Two-stage Enzymatic Hydrolysis of Soybean Concentrate Phospholipid to Prepare Glycerylphosphorylcholine: Optimized by Response Surface Methodology

Shaohua Liang, Yameng Liu, Yannan Meng, and Cong Sun*

College of Food Science and Engineering, Henan University of Technology, Lianhua Road 100, Zhengzhou 450001, Henan Province, P. R. CHINA

Abstract: A two-stage enzymatic hydrolysis method, in which phospholipase A₁ (PLA₁) was added after phospholipase A₂ (PLA₂) was added for a certain time, was successfully carried out to prepare glycerylphosphorylcholine (GPC) from soybean concentrated phospholipid. Effects of reaction variables on hydrolysis reaction were optimized using response surface methodology, and the optimal conditions were as follows: PLA₂ load of 1.25%, PLA₁ load of 0.70%, substrate concentration of 13%, reaction temperature of 41°C, and stirring rate of 680 rpm. Under the optimal conditions, the GPC yield reached 83.07%, which is close to the predicted value by the fitted model. This paper not only provides an efficient and low-cost method to prepare GPC, but also improves the high-value utilization of soybean concentrated phospholipid.

Key words: glycerylphosphorylcholine, soybean concentrated phospholipid, phospholipase A₂, phospholipase A₁, response surface methodology

1 Introduction

Glycerylphosphorylcholine (GPC) is a hydrolysis product of phosphatidylcholine (PC). GPC is not only a precursor of the neurotransmitter acetylcholine and PC, but also a precursor of membrane phospholipid. Some studies have shown that the insufficient intake of choline will affect the structures and functions of human brain, and even lead to some diseases such as neural tube defects. GPC can treat Alzheimer’s disease by promoting the secretion of growth hormone, and can also be used to treat cognitive disorders, schizophrenia, and affective disorders by increasing individual hormone release. In addition, GPC also has the functions of treating cardiovascular diseases and promoting non-REM sleep. Therefore, GPC is considered an essential nutrient for humans, and is recommended for daily intake.

With the further studies on the clinical value of GPC in medical care, many methods of GPC preparation have been discovered, the representatives of which are chemical preparation and enzymatic hydrolysis. Although chemical preparation is characterized by high recovery and purity, there are some deficiencies, such as complex synthesis process, long reaction time, and high-impurity compounds. GPC is prepared by enzymatic hydrolysis of phospholipase and modification of phospholipid structure by specific acyl receptors and donors, which has the characteristics of environmental protection and high efficiency. Therefore, there has been increasing interest in the preparation of GPC by enzymatic hydrolysis in recent years. In general, single phospholipase or lipase is used for GPC preparation. Bang et al. mixed n-hexane-water and PC 9:1 in a certain proportion (21.3 g/100 mL), and then added 13% phospholipase A₁ (PLA₁; according to PC weight) at 50°C for 30 h to prepare GPC. In order to improve the solubility of PC in the aqueous phase, Lu et al. investigated the effects of various surfactants on the GPC preparation using PLA₁ as catalyst, and found that Tween 20 was the most effective surfactant. Kim et al. hydrolyzed soy phosphatidylcholine or a fractionated soy lecithin via Novozyme 435 in hexane-water biphasic media for GPC preparation. Zhang et al. used Rhizopus chinensis lipase to catalyze the deacylation of PC for GPC preparation. Although high yield of GPC could be obtained with single enzyme, high-purity raw material was required, which would increase the production cost and was not conducive to industrial production. In addition, the catalysis of single enzyme is ineffi-
cient in theory due to the occurrence of acyl migration\textsuperscript{20}. Therefore, some studies have attempted to prepare GPC under the concerted catalysis of phospholipase and lipase, but the reaction time was quite long\textsuperscript{17}. In theory, PLA\textsubscript{1} and phospholipase A\textsubscript{2} (PLA\textsubscript{2}) are sn-1 and sn-2 specific phospholipases, the combinational catalysis of which is effective for GPC preparation\textsuperscript{21, 22}. According to our previous study, compared with the concerted catalysis of PLA\textsubscript{1} and PLA\textsubscript{2}, the catalytic efficiency of the method, in which PLA\textsubscript{1} was added after PLA\textsubscript{2} was added for a certain time (PLA\textsubscript{2}→PLA\textsubscript{1}), was more beneficial to improve the GPC yield and shorten the reaction time\textsuperscript{20}.

