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Short Paper

Production of docosahexaenoic acid bounded phospholipids via phospholipase A_2 mediated bioconversion

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Many functionalities of docosahexaenoic acid-rich phospholipids (DHA-PL) are beginning to be exploited by American and Japanese scientists. There is no doubt that the nature of the fatty acid and the position of attachment to the glycerol are important, especially for medical applications. In fact, it has been borne out through many studies that DHA should reside at *sn*-2 position of the glycerol backbone to exert beneficial functionalities. There are several alternatives to obtain PL with DHA bounded on position *sn*-2. Natural sources can provide only moderately DHA-concentrated PL. Other alternatives to produce DHA-PL are the chemical or enzymatic syntheses. Chemical synthetic methods would provide high yield, but they often accompany undesirable side reactions. From these points of view, bioconversion must be a better choice, because there are very little risks of side reactions, easy to incorporate DHA exclusively into position *sn*-2 when phospholipase A_2 is used, and would require only a simple production line. The only, but serious, drawback of the bioconversion method has been the poor yield. Nakasato *et al.*[1] first overcame this problem by developing a transacylation method between soy lecithin and DHA-rich monoglyceride using non-positional specific lipases. They succeeded in obtaining DHA-PL up to 97% yield. However, not only the PL with DHA bounded on position *sn*-2, but also the poorly functional PL with DHA bounded on *sn*-1 was produced. To obtain exclusively, the *sn*-2 DHA resided PL, phospholipase A_2 must be used. The esterification mediated by phospholipase A_2 can provide an exclusively polyunsaturated fatty acid (PUFA)-enriched PL by using free PUFA with very high purity as an acyl donor. In 1995, Hosokawa *et al.* reported that in the enzymatic synthesis of icosapentaenoic acid (EPA)-rich PL, appropriate amount of formamide (as an enzyme activator) was useful in suppressing the undesirable hydrolysis that seriously impaired the yield.[2] However, the maximum yield still remained around 60% in weight base at the most, and when DHA was used as an acyl donor, the yield decreased to almost 40% in weight base.[3] In this present study, the authors have succeeded in producing the *sn*-2 DHA bounded PL with over 90 mole percentage yield via phospholipase A_2 mediated esterification by combining a decompression condition using formamide as an enzyme activator.

Phospholipase A_2 was prepared with a freeze-dryer after dialyzing Lecitase 10 L (Novo Nordisk A/S, Bagsvaerd, Denmark). Soy lysolecithin (LPC), obtained from soy lecithin hydrolysate (Kyowa Hakko Kogyo, Tokyo, Japan), was purified up to 95% with a silica gel column chromatography. As for the acyl donor substrate, DHA (97% purity) was supplied from Ikeda Tohka Industries (Fukuyama, Japan). Silica gel 60 (E. Merck) plates with 0.5 mm and 0.25 mm thickness were used for thin layer chromatography (TLC). All solvents and chemicals used were reagent-grade. The Karl Fischer method was used for moisture content analyses. The

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amount of formamide was determined on a Shimadzu GC-9A Gas Chromatograph (Shimadzu, Kyoto Japan) with Sunpak-A Column containing Thermon 3000 (5%) and potassium hydroxide (KOH) (1%) (Shinwa Chemical Ind., Kyoto, Japan). Injection temperature was 240°C, and column temperature was maintained at 220°C. Flame inonization detector (FID) was used as a detector. Carrier nitrogen gas was set to 50 mL/min. Hitachi 163 gas chromatograph (Hitachi, Tokyo) equipped with G-300 column (40 m × 1.2 mmφ) with 0.5 μm polyethylene glycol (PEG) 20 M liquid phase coating (Chemicals Evaluation and Research Institute, Saitama, Japan) and FID were used for fatty acid composition analysis. Column temperature was maintained at 170°C and injection temperature was set to 250°C. Flow rate of helium used as a carrier gas was 10 mL/min. Methyl esterification was carried out for the individual lipid class prior to the gas chromatographic analysis, following the method of Christopher and Glass described by Prevot and Mordret.[4] Then, 110 mg of LPC and 180 mg of DHA was mixed in 5500 mg of glycerol, then purged with argon gas and kept for overnight under 4°C to prepare a thoroughly homogeneous substrate. Enzyme solution was prepared by dissolving 60 mg of phospholipase A_2 and 3 μmol calcium chloride in 0.5 mL formamide. Phospholipase A_2-mediated synthesis of DHA-PC was initiated by adding the enzyme solution to a round bottom reaction flask containing the above resulting mixture substrate. Reactions were carried out at 40°C under varying atmospheric conditions. The apparatus used for the synthetic reaction consisted of a rotary evaporator connected with a molecular sieve trap, and a nitrogen gas cylinder to derive dried nitrogen gas into the bottom of the reaction flask. A device for cooling the nitrogen gas was also set up. Time courses of the formamide and moisture contents in the reaction mixture, and the yield, were monitored.

