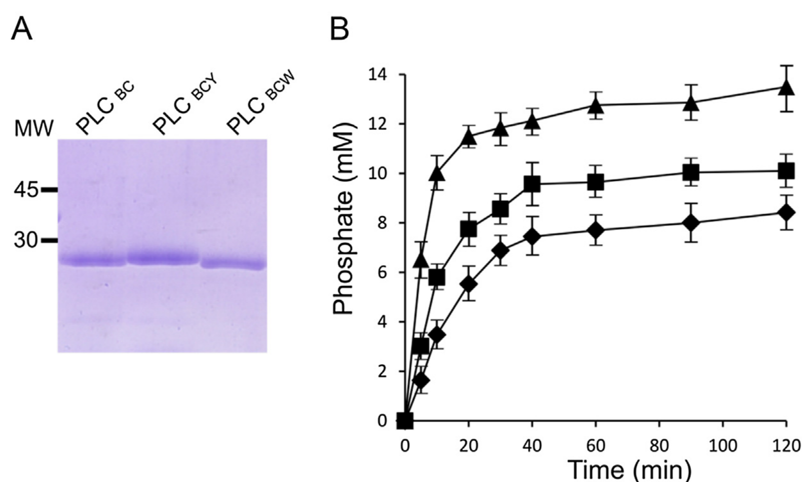

Fig. 1. PLC expression analysis and activity tests in crude soybean oil. A. SDS-PAGE analysis of culture supernatants from *Pichia pastoris* strains carrying plasmids pJ912:PLC_{BC} (PLC_{BC}, lane1), pJ912:PLC_{BCY} (PLC_{BCY}, lane2) and pJ912:PLC_{BCW} (PLC_{BCW}, lane 3). MW stands for molecular weight marker. B. Phospholipase activity of PLC_{BC} (diamonds) and mutants PLC_{BCY} (triangles) and PLC_{BCW} (squares) determined in crude soybean oil as phosphate concentration. 15 μg of each enzyme was used for oil degumming in 3 g of crude oil samples. Inorganic phosphate values are proportional to PLC phospholipids hydrolysis. Values shown are means of three independent experiments. The standard deviations were in all the cases less than 10% of the corresponding means.

2.6. NMR analysis of crude and treated oil

Treated oil was emulsified using an Ultra-Turrax T 50 Homogenizer (IKA) for 1 min before taking 300 mg samples for further analysis. Oil samples were extracted with 900 μl of NMR solution (100 mM Tris-HCl pH 10.5, 50 mM EDTA, 2.5% sodium deoxycholate) during 1 h at 37 °C with constant agitation [26]. Finally, the resulting aqueous phase was extracted with 600 μl hexane and analyzed by NMR. NMR spectra were acquired using a Bruker Avance II 600 MHz spectrometer. Samples of phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid and phosphatidylinositol were run as standards. NMR spectra were integrated to determine peak areas using TopSpin 3.5 pl5 software. PI was used as an internal standard for calculation of the remaining phospholipids fractions. Values in crude oil were considered as 100% to estimate the percentage of hydrolyzed PC and PE in enzyme treated oil.

3. Results

3.1. Oil degumming with B. cereus wt PLC

B. cereus wt PLC (PLC_{BC}) is a highly stable protein with an optimum activity temperature of 50–60 °C, two appealing features for an enzyme to be used in industrial enzymatic oil degumming [13,27,28]. Although not all phospholipids present in crude soybean oil are substrate of PLC_{BC}, PC and PE account for approximately 70% of total soybean oil phospholipids and can be hydrolyzed by this enzyme. Based on a previous techno-economic analysis (unpublished data), we assumed that to establish a cost efficient degumming process, the enzyme must be dosed at a concentration below 5 g per ton of oil and the hydrolysis should be complete in less than 2 h.

PLC_{BC} was produced as a secreted protein in high cell density cultures of *P. pastoris* as a fusion to α-mating factor and recovered from the culture supernatant by ultrafiltration and diafiltration giving a highly pure enzyme preparation (Fig. 1A).

