Enzyme Engineering for Lipids

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Abstract

Lipids (edible fats and oils, phospholipids and glycolipids, etc) are one of major important food resources, in addition to carbohydrate (starch, sugar, etc.) and protein resources. Edible fats and oils are produced mostly from agricultural products such as plant seeds and animals. Animal-derived fats and oils are also utilized as energy sources in human nutrition. Fish oils have some functional fatty acids categorized as n-3 polyunsaturated fatty acids (PUFA) with 5 and 6 unconjugated methene double bonds. Phospholipids are utilized as food and cosmetic emulsifier (pure grade), and animal feed (crude). Enzymes, which are involved as catalysts (biocatalysts) in all metabolisms and biochemical reactions in living cells, are very tightly connected with all life phenomena. Two novel engineering disciplines relating to effective enzyme utilization have emerged in the late 20th century: enzyme engineering and protein engineering. Enzyme engineering is a branch of bioprocess engineering devoted to increasing efficacy of enzyme utilization as biocatalysts for various bioconversions. Protein engineering is one of major branches of biomolecular engineering, aiming at creation of enzymes of better nature through various genetic engineering techniques such as site-directed mutagenesis, error-prone PCR, DNA shuffling, molecular evolution technique, etc. Natural lipids are a mixture of hundreds or thousands of triacylglycerols when looked at from the viewpoint of organic chemistry. Recently, the so-called 'structured lipids' have attracted much attention. In the strictest sense, the structured lipids are those having definite chemical structure. From a number of clinical experiments in lipids nutrition science, structured triacylglycerols (sTAG) have been shown to confer various enhanced nutritional values so that they are useful as functional foods or nutritional supplements. Taking advantage of enzyme's properties, the author and his co-researchers have been studying enzyme engineering for lipids bioconversions for many years. Recent studies showed success in making feasible the industrial production of 1,3-dioctanoyl -2-docosahexaenoyl-sn-glycerol by the action of immobilized lipases following a two-step process, i.e. ethanolysis of fish oil by immobilized *Candida antarctica* lipase followed by re-esterification of the resulting 2-monoacylglycerol with n-octanoic acid by immobilized *Rhizomuco miehei* lipase in neat system. Phosphatidylserine (PS), which is one of structured phospholipids (sPL), is found neither in soy- nor in egg-lecithin, but is contained highly in mammalian cerebral cortex, and is claimed to be effective for Age-Associated Memory Impairment (AAMI). Recently, an efficient high-yield synthesis technique of PS from lecithin by the transphophatidylation action of *Streptomyces* phospholipase D in non-solvent system has been developed.

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1. Lipids as Renewable Resource

There are three major food resources: carbohydrate, protein, and lipids. For the lipids, edible fats and oils are most predominant, but phospholipids and glycolipids are also included. When people talk about biomass, it usually includes starch, cellulose, chitin, xylan, and lignin. The author, however, would like to emphasize that lipids are also important, inexpensive, renewable, biodegradable, and energy-rich natural resources. Lipid resources are one of the major bio-resources and hence, are included in the green chemistry strategy. As for the plant seed oils, the major oils include palm oil, soybean oil, rapeseed oil, sunflower oil, peanut oil, cottonseed oil, olive oil, safflower oil and others.

The total consumption of these seed oils in 2001 was as huge as 118 million tones in the world. In some Southeast Asian countries such as Malaysia and Indonesia, very large-scale plantations of oil palm trees are widely seen. About 23 million tones of palm oil were produced in 2001 worldwide, of which Malaysia produced 12 millions tones (52 %). Glycerol and fatty acid are obtained from fats and oils by hydrolysis (chemically or lipase-catalyzed). The former is a starting material for nitroglycerine and propylene oxide, while fatty acids can be converted to methyl esters that

are utilizable as automobile diesel fuels, and to amides, acid chlorides, and alcohols, all of which are raw materials of various commodity and fine chemicals.

People utilize animal fats and oils as well, such as beef tallow, lard, milk fat, and fish oil. In 1998, the total consumption of animal fats and oils in the world was 18 million. This figure is huge but is much less than that of plant seed oil.