Yield was calculated through two steps as shown below:

$$D(cm^2) = D'\ (cm^2) \times S_{TLC}(cm^2)/S_{SC}(cm^2)$$

where D is the corrected area of the synthesized DHA-PC spot on TLC plate, D′ is the spot area of the synthesized DHA-PC measured on Densitometer (Model F-808; Cosmo, Tokyo, Japan). S_{TLC} is the spot area on the same TLC plate of known amount of DHA-PC measured on Densitometer, and S_{SC} is the area calculated from a calibration curve showing correlation between the spot area of DHA-PC and the actual weight of DHA-PC previously prepared. From D and the calibration curve showing correlation between the spot area of DHA-PC *versus* the actual weight of DHA-PC, D was converted to weight. Then, the yield was calculated according to the following formula:

$$\text{DHA-PC yield (mol\%)} = \text{Synthesized DHA-PC/Substrate LPC} \times *1.53$$

*1.53 designates a molar ratio of the theoretical maximum when the applied LPC is fully converted to DHA-PC.

Excess moisture in lipase and phospholipase-mediated esterifications seriously impairs the yield of the desired lipids.[5,6] Moisture restriction is crucial for the reactions in non-aqueous media. However, an extremely dried condition often results in an impractically time-consuming reaction period. Reslow *et al.* discovered that a so-called water mimics is useful in lipase mediated reaction to improve the yield with practical reaction period.[7] Hosokawa *et al.* first applied the water mimics to produce EPA-bound phospholipids.[8] But when DHA was used as an acyl donor, the maximum yield was approximately 40% in weight base which corresponded to only less than 30 mole percentage. We considered that although formamide is essential to attain a practical reaction velocity at the early stage of the synthetic reaction, it must be removed in the latter stage of the acyl donor-incorporating reaction.

To prove this prediction, the authors compared the yield of the desired DHA-PC under three different conditions. Under the atmospheric pressure (without decompression), with only stirring, the yield of the desired DHA-PC remained 43.1 mole percentage. When vacuum was provided with a rotary pump, the yield increased to 58.5 mole percentage. Under this condition, dews appeared on the inner walls of the round bottom flask and stopcock. After removing these dews with ethanol-spread cotton, the reaction was continued for another 24 h under decompression. It was possible to get a yield to reach 76.1 mole percentage. The amount of the appeared dews was apparently much more than the theoretical moisture amount generated during the esterification reaction. Therefore, it was considered that formamide had condensed on the inner walls of the flask and stopcock. Dried nitrogen gas was purged to remove excess moisture and the formamide. The authors obtained 87.1 mole percentage yield without a condenser, and 90.2 mole percentage with a condenser in the nitrogen gas flow. As illustrated in Figure 1, there was an inverse relationship between the formamide content in the reaction system and the yield of DHA-PC at 132–165 Torr. The decrease in formamide was larger than that of moisture, implying that not only the moisture, but also the formamide, must be removed to get high yield. Under this optimum condition, DHA was

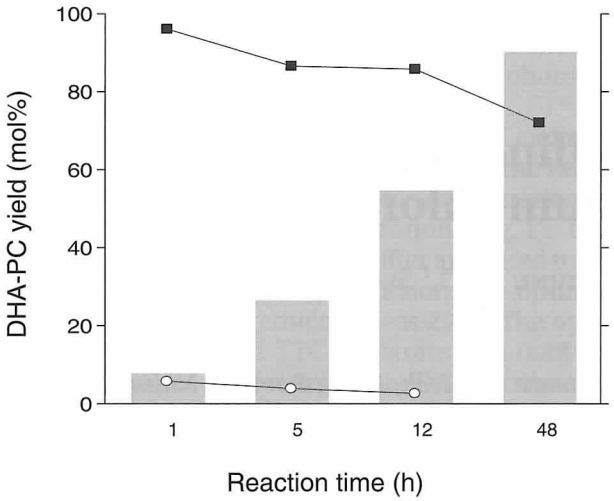

Fig. 1 Relationships between docosahexaenoic acid-rich docosahexaenoic acid-rich phosphatidylcholine (DHA-PC) yield *versus* moisture (○) and formamide (■) content. Reaction mixture: Soy lysolecithin 110 mg, DHA 180 mg, glycerol 5500 mg, PLA2 60 mg, $CaCl_2$ 3 μmol, formamide 0.5 mL. Condition: 40°C, 48 h, decompression (132–165 Torr).

incorporated into the LPC backbone nearly to its theoretical maximum (i.e. $(43.4/[97 \div 2]) \times 100 = 89.5\%$).

It can be concluded that although formamide is essential to obtain high velocity at the early stage of the synthetic reaction, it must be removed as soon as the acyl donor is incorporated into the glycerol backbone. Otherwise, hydrolysis would also be activated by the excessive formamide so as to clog up the synthetic reaction.

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