In terms of GPC preparation, high-purity soybean powder phospholipid or lecithin phospholipid is generally used as raw material\textsuperscript{24}, which leads to high cost. In comparison, soybean concentrated phospholipid (SCP), which is a primary by-product of soybean oil processing, has high yield and low price. Moreover, phospholipid is quite unstable when exposed to air or sunlight, and is easily oxidized and rancid. The oil in SCP can prevent phospholipid from oxidation and rancidity, which is beneficial to the storage\textsuperscript{25}. Therefore, the preparation of GPC from SCP can not only reduce the production cost and broaden the comprehensive utilization of SCP, but also improve the oxidative stability and thermal stability of phospholipid.

In this paper, the processing conditions of GPC preparation with the PLA\textsubscript{2}→PLA\textsubscript{1} method were optimized with SCP as the raw material and GPC yield as the result index. The effects of reaction time, reaction temperature, stirring speed, substrate concentration, PLA\textsubscript{1} load, and PLA\textsubscript{2} load on the preparation of GPC were investigated and evaluated using response surface methodology (RSM).

### 2 Experimental Procedures

#### 2.1 Materials

SCP was supplied by COFCO Jiayue Co., Ltd. (Tianjin, China). PLA\textsubscript{1} was purchased from Novozyme Biotechnology Co., Ltd., and PLA\textsubscript{2} was purchased from DuPont Danisco Co., Ltd. GPC standard (purity ≥ 98\%) was purchased from Sigma-Aldrich Chemical Co., Ltd. Chloroform and methanol were chromatographic grade (Kernio Chemical Reagant Co., Ltd., Tianjin, China). All other solvents were of analytical grade (Tianjin Chemical Reagent Co., Ltd., Tianjin, China). Ultrapure water was made in laboratory.

#### 2.2 Enzymatic hydrolysis reaction

SCP and water were mixed in a 250 mL round bottom flask with stirring for 30 min, then the mixture was homogenized at 10000 rpm for 5 min with a high-speed shear dispersion emulsifier. After adjusting the temperature and pH, PLA\textsubscript{1} was added to start the reaction. When the reaction was performed for a certain time, PLA\textsubscript{2} was added to continue the hydrolysis reaction for some times.

#### 2.3 GPC analysis

Once the reaction was finished, the reaction product was taken to dehydrate in vacuum at 80°C. Then, it was washed 3 times using acetone. After the oil in the product was removed, the solvent was evaporated in vacuum at 40°C. The mixed solvents (chloroform/methanol, v/v, 2:1) were added with shaking, then it was centrifuged at 10000 rpm for 10 min. The upper layer was filtered by a 0.22 μm polypropylene filter and analyzed by high-performance liquid chromatography (HPLC).

GPC was analyzed by HPLC (Agilent 1260) equipped with an evaporative light scattering detection (ELSD). A SunFire\textsuperscript{TM} Prep silica column (5 μm, 4.6 × 250 mm, Agilent, USA) was employed to analyze samples, and the column temperature was set at 35°C. The evaporation and atomization temperatures of ELSD were respectively set at 40 and 65°C with a nitrogen gas flow rate of 1.6 L/min. The mobile phase consisted of methanol (A) and water (B) at a flow rate of 1.0 mL/min. A gradient elution was used starting with 85% phase A, then reached to 75% in 7 min and decreased to 70% in 6 min, returned to the initial 85% in 0.1 min and held for 6.9 min.

The concentration of GPC was measured by the external standard method. The yield of GPC was calculated as follows:

$$\text{GPC yield} \% = \left( \frac{C_{\text{arc}} \times V}{m_{\text{arc}}} \right) \times 100\%$$

Where, $C_{\text{arc}}$ is the concentration of GPC in the sample (mg/mL), $V$ is the sample volume (mL), and $m_{\text{arc}}$ is the theoretical yield of GPC (mg).