Among the edible oils, fish oils are quite unique because they have the so-called n-3 polyunsaturated fatty acid, which is also called PUFA for short. In addition to the conventional plant seed oils and animal fats, novel edible oils have been exploited. These are single cell oils (SCO), which are produced by microorganisms. The SCO producers are *Mortierella* sp., *Schizochytrium* sp., and *Crypthecodinium* sp. These microorganisms have been screened as producers of oils containing specific PUFAs.

Table 1 summarizes the lipids bio-resources in the world. PUFAs are classified into two (Figure 1): n-6 PUFA such as eicosatrienoic acid , and eicosapentaenoic acid (commonly called arachidonic acid, ALA), and n-3 PUFA such as eicosapentaenoic acid and docosahexaenoic acid. They are shortly named EPA, and DHA, respectively. From many clinical tests, these PUFAs have a number of physiological benefits so that these may be called functional fatty acids or neutaceuticals. Because of many double bonds franking active methylene groups, PUFAs are very unstable. They are easily oxidized, isomerized from E configuration to Z configuration, and polymerized. Therefore, any reactions, whether or not chemical or biochemical, should be carried out under mild conditions without oxygen.

Major natural resources of phospholipids are soybean lecithin, and egg yolk lecithin. They are obtained as byproducts of soybean oil and egg products. The annual production of soybean lecithin and egg yolk lecithin in Japan in 2001 was 6500 tones, and 250 tones, respectively. Although they are now utilized mainly as food and cosmetic emulsifiers as well as fodder feed, they have great potential as resources for neutraceuticals.

Table 1. Lipid bio-resources in the world

1) Triacylglycerols

Plant seed oils	Animal oils	Single cell oils
Palm oil	Lard	EPA oil
Rape seed oil	Milk fat	DHA oil
Sunflower oil	Fish oil	Others
Peanut oil	Others	
Cotton seed oil		
Olive oil		
Safflower oil		
Others		

2) Phospholipids
Soybean lecithin
Egg yolk lecithin

3) Glycolipids
glycoglycerolipids (galactosyldiacylglycerol, etc)
glycosphingolipids (gangliosides, etc)

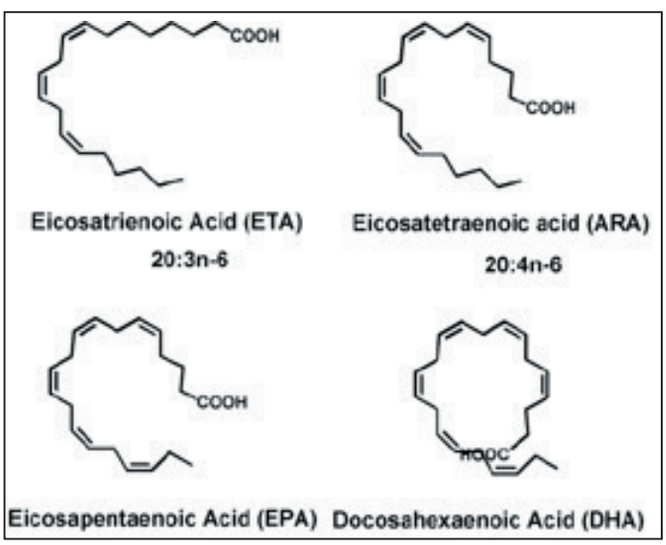

Fig 1. Structures of Major Polyunsaturated Fatty Acids (PUFAS)