In order to evaluate the suitability of this enzyme for oil degumming reactions, experiments in small scale using 3 g of crude soybean oil were performed, simulating the conditions used for enzymatic oil degumming on industrial scale (3% H_2O, 55 °C, 120 min with constant agitation). Quantification of inorganic phosphate generated from polar heads groups of hydrolyzed phospholipids was used as a direct measure of phospholipase C activity as described in Materials and Methods. After one hour of PLC_{BC} treatment at 55 °C there was no significant increase in phosphate concentration (Fig. 1B).

NMR analysis was performed to analyze the composition of the remaining phospholipid fraction. The NMR spectra for treated oil and a control where no enzyme was added are shown in Fig. 2. In these spectra, phospholipids and their hydrolysis products (phosphocholine (pCho) and phosphoethanolamime (pEt)) can be quantified. It can be observed that upon treatment with PLC_{BC} the peak corresponding to PC is no longer detectable indicating that PC hydrolysis was complete. However, 52% of PE remained in oil demonstrating that enzymatic treatment with PLC_{BC} was not equally efficient for PE removal (Fig. 2B).

3.2. Activity assessment of PLC_{BC} F66Y and F66W mutants

PLC_{BC} mutants F66Y and F66W have been shown to be more efficient than the *wild type* enzyme at hydrolyzing 1,2-dihexanoyl-sn-*glycero*-3-phosphoethanolamine (C6PE) in aqueous reactions, a substrate analogous to PE [14,16]. We therefore decided to replace PLC_{BC} residue F66 with W or Y and explore their PLC activity in crude soybean oil with natural phospholipids. With this purpose, the genes encoding for these enzymes were engineered by site-directed PCR mutagenesis and the mutant proteins, PLC_{BCW} and PLC_{BCY}, produced in *P. pastoris* and purified as described in materials and methods section (Fig. 1A).

To initially characterize the engineered variants, kinetic parameters for the three enzymes were determined in an aqueous assay with *O*-(4-nitrophenylphosphoryl) choline as substrate. As shown in Table 1, both mutant enzymes showed better catalytic efficiency than the *wild type* PLC_{BC}, defined by a significant increase in the k_{cat}/K_m parameter, with PLC_{BCW} showing a slightly higher catalytic efficiency.

Table 1
Kinetic parameters of PLC_{BC}, PLC_{BCW}, PLC_{BCY} with *O*-(4-nitrophenylphosphoryl) choline in aqueous media.

	PLC_{BC}	PLC_{BCW}	PLC_{BCY}
K_m (mM)	39.5 ± 0.4	14.8 ± 0.2	17.4 ± 0.2
k_{cat} (s^{-1})	3.9 ± 0.3	5.4 ± 0.1	4.8 ± 0.1
k_{cat}/K_m (s^{-1} M^{-1})	98 ± 7	365 ± 8	276 ± 6