#### 2.4 Experimental design for RSM

A three-level-five-factor Box-Behnken design was employed to evaluate the interaction effects of reaction variables on the GPC yield. The factors and levels were as follows: reaction temperature (30, 40, and 50°C), substrate concentration (1, 10, and 15\%), PLA\textsubscript{1} load (0.45, 0.60, and 0.75\%, relative to the weight of total substrates), PLA\textsubscript{2} load (0.75, 1.00, and 1.25\%, relative to the weight of total substrates), and stirring rate (300, 500, and 700 rpm) (Table S1).

#### 2.5 Statistical analysis

The experimental data were analyzed by Design-Expert 8.0. The mathematical models between the reaction variables with the responses can be obtained by the following quadratic polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j$$

Where $Y$ is the predicted response (GPC yield), and $X_i$ and $X_j$ represent the independent variables. $\beta_0$, $\beta_i$, and $\beta_{ij}$ are the intercept, linear, quadratic, and interaction

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3 Results and Discussion

The SCP product mainly contained PC (17.02%), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), and a small amount of sn-2-lyso-phosphatidylcholine (sn-2-LPC, Fig. S1). After enzymatic hydrolysis, the generated GPC was quantified by external standard method.

3.1 Enzymatic preparation of GPC

3.1.1 Effect of reaction time

According to our previous study, the total reaction time of at least 120 min was required for PC to be adequately hydrolyzed by PLA₂ and PLA₁ for obtaining a high yield of GPC. The influence of the respective time of PLA₂ and PLA₁ on the GPC yield was shown in Fig. 1. As PLA₂ time increased, GPC yield increased, followed by flattening. In theory, when PLA₂ is initially added to act on PC, a large amount of sn-1-LPC are generated, which is relatively difficult to undergo acyl migration \(^{26,27}\). When the reaction time of PLA₂ is insufficient, less sn-1-LPC is generated, which leads to reduce the catalytic efficiency of subsequent PLA₁. When the reaction time of PLA₂ was 60 min, the GPC yield reached equilibrium (68.50%). This was consistent with the results reported by Vikbjerg et al. \(^{20}\), who pointed out that although increased reaction time promoted the hydrolysis rate, acyl migration was also difficult to be avoided. Therefore, the total reaction time was preferably 120 min, and the times of PLA₂ and PLA₁ were both 60 min.

3.1.2 Effect of reaction temperature

The influence of reaction temperature on the GPC yield was shown in Fig. 2. The GPC yield increased from 32.04 to 58.68% when the reaction temperature increased from 30 to 40°C. However, when the temperature further increased from 40 to 70°C, the GPC yield remained constant, followed by a significant decrease. These results indicated that the activity of PLA₂ is relatively high at 40-50°C, thus increasing PC conversion and providing sufficient reactants for subsequent PLA₁; when temperature continues to rise, enzyme activity decreases and acyl migration is promoted \(^{27}\). Similar results were also observed in some studies \(^{24,28,29}\). Hence, considering the production cost, the optimal temperature was chosen at 40°C.

3.1.3 Effect of substrate concentration

As shown in Fig. 3, when the substrate concentration increased from 5 to 10%, the GPC yield increased by 14.58%. However, the GPC yield sharply decreased from 67.20 to 33.16% with the increase of the substrate concentration from 10 to 25%, which might be due to the fact that when substrate concentration is relatively low, the viscosity of reaction system is low, where phospholipid exists in the form of single molecule or small aggregates to obtain a large reaction interface area. Although excessive phospholipids can theoretically accelerate the equilibrium of enzymatic hydrolysis reaction, the number of phospholipase active sites is limited. Therefore, when substrate concentration exceeds a certain range, single and dispersed phospholipids gather and accumulate, resulting in the decrease of reaction system dispersion and reaction interface and subsequent decrease of GPC yield \(^{30}\). Liu et al. also found a similar trend, which the inhibitory effect of microenvironment and products on enzymatic hydrolysis increased with the increase of substrate concentration \(^{31}\). Considering...
these results, the optimum substrate concentration was 5%.