2. Enzyme as catalyst

Enzymes are involved in all the metabolism and biochemical reactions in living cells so that they are very tightly connected with all life phenomena. From the viewpoint of being a catalyst, enzymes are characterized by high molecular activity, being active under mild conditions, and high specificity (or selectivity) (substrate specificity, reaction specificity, enantiospecificity, sequence specificity, positional specificity, etc). Enzymes are also "tender to the earth" because they are proteins, non-toxic, and quickly decompose in natural environment. Because of these merits, people have utilized a number of enzymes in various fields since early civilization. Among these merits, specificity or selectivity is the most important characteristic of enzyme. If one wants to synthesize a chemical compound or to modify it enzymatically, he has to take maximum advantage of enzyme specificity. In the next section, the author is going to introduce his work on syntheses of particular lipid using lipase and phospholipase so that specificity of both lipases and phospholises are mentioned briefly. Lipases, which split ester bonds of triacylgylcerol, are classified into two: 1,3-specific, and non-specific. There are five phospholipases depending on their position of splitting. Phospholipase A_1 hydolyses *sn*-1 ester bond, phospholipase A_2 does *sn*-2 bond, phospholipase C cleaves O-P bond of *sn*-3 position, and finally phospholipase D splits P-X bond of phosphate diester, respectivity. Phospholipase B hydorolyzes ester bond either on sn-1 or sn-2, or both. Thanks to these specificities, one can carry out particular bioconversion of lipids without the introduction of a protecting group and its deprotection.

Two novel engineering disciplines relating to effective utilization of enzymes emerged in the late 20th century. They are enzyme engineering and protein engineering.

Enzyme engineering is a branch of bioprocess engineering devoted to the increasing efficacy of enzyme utilization as biocatalysts for various bioconversions. Enzyme screening, production, purification, immobilization, bioreactor system, are some of technologies involved in enzyme engineering. Among them enzyme immobilization is notably effective for its reuse and continuous reaction.

Protein engineering is one of the major disciplines under biomolecular engineering, which aims at creation of enzymes of a better nature. It involves various genetic engineering techniques such as site-directed mutagenesis, saturation mutagenesis, combinatorial mutagenesis, error-prone PCR, overlapping PCR, DNA shuffling, molecular evolution technique, high throughput screening, etc.

3. Enzymatic syntheses of 'structured lipids' (1,2)

Natural lipids are simply a mixture of hundreds or thousands of chemical compounds when they are looked at from the viewpoint of organic chemistry. Natural edible fats and oils are simply a mixture of a number of triacylglycerols (TAGs) that are different in terms of both fatty acid species and their distribution along the glycerol backbone. Recently, the so-called 'structured lipids' have attracted much of the people's attention. In contrast to natural edible lipids, the structured lipids are, in the broadest sense, lipids that have been restructured to change positions of fatty acids and/or have been modified chemically or enzymatically to change fatty acid compositions from the native state to either the type of fatty acid or the position of the fatty acids. In a less broad sense, structured triacylglycerols (sTAGs) are structured TAGs containing mixtures of either short-chain fatty acids, or medium-chain fatty acids (MCFA), or both, and long-chain fatty acids, in the same glycerol molecule. A number of studies have been carried out for the synthesis of sTAGs having (medium chain)-(lon g-chain)-(medium-chain)-type fatty acids. This type sTAG is sometimes called symmetrical structured triacylglycerol (SST). These sTAGs are claimed to provide fewer metabolizable calories per gram than do traditional fats and oils, and to be efficient food sources for patients with pancreatic insufficiency and other forms of malabsorption. Cocoa-butter substitute, which consists predominantly of stearoyl-oleoyl-stearoy lglycerol (SOS) or more generally SUS (saturated fatty acid)-(unsaturated fatty acid)-(saturated fatty acid) type TAG, and 'Betapol' manufactured by Uniliver Company, which has the structure of oeo yl-palmitoyl-oleoylglycerol (OPO), are included in this category of sTAGs.

In recent years, the concept of sTAGs has been extended such that sTAGs are designer TAGs with desired fatty acids in terms of their structures and positions as 'nutraceuticals, functional

food, or pharmaceuticals' to target the optimal nutrition, better metabolic conditions, and specific diseases. Along this trend, in the strictest sense, the term 'Structured TAG' is given to a TAG with a particular fatty acid at a specific position of glycerol hydroxyl moieties. The strictest definition of sTAG can be referred to its classification as shown in Table 2: sTAG is either monoacid sTAG, diacid sTAG, or triacid sTAG. Note that in Table 2, fatty acids are shown in the order of their positions located at *sn*-1, *sn*-2 or *sn*-3 of the glycerol backbone. Thus AAB is not identical to BAA, but they are enantiomers. AAB (or BAA) type diacid sTAGs and all triacid sTAGs are chiral.