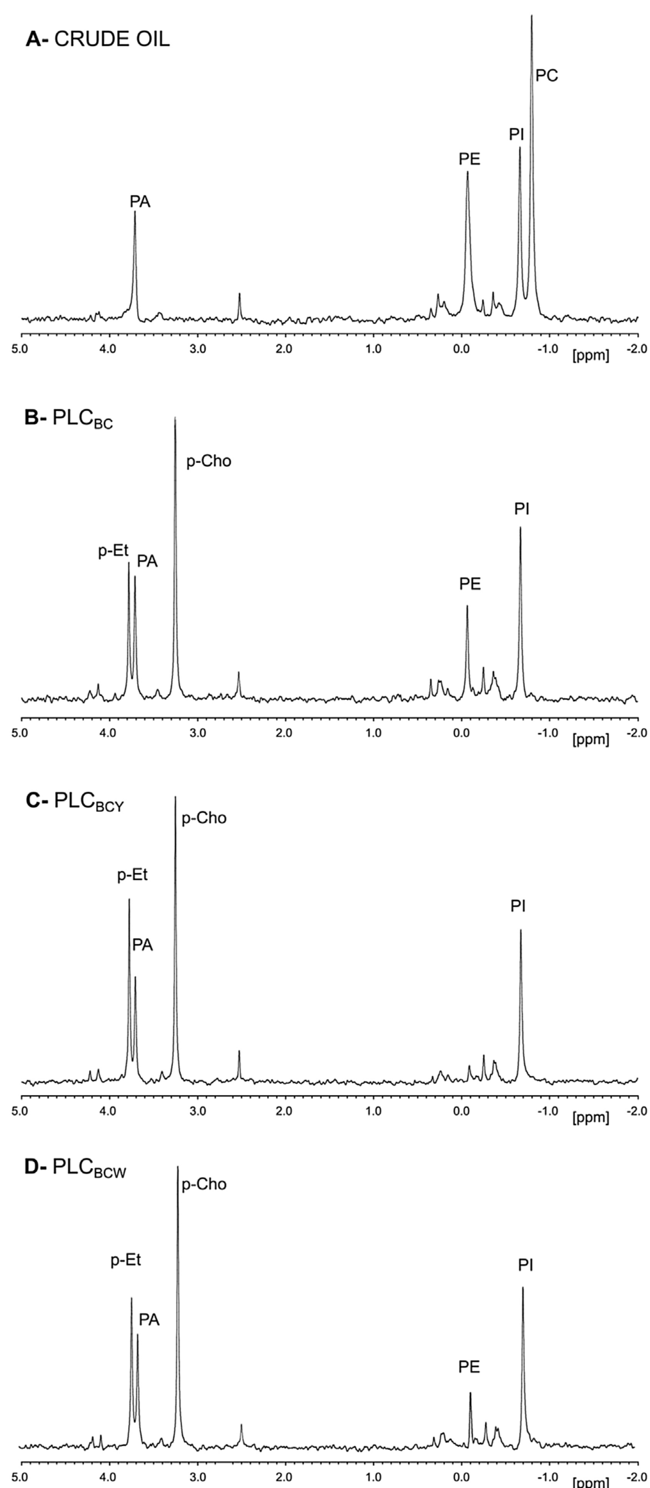

Fig. 2. NMR analysis of soybean oil phospholipids. Crude oil (A) or oil treated for 120 min with PLC_{BC} (B), PLC_{BCY} (C) or PLC_{BCW} (D). Phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidic acid (PA). Hydrolysis products: phosphocholine (pCho) and phosphoethanolamime (pEt).

These results prompted us to evaluate the performance of these mutants in edible oil treatment. For this, enzymatic assays of PLC_{BC} and mutants were performed in crude oil and measured via inorganic phosphate quantification as described above. The results shown in Fig. 1B demonstrate that both mutant enzymes displayed a higher activity in oil than *wild type* PLC_{BC}. In this reaction medium, PLC_{BCY} showed superior catalytic properties, indicating that the activity in aqueous environment is not an accurate predictor of the efficiency of the mutant enzymes for the enzymatic degumming of oils.

A time course NMR analysis was performed to analyze the specificity of the reaction catalyzed by the different enzymes through the evaluation of the remaining phospholipid fraction after treatment (Fig. 3 and Supplementary Figs. 1–3 in the online version at DOI: 10.1016/j.procbio.2017.01.011). Fig. 3 shows that degumming with PLC_{BC} or either mutant enzyme completely hydrolyzed PC after 60 min. However, when analyzing short reaction times (10 or 20 min) it can be observed that mutants PLC_{BCY} or PLC_{BCW} showed a higher reaction rate compared to the *wild type* PLC_{BC}. For PE hydrolysis, PLC_{BCY} showed a higher efficiency, hydrolyzing 91% of PE at 120 min compared to 68% for PLC_{BCW} and 49% for PLC_{BC} under identical conditions (Fig. 3). Taken together, these results indicate that both engineered enzymes were more efficient than the *wild type* PLC_{BC} hydrolyzing PC and PE in crude oil, being PLC_{BCY} the best candidate to be evaluated in larger scale experiments.