3.1.4 Effect of stirring rate

As the reaction system is heterogeneous, the mixing strength of SCP-water might affect the rate of enzymatic reaction. Effect of stirring rate on the GPC yield in enzymatic hydrolysis reaction was shown in Fig. 4. With increasing stirring rate, GPC yield increased initially and then flattened with the highest level of 65.07% at 500 rpm. The results could be explained that high stirring rate increases the interface area of SCP at the aqueous phase and the probability of phospholipase active sites on SCP, resulting in the increase of the GPC yield. However, when stirring rate is too high, the centrifugal force on the SCP-water mixture and phospholipase is so large that they are thrown onto the inner wall of the reactor, which reduces the amounts of SCP-water mixture and phospholipase in the reaction, and slightly reduces the GPC yield. Thus, the stirring rate was preferably 500 rpm.

3.1.5 Effect of phospholipase A2 load

PLA2 load directly determines the amount of primary hydrolysate (sn-2-LPC), which indirectly affects the catalytic efficiency of PLA1 in the second stage. The influence of PLA1 load on the GPC yield was shown in Fig. 5. When the PLA1 load increased from 0.25 to 1.00%, the GPC yield increased from 30.78 to 75.07%. The phenomenon indicated that the addition of more PLA2 in the early stage benefited generation of more sn-1-LPC, which led to a high GPC yield. It is due to the fact that the catalytic rate increases with the increase of enzyme load per unit volume. Nevertheless, excessive PLA2 had no positive effect on improving GPC yield, and it might even have a negative effect on the reaction interface of substrate and enzyme. Some studies have reported that the rate of enzymatic reaction sometimes does not increase with the increase of enzyme concentration. Overall, the PLA2 load was selected to be 1.00%.

3.1.6 Effect of phospholipase A1 load

The amount of phospholipase directly affects GPC yield. As shown in Fig. 6, the trend of GPC yield with the increase of PLA1 load was similar to that of PLA2. The GPC yield increased from 42.02 to 64.97% with the PLA1 load from 0.15 to 0.60%, whereas there was no significant difference in the GPC yield with the PLA1 load from 0.60 to 0.90%. In the primary stage of hydrolysis reaction, a large number of sn-1-LPC are generated. In the second stage, the addition of more PLA1 generates more active sites per unit volume, which accelerates the enzymatic reaction. However, sn-1-LPC generated is limited, excessive PLA1 has no an obvious effect on enzymatic reaction when the active sites and substrates of PLA1 reach saturation. Therefore, the PLA1 load was chosen at 0.60%.

3.2 Model fitting

In this work, RSM was employed to examine the effects
of reaction temperature, substrate concentration, stirring rate, PLA₁ load, and PLA₂ load on the GPC yield (Table S1). According to the statistical method, the data of 46 experimental runs were analyzed to simulate multiple response values of regression analysis. As shown in Table 1, the quadratic regression model was significant (p < 0.0001), which indicated that the model was predominant and adequate to predict the actual relationship between these reaction parameters and GPC yield. The multinomial regression equation of GPC yield was obtained as follows:

\[
\text{GPC yield (\%) = 72.93 + 5.57A - 0.28B + 4.47C + 4.64D + 4.34E + 3.70AB - 2.93AC + 3.36AD - 4.49AE + 2.43BC + 11.5BD + 2.97BE + 3.19CD + 3.23CE + 8.39DE - 10.47A^2 - 10.08B^2 - 6.70C^2 - 8.79D^2 - 6.78E^2}
\]

The interaction of reaction variables can be better un-

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**Table 1**  Analysis of variance (ANOVA) for quadratic model for GPC yield.