The objective to produce 'structured lipids' is to enhance their functionalities. Lipid's functionalities are, i) physical properties such as melting point, polymorphism of crystals, etc, ii) chemical properties such as stability against oxidation, and iii) nutritional properties such as calorie, absorption through intestine, digestion, pharmacological effects, etc. From a number of clinical experiments in lipids nutrition science, sTAGs have been shown to confer various enhanced nutritional values so that they are useful as functional foods or nutritional supplements.

STAGs containing PUFAs such as EPA, DHA or ALA, have become of great interest because of various pharmacological effects of these fatty acids. These effects include several health benefits on cardiovascular diseases, immune disorders and inflammation, renal disorders, allergies, diabetes, cancer, etc. These fatty acids may also be essential for brain and retina development in humans.

Among sTAGs containing PUFA, those containing one molecule PUFA and two molecules of medium-chain fatty acids are very noticeable. Several studies have been performed for the synthesis of sTAG containing PUFA at specific sites of the glycerol backbone. The absorption of PUFA into the body depends upon the position of PUFA along the glycerol backbone, i.e. at sn-1 (or 3) or *sn*-2 position. Those sTAGs containing PUFA at *sn*-2 position and MCFA at *sn*-1 and –3 positions can be hydrolyzed into 2-monoacylglycerol (2-MAG) containing PUFA and FA by pancreatic lipase and are efficiently absorbed into intestinal mucosa cells in normal adults. It is to be noted that mammalian pancreatic lipases hydrolyze the ester linkages at the *sn*-1 and *sn*-3 positions with a preference for MCFA over long-chain ones. Therefore, for dietary supplement for adult health, sTAG containing PUFA at *sn*-2 position and MCFA at the *sn*-1 and *sn*-3 positions may be suitable. On the other hand, due to the antiatherogenic, antineoplastic, and anti-inflammatory effects of n-3 PUFA, their intake is important for newborns for eicosanoid synthesis as well as for normal neonatal brain nervous system development and cell membrane structure. Although PUFA are essential to the neonate for both normal growth and metabolism, neonatal intestinal function is immature, resulting in reduced levels of pancreatic lipase and bile acid salts. In addition, pancreatic lipase does not hydrolyze ester bonds containing long-chain n-3 PUFA. Therefore, in the case of the newborn, PUFA absorption by pancreatic lipase is not feasible. However, for neonatal adsorption of PUFA, there is an alternative mechanism. PUFAs are released from gastric digestion, and gastric lipase exhibits stereospecificity for the α -position of TAG and hydrolyzes the sn-3 position twice as fast as the sn-1 position. Therefore, for PUFA therapy for neonates, sTAG containing PUFA at *sn*-1 (or -3) position and MCFA at the other sites may be suitable.

The sTAG can be synthesized either chemically or enzymatically. However, an enzymatic synthesis of sTAG is more advantageous over a chemical process with regard to several aspects. Enzymes are generally specific, thus, giving rise to less or no byproducts, and exhibiting catalytic action under mild conditions. Enzymatic reactions have another advantage for the synthesis of sTAG containing PUFA because PUFAs are very unstable as mentioned before. They are prone to be easily isomerised, oxidised and polymerised. These properties necessitate the use of as mild conditions as possible, especially oxygen-free conditions.

Table 2. Classification of structured triacylglycerols (sTAG).

No. of different FA	Type	Chirality	Stereoisomer
Monoacid-	AAA	Achiral	
Diacid-	ABA	Achiral	
	AAB, BAA	Chiral	Enantiomers
Triacid-	ABC, CBA	Chiral	Enantiomers
	BCA, ACB	Chiral	Enantiomers
	CAB, BAC	Chiral	Enantiomers

Taking advantages of the enzyme's properties, the author and his coworkers have been studying enzyme engineering for lipids bioconversions for many years. Enzymes involved in lipids bioconversion are mostly hydrolytic enzymes such as esterases, lipases and phospholipases. One must take some points into consideration in order to carry out enzyme-catalyzed bioconversion of lipids effectively, such as water-insolubility of the substrates, instability of the substrate, effect of trace amount of water in both ester synthesis and transesterification reactions, etc.