3.3. Enzymatic oil degumming with PLC_{BCY}

To further assess the performance of the recombinant PLC_{BCY} and analyze the extra yield of oil provided by the enzymatic treatment, larger scale degumming experiments were performed. Two kg of crude soybean oil containing 2.5% phospholipids (1000 ppm phosphate) were treated with 10 mg of recombinant PLC_{BCY}. The oil-enzyme mixture was emulsified with a high-shear mixer and incubated at 55 °C for two hours with constant agitation. After treatment, the enzyme was inactivated at 85 °C for 10 min and the reaction efficiency was first evaluated measuring 1,2-DAG concentration (Fig. 4A). The increase in 1,2-DAG concentration up to 1,13% is in accordance with the theoretical value of 1,18% calculated for 100% hydrolysis of PC and 91% hydrolysis of PE obtained in the small scale experiments. The increase in oil yield, measured as the difference between the weight of gums obtained with water degumming and enzymatic treatment, gave a value equivalent to 1.84% (Fig. 4B). This extra yield results from the mass of 1,2-DAG generated from the hydrolysis of PC and PE that remain in the oil phase (1,13%) plus an additional increase due to the oil released as a consequence of having a lower volume of gums. NMR analysis shows that PC hydrolysis was complete, while 87% PE was hydrolyzed, indicating that similar reaction efficiencies were obtained at both scales (Fig. 4C). Finally, residual phosphorous levels as well as free fatty acid content were determined in the enzyme treated oil. The values obtained (91 ppm phosphate and 0.95% free fatty acid) are within the commercial specifications for degummed oil.

4. Discussion

The use of enzymes as an alternative to chemical processes often provides a cleaner solution for industrial processes [29,30]. However, natural enzymes do not always display the characteristics required to establish a robust industrial process. Enzyme features such as catalytic efficiency, substrate selectivity, stability, optimal pH and temperature, can be altered using protein engineering, which represents a key tool in modern biotechnology [31]. The use of enzymes as auxiliary agents in vegetable oil degumming provides an increased oil yield in the refining process with the consequent economic impact [3]. With PLC enzymes, phospholipids are hydrolyzed to oil-soluble diacylglycerol and water-soluble phosphate esters. Since diacylglycerols remain in the oil during refining, they contribute to the oil yield. In addition, enzymatic treatment decreases the amount of gums and therefore less oil is retained also contributing to an improved oil yield.

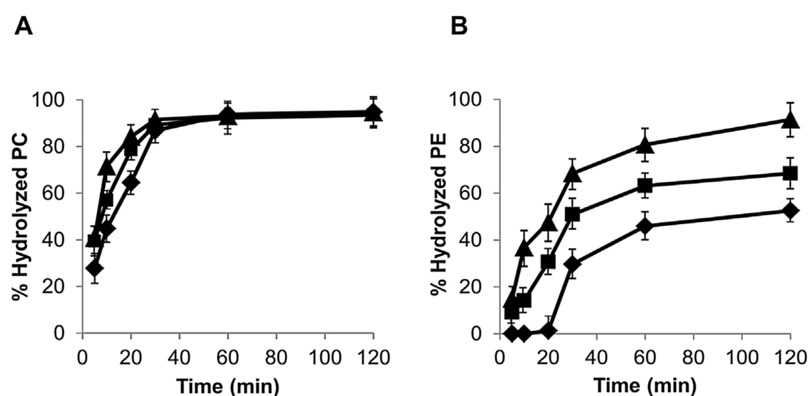

Fig. 3. Time course NMR analysis of soybean oil phospholipids. Hydrolyzed phosphatidylcholine (A) or phosphatidylethanolamine (B) was quantified from NMR spectra. Crude soybean oil was treated with PLC_{BC} (diamond), PLC_{BCY} (triangle) or PLC_{BCW} (square), samples were taken at 5, 10, 20, 30, 60 or 120 min of reaction and remaining phospholipids were analyzed by NMR.

Although PLC_{BC} is a robust enzyme with high thermostability, the activity of this phospholipase is suboptimal for industrial oil degumming processes. Through site-directed mutagenesis, we constructed the mutant F66Y (PLC_{BCY}) which displayed a higher efficiency at hydrolyzing PC and PE in oil. Our findings show that PLC_{BCY} mutant can hydrolyze 40% more PE than the wild type using the same dose of enzyme, which provides a significant economic benefit for industrial oil degumming.