| Source | Sum of squares | Degrees of freedom | Mean squares | \(F\) value | Prob > \(F\) |
|--------|----------------|--------------------|--------------|-------------|-------------|
| Model  | 4314.54        | 20                 | 215.73       | 12.82       | <0.0001     |
| A      | 495.97         | 1                  | 495.97       | 29.49       | <0.0001     |
| B      | 1.25           | 1                  | 1.25         | 0.07        | 0.7873      |
| C      | 320.07         | 1                  | 320.07       | 19.03       | 0.0002      |
| D      | 345.05         | 1                  | 345.05       | 20.51       | 0.0001      |
| E      | 301.80         | 1                  | 301.80       | 17.94       | 0.0003      |
| AB     | 54.76          | 1                  | 54.76        | 3.26        | 0.0832      |
| AC     | 34.22          | 1                  | 34.22        | 2.03        | 0.1661      |
| AD     | 45.09          | 1                  | 45.09        | 2.68        | 0.1141      |
| AE     | 80.54          | 1                  | 80.54        | 4.79        | 0.0382      |
| BC     | 23.52          | 1                  | 23.52        | 1.40        | 0.2481      |
| BD     | 531.30         | 1                  | 531.30       | 31.59       | <0.0001     |
| BE     | 35.24          | 1                  | 35.24        | 2.10        | 0.1602      |
| CD     | 40.66          | 1                  | 40.66        | 2.42        | 0.1326      |
| CE     | 41.80          | 1                  | 41.80        | 2.48        | 0.1275      |
| DE     | 281.57         | 1                  | 281.57       | 16.74       | 0.0004      |
| A²     | 955.79         | 1                  | 955.79       | 56.82       | <0.0001     |
| B²     | 887.48         | 1                  | 887.48       | 52.76       | <0.0001     |
| C²     | 391.77         | 1                  | 391.77       | 23.29       | <0.0001     |
| D²     | 673.55         | 1                  | 673.55       | 40.04       | <0.0001     |
| E²     | 400.86         | 1                  | 400.86       | 23.83       | <0.0001     |
| Residual | 420.52     | 25                 | 16.82        |             |             |
| Lack of fit | 357.00   | 20                 | 17.85        | 1.40        | 0.3783      |
| Pure error | 63.52    | 5                  | 12.70        |             |             |
| Total   | 4735.06       | 45                 |              |             |             |

\(R^2=0.9112\)
Fig. 7
Preparation of Glycerylphosphorylcholine by Two-stage Enzymatic Hydrolysis Reaction

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The average GPC yield of triplicate under these conditions was 83.07% (Fig. S2), which was in accordance with the predicted value.

4 Conclusions

In this work, a two-stage enzymatic hydrolysis of SCP catalyzed by PLA₂→A₁ was successfully carried out with RSM for optimizing processing parameters. The reaction conditions were optimized as follows: PLA₁ load of 1.25%, PLA₂ load of 0.70%, substrate concentration of 13%, reaction temperature of 41°C, and stirring rate of 680 rpm. Under these conditions, the GPC yield reached 83.07%, which was in agreement with the predicted value. This paper not only provides an efficient and low-cost method for GPC preparation, but also improves the high-value utilization of SCP. However, further purification of the obtained crude GPC is required in further study.

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Supporting Information

This material is available free of charge via the Internet at http://dx.doi.org/jos.70.10.5650/jos.ess20261

3.3 Optimal conditions and model verification

The optimal process conditions were obtained by RSM as follows: reaction temperature of 40.73°C, substrate concentration of 12.98%, PLA₁ load of 1.24%, PLA₂ load of 0.71%, and stirring rate of 676.97 rpm. Under the conditions, the predicted yield of GPC was 85.16%. Considering the practical application of industry, the processing parameters were adjusted as follows: reaction temperature of 41°C, substrate concentration of 13%, PLA₁ load of 1.25%, PLA₂ load of 0.70%, and stirring rate of 680 rpm. The average GPC yield of triplicate under these conditions was 83.07% (Fig. S2), which was in accordance with the predicted value.

References

1. Brownawell, A.M.; Carmines, E.L.; Montesano, F. Safety assessment of AGPC as a food ingredient. Food Chem. Toxicol. 49, 1303-1315 (2011).
2. Tayebati, S.K.; Tomassoni, D.; Stefano, A.D. Effect of choline-containing phospholipids on brain cholinergic transporters in the rat. J. Neurol. Sci. 302, 49-57 (2011).
3. Lee, S.H.; Choi, B.Y.; Kim, J.H. Late treatment with choline alfoscerate (l-alpha glycerylphosphorylcholine, α-GPC) increases hippocampal neurogenesis and provides protection against seizure-induced neuronal death and cognitive impairment. Brain Res. 1654, 66-76 (2017).
4. Brambilla, G.; Martelli, A. Update on genotoxicity and carcinogenicity testing of 472 marketed pharmaceuticals. Mutat. Res/Rev. Mutat. Res. 681, 209-229 (2009).
5. Grimm, M.O.W.; Grössgen, S.; Riemenschneider, M.; Tanila, H.; Grimm, H.S.; Hartmann, T. From brain to food: Analysis of phosphatidylcholines, lysophosphatidylcholines and phosphatidylcholine–plasmalogens derivates in Alzheimer’s disease human post mortem brains and mice model via mass spectrometry. J.
Ceda, G.P.; Ceresini, G.; Denti, L.; Marzani, G.; Piovani, E.; Banchini, A.; Tarditi, E.; Valenti, G. alpha-Glycerophosphorylcholine administration increases the GH responses to GHRR of young and elderly subjects. *Horm. Metab. Res.* **24**, 119-121 (1992).