1) Synthesis of 1,3-dioctanoyl-2-docosahexaenoyl-sn-glycerolfrom fish oil (3)

Fish oil is an inexpensive source of PUFA - containing TAGs. The content of DHA and EPA and also their positional distribution in TAGs varies among the fish species. Fish oils with a high content of DHA and EPA at the second position can be used as starting materials for production of nutritionally valuable symmetrical structured triacylglycerol (SST) with medium chain fatty acids at the primary positions.

Some research groups used fish oil or DHA-rich oils such as single cell oil for the production of SST by acidolysis with octanoic acid catalyzed by 1,3-regiospecific lipases. DHA residues situated at the outer positions could not be exchanged (due to the low specificity of 1,3-regiospecific lipases for DHA) resulting in a limited yield of SST. The same problem was encountered in another work where SSTs were obtained in two steps. 2-MAGs were obtained by ethanolysis of TAGs in an organic solvent with a 1,3-regiospecific lipase and then reesterified with oleic acid. The yields of ethanolysis were less than 40% for fish oils with 8% DHA content or more. A large amount of 1-MAGs was formed probably due to acyl migration.

Recently the author and his coworkers succeeded in a fast and straightforward two-step method for synthesis of SST with octanoic acid residues at the outer positions from DHA and EPA-rich bonito oil. This is industrially feasible production of 1,3-dioctanoyl-2-docosahexaenoyl-sn-glycerol, which is classified as a 'MCFA-PUFA-MCFA'. Fish oil TAGs (bonito oil) were subjected to ethanolysis with immobilized *Candida antarctica* lipase (Novozym 435) to yield 2-MAGs, which were subsequently reesterified with ethyl octanoate catalyzed by immobilized *Rhizomucor miehei* lipase (Lipozyme IM) to form SST in neat system. Typical time course of this reaction process is shown in Figure 2.

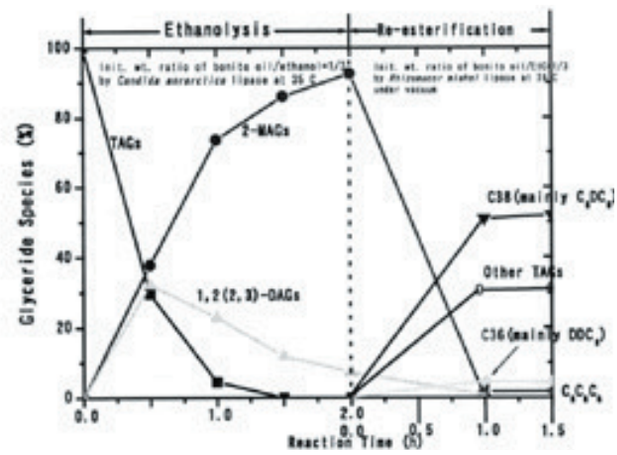

Fig 2. sTAG production from bonito oil via 2-MAG

The ethanolysis reaction was very fast. The acylglycerol composition at 2 h was 92.5% 2-MAG and 7.5% 1,2(2,3)-DAG (area %). The TLC analysis of the ethanolysis product showed that the formed MAGs were 100% 2-MAGs. No spot of 1-MAGs or 1,3-DAGs was detected. The reaction mixture composition (area %) at 2 h was 69.55% ethyl esters, 2.3% 1,2(2,3)-DAGs, and 28.25% 2-MAGs determined by TLC-FID. The FID response for ethyl esters is higher than for partial acylglycerols so that its actual weight % might be lower. No free fatty acids were detected in the reaction mixture.

The fatty acid composition of 2-MAGs resulting from ethanolysis was in good agreement with the composition of 2-MAGs obtained by Grignard degradation of the initial oil except EPA and DHA contents. Similar differences of the results obtained by enzymatic hydrolysis and Grignard degradation applied for fish oil were also mentioned in another work. The composition of ethyl esters resulting from ethanolysis was also determined. These ethyl esters are theoretically removed only from the primary positions of TAGs and thus the fatty acid composition of the initial TAGs might be recalculated using the fatty acid compositions of 2-MAGs and ethyl esters resulting from ethanolysis. The result of these calculations was very close to the initial composition determined directly after derivatization of initial TAGs to fatty acid methyl esters and thus the above hypothesis was confirmed. The analytical procedure using Grignard degradation might cause partial damage of PUFA resulting in the observed difference of the results for 2-MAGs composition determined by the two methods.