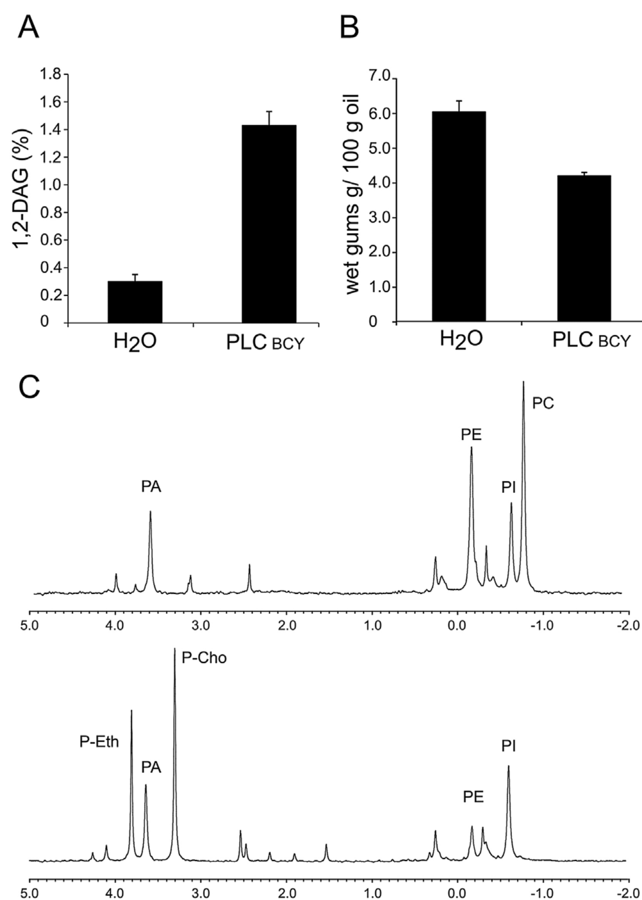

Fig. 4. Evaluation of soybean oil degumming by PLC_{BCY}. A – 1,2 DAG content in soybean oil treated with water (control) or PLC_{BCY}. B – Residual wet gum percentage after water or enzymatic treatment of crude soybean oil. C – Remaining phospholipids profile obtained by NMR analysis. In all cases, 2 kg of crude soybean oil were treated with PLC_{BCY} and different aliquots were taken for the respective analysis. Control sample without the addition of the enzyme was run in parallel as control. Values shown are means of three independent experiments. Error bars correspond to standard deviations.

The time course NMR analysis shows that PE hydrolysis by PLC_{BC} occurs after most PC has been already hydrolyzed, demonstrating the substrate preferences of the enzyme in the heterogeneous reaction environment. In contrast, both mutant enzymes can hydrolyze PE even when PC is still present, thus establishing the alteration in substrate selectivity brought about by the mutation. All three enzymes are able to hydrolyze both PC and PE, but the higher catalytic efficiency and the substrate promiscuity of PLC_{BCY} and PLC_{BCW} allow for a larger extent of the reaction to take place in a fixed amount of time. Although PLC_{BC} has been shown to be stable at 50–60 °C, at this reaction temperature (55 °C) the PLC enzymes become partially inactivated when incubated for long periods. Less than 10% residual PLC activity was recovered after two hours in oil at 55 °C for any of the three PLC proteins (data not shown). This could explain the different extent in PE hydrolysis obtained for PLC_{BC}, PLC_{BCY} and PLC_{BCW} after two hours of reaction at 55 °C.

PLC_{BC} mutant enzymes F66Y and F66W showed different behavior with the colorimetric substrate *O*-(4-nitrophenylphosphoryl) choline and natural soybean oil phospholipids. While mutant F66W showed better kinetic parameters in the aqueous assay, F66Y was the most efficient hydrolyzing phospholipids in oil. These results show that enzyme screening methods based on an artificial substrate cannot guarantee their efficacy towards the natural substrate. Small scale oil degumming assays using soybean oil may provide a new alternative for phospholipase screening and selection.

In order to obtain an economical benefit from enzymatic oil degumming, the oil yield increase in the degumming process must be greater than the enzyme cost plus additional energy and capital costs required in the plant. Enzymatic degumming with PLC_{BCY} resulted in extra yield of oil recovery of 1.84% compared to water degumming process. At the current oil price ($800 per ton), the treatment using this novel enzyme would provide an additional benefit of near $14.7 per ton of oil. We have recently designed a process for the efficient production of this novel enzyme for oil degumming, by combining strain engineering and fed batch fermentation development [20].

Scaling up of this fermentation process should provide large quantities of this novel enzyme at affordable prices (PLC dose for one ton of oil below $1) for the adoption of enzymatic oil degumming on industrial oil refining plants. Considering operative and capital costs for oil degumming, the net benefit is estimated to be above $10 per ton of treated oil, representing an appealing advantage for the adoption of enzymatic oil degumming on industrial oil refining plants.

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