7. Parnetti, L.; Amenta, F.; Gallai, V. Choline alphoscerate in cognitive decline and in acute cerebrovascular disease: an analysis of published clinical data. *Mech. Ageing Dev.* **122**, 2041-2055 (2001).

8. Hibino, H. Preparation of deacylated phospholipid: Glycerophosphocholine and central nervous system activated functional development. *Oleoscience* **7**, 399-411 (2007).

9. Zeisel, S.H. Dietary choline: Biochemistry, physiology, and pharmacology. *Annu. Rev. Nutr.* **1**, 95-121 (1981).

10. Marrapu, B.; Mallampalli, L.K.; Kaki, S.S.; Rachapudi, B.N.P. A novel method to synthesize 1-acyl-sn-glycero-3-phosphocholine and 1,2-diacyl-sn-glycero-3-phosphocholine. *Eur. J. Lipid Sci. Tech.* **117**, 1049-1055 (2015).

11. Blasi, F.; Cossignani, L.; Maurizi, A.; Simonetti, M.S.; Damiani, P. Enzymatic synthesis of structured 1,2-diacyl-sn-glycero-3-phosphocholines from glycerol-sn-3-phosphocholine. *Ital. J. Food Sci.* **20**, 39-47 (2008).

12. Park, J.M.; Castro, K.A.D.; Ahn, H.S. Facile syntheses of L-α-glycero-phosphoryl choline. *Bull. Korean Chem. Soc.* **31**, 2689-2691 (2010).

13. Li, H.Y.; Zhang, T.T. L-α-glycerylphosphorylcholine from natural lecithin via transesterification catalyzed by propylamine. *Adv. Mater. Res.* **997**, 73-76 (2014).

14. Li, H.Y.; Zhang, X.L.; Bai, W.L.; Zhao, B.X. Preparation of L-α-glycerophosphocholine from natural lecithin catalyzed by tert-butylamine. *Adv. Mater. Res.* **641-642**, 148-151 (2013).

15. Song, Y.S.; Song, E.S.; Kang, D.S.; Song, I.W.; Kang, P.G.; Oh, S.S. A process for preparation of L-alpha-glycerophosphoryl choline. WO2007145476 A1 (2007).

16. Zhang, K.; Wang, X.; Liu, Y. Aqueous medium enzymatic preparation of l-alpha-glycerophosphorylcholine optimized by response surface methodology. *Eur. Food Res. Technol.* **234**, 485-491 (2012).

17. Bang, H.-J.; Kim, I.-H.; Kim, B.H. Phospholipase A1-catalyzed hydrolysis of soy phosphatidylcholine to prepare l-α-glycerophosphorylcholine in organic-aqueous media. *Food Chem.* **190**, 201-206 (2016).

18. Lu, Y.; Zhang, A.; Wang, X.; Hao, N.; Chen, K.; Ouyang, P. Surfactant enhanced l-α-glycerophosphorylcholine production from phosphatidylcholine using phospholipase A1 in the aqueous phase. *Biocatal. Biotransform.* **37**, 361-366 (2019).

19. Kim, J.; Song, Y.; Lee, S.J.; Lee, J.E.; Chung, M.; Kim, I.; Kim, B.H. Enzymatic preparation of food-grade l-α-glycerophosphorylcholine from soy phosphatidylcholine or fractionated soy lecithin. *Biotechnol. Prog.* **36**, e2910 (2019).

20. Vikberg, A.F.; Mu, H.; Xu, X. Elucidation of acyl migration during lipase-catalyzed production of structured phospholipids. *J. Am. Oil Chem. Soc.* **83**, 609-614 (2006).