The results of fatty acid composition analysis of the ethanolysis reaction products (2-MAGs

and ethyl esters) demonstrate that Novozym 435 displays very strict 1,3-regiospecificy in this reaction under the employed reaction conditions. Bonito oil is a very complex mixture of TAGs with regard to the large variety of fatty acid species and their positional distribution in TAG and therefore it is an appropriate substrate to test the fatty acid specificity of Novozym 435 in ethanolysis. The fatty acid composition of 2-MAGs and ethyl esters formed after 2 h of ethanolysis indicated no distinguishable preference of Novozym 435 for any of the fatty acid species present in TAGs. The high conversion of the original TAGs to 2-MAGs at 2 h might have alleviated the effect of enzyme's fatty acid specificity. The acyl migration reactions were limited by the low water content of the ethanolysis reaction mixture (only the water contained in the enzyme preparation and reactants), the absence of fatty acids, and the low reaction temperature. Therefore, ethanolysis did not proceed to the removal of fatty acid residues of the second position of TGs, which usually affects analytical methods based on TAG hydrolysis with 1,3-specific lipases. Reduced acyl migration, strict 1,3-regiospecificity and good activity of Novozym 435 on PUFA enable the use of the ethyl esters resulted from ethanolysis for the determination of fatty acid composition of the secondary position of TAGs.

SSTs were obtained by reesterification with ethyl ocanoate of 2-MAGs formed in the first step. The ethanolysis reaction mixture was used directly in the second step after the catalyst was filtered and the excess of ethanol was removed. Novozym 435 showed no regiospecificity in this reaction so that it had to be replaced with a 1,3-regiospecific lipase, which can work at low water concentrations of the reaction medium. Lipozyme IM gave good results for a similar reaction and therefore, it was used also for this work. The value of ethyl octanoate excess adopted in the reesterification step was chosen according to some previous results showing that higher ethyl ocanoate/partial acylglycerol ratios improve the final reaction yield. The reaction equilibrium was pushed to high yields by removing the resulting ethanol under reduced pressure.

The reaction was completed after 1 h as shown by the disappearance of the 2-MAG and 1,2-DAG spots in TLC analysis. The separation of the finally purified SSTs by high-temperature GC was much better than that of the initial TGs. The final SST had lower molecular weight and therefore, higher volatility resulting in shorter retention times and better resolution. The area percentages of C38 (TAGs with two octanoic acid residues and one 22:6, 22:5 or 22:4 residue), and C36 (TAGs with two octanoic acid residues and one 20:5, 20:4, 20:1 or 20:0 residue) were: 51.0 and 4.5%, respectively. The percentage of trioctanoylglycerol was 1.7 %.

2) Synthesis of phsphatidylserine (4)

Phosphatidylserine (PS) is one of the structured phospholipids (sPL). PS is found neither in soybean nor in egg yolk lecithin, but is contained highly in mammalian cerebral cortex, and is claimed to be effective for Age-Associated Memory Impairment (AMMI). In 1983, Weihauch and Son determined PS contents of some food materials. According to their data, bovine brain has the highest PS content. Since then, PS was prepared by extraction of bovine brain. However, PS content is still quite small (only 1g/bovine head) so that PS is very expensive. Moreover, PS prepared from bovine brain was said to possibly cause BSE (Bovine Spongeform Enphalopasy). Due to these reasons, people searched for other cheaper and safer biomaterials.

By nature, phospholipase D (PLD) catalyzes hydrolysis of lecithin such as phosphatidylcholine (PC) to give rise to phosphatidic acid (PA). In addition to this, PLD catalyzes transphosphatidylation reaction, which is a base-exchange reaction (Figure 3). In the presence of excessive serine, PS can be easily produced by the action of PLD.