21. Alekseeva, A.S.; Volynsky, P.E.; Krylov, N.A.; Chernikov, V.P.; Vodovozova, E.L.; Boldyrev, I.A. Phospholipase A1: way to hydrolysis: Dint formation, hydrophobic mismatch, and lipid exclusion. *Biochim. Biophys. Acta 1863*, 183481 (2021).

22. Zhao, T.; No, D.S.; Kim, B.H.; Garcia, H.S.; Kim, Y.; Kim, I.-H. Immobilized phospholipase A1-catalyzed modification of phosphatidylcholine with n-3 polyunsaturated fatty acid. *Food Chem.* **157**, 132-140 (2014).

23. Liang, S.H.; Wang, S.K.; Meng, Y.N.; Sun, C. Enzymatic preparation of glycerophosphatilcholine catalyzed by combinational phospholipases: a comparative study of concerted versus stepwise catalysis. *RSC Adv.* **10**, 38727-38735 (2020).

24. Zhang, K.; Liu, Y.; Wang, X. Enzymatic preparation of L-α-glyce-rophosphorylcholine in an aqueous medium. *Eur. J. Lipid Sci. Technol.* **114**, 1254-1260 (2012).

25. Wang, P.; Cao, X.; Chu, Y.; Wang, P. Ginkgolides-loaded soybean phospholipid-stabilized nanosuspension with improved storage stability and in vivo bioavailability. *Colloids Surf. B* **181**, 910-917 (2019).

26. Adlercreutz, D.; Budde, H.; Wehtje, E. Synthesis of phosphatidylcholine with defined fatty acid in the sn-1 position by lipase-catalyzed esterification and transesterification reaction. *Biotechnol. Bioeng.* **78**, 403-411 (2002).

27. Hong, S.I.; Kim, Y.; Kim, C.-T.; Kim, I.-H. Enzymatic synthesis of lysophosphatidylcholine containing CLA from sn-glycero-3-phosphatidylcholine (GPC) under vacuum. *Food Chem.* **129**, 1-6 (2011).

28. Primozič, M.; Habulin, M.; Knez, Ž. Parameter optimization for the enzymatic hydrolysis of sunflower oil in high-pressure reactors. *J. Am. Oil Chem. Soc.* **80**, 643-646 (2003).

29. Poisson, L.; Devos, M.; Godet, S.; Ergan, F.; Pencreach, G. Acyl migration during deacylation of phospholipids rich in docosahexaenoic acid (DHA): An enzymatic approach for evidence and study, *Biotechnol. Lett.* **31**, 743-749 (2009).

30. Finkle, P.; Draper, H.D.; Hildebrand, J.H. The theory of emulsification1. *J. Am. Chem. Soc.* **45**, 2780-2788 (1923).

31. Liu, L.; You, Y.; Deng, H.; Guo, Y.; Meng, Y. Promoting hydrolysis of apple pomace by pectinase and cellulase to produce microbial oils using engineered *Yarrowia lipolytica*. *Biomass Bioenergy* **126**, 62-69 (2019).

32. Li, H.; Cao, X.; Lu, Y.; Ni, Y.; Wang, X.; Lu, Q.; Tan, W. Alkaline modification of a metal–enzyme–surfactant complex for the production of phospholipids. *Biotechnol. Bioeng.* **99**, 1148-1159 (2007).

8. *J. Oleo Sci.*
nanocomposite to enhance the production of L-α-glycerylphosphorylcholine. Catal. 9, 237 (2019).

33) Kiełbowicz, G.; Gladkowski, W.; Chojnacka, A.; Wawrzeńczyk, C. A simple method for positional analysis of phosphatidylcholine. Food Chem. 135, 2542-2548 (2012).

34) Tabuchi, K.; Ito, Z.; Tsuji, S.; Wada, T.; Takahashi, K.; Hara, A.; Kusakari, J. The contribution of phospholipase A2 to the cochlear dysfunction induced by transient ischemia. Hear. Res. 144, 1-7 (2000).

35) Morris, A.J.; Engebrecht, J.; Frohman, M.A. Structure and regulation of phospholipase D. Trends Pharmacol. Sci. 17, 182 (1996).

36) Šližytė, R.; Rustad, T.; Storrø, I. Enzymatic hydrolysis of cod (Gadus morhua) by-products. Process Biochem. 40, 3680-3692 (2005).