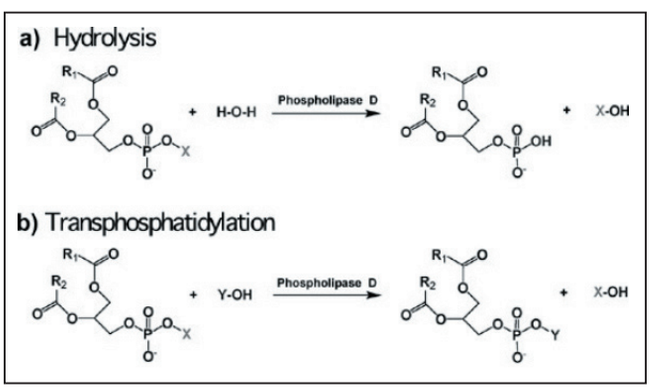

Fig3. Catalytic Action of Phospholipase D (PLD)

Lecithin is not soluble in water so that conventional PLD-catalyzed reaction is carried out in two-liquid phase system. That is, lecithin is dissolved in an organic solvent such as ethyl ether or ethyl acetate or chloroform, and serine and PLD are dissolved in water phase. These two-liquid

phases are then mixed vigorously. The product PS can be produced with as high yield as 85% in 20-30 minutes. However, use of these organic solvents makes the reaction system unsuitable for the practical food product. It is highly advisable not to use such organic solvents from the viewpoints of the health of both consumers and the workers in factory. The n-hexane is one of several safe solvents that are allowed for use in the food industry. However, unfortunately, no transphosphatidylation reaction occurs in hexane-water system.

The author and his coworkers have recently developed an efficient high-yield synthesis technique of PS from abundantly safe lecithin such as soybean lecithin by the transphosphatidylation of *Strteptomyces antibioticus* phospholipase D in non-solvent system.

The reaction in aqueous system without any organic solvents resulted in as low yield as 20 %. The author and his coworkers thought that the low yield of PS formation would be due to poor dispersion of lecithin, and tried the use of lecithin adsorbed on silica gel particles. First, lecithin was dissolved in ethanol, and then silica gel particles were added, followed by removal of ethanol by reduced pressure to get lecithin-silica gel complex. The complex was added in aqueous buffer containing serine and PLD. About 40% of PS was obtained in one hour. The effect of ratio of lecithin per silica gel particles was then examined. Serine concentration was also increased up to its saturation. The ratio of 1/5, and the increased serine concentration gave us 80 % yield in 24 hours. In order to seek for recovery procedure of PS after the reaction, the author and his coworkers tried to determine the location of the PS. Initially, the PC was on the surface of silica gel particles as expected. After the reaction, it was found that PS was in the supernatant, not in the precipitates, that is, not on the surface of silica gel particles. This makes it difficult to separate PS from the remaining serine existing in excess. A number of solid carrier particles were then screened to select better ones, including $CaCO_3$, $CaSO_4$, $Ca_3(PO_4)_2$, active carbon, etc. Among them, Calcium sulfate fine powder gave the best result. It yielded more than 99 % of conversion, more than 80 % of PS yield, and less than 10 % of PA (byproduct) content. The author and his coworkers had the same question as for calcium sulfate fine powders: where was PS located when lecithin adsorbed on calcium sulfate was used? At time zero, unexpectedly PC was dispersed in the supernatant. This means that PC was not adsorbed on $CaSO_4$ particles. At 24 hours, PS was in the precipitates, indicating that PS was selectively adsorbed on $CaSO_4$ particles. This makes easy separation of PS after the reaction. Then, the reaction operation was simplified. In the preceding experiments, lecithin was first dissolved in ethanol, followed by the addition of fine solid particles. In this experiment, the author and coworkers just added lecithin and CaSO4 powder to the serine-saturated aqueous solution containing acetate buffer and PLD to start the reaction. The time course of the reaction is shown in Figure 4. It is almost the same as in the old operation. PC was completely converted to PS in 20 minutes. The next attempt was to improve volumetric productivity by examining the effect of lecithin concentration in the reaction mixture while keeping the same amounts of $CaSO_4$, serine, and PLD. It was found that the higher its concentration, the slower the PS formation rate, but the final PS content of 80 % was still possible even though it took longer time. The final experiment of the author and his coworkers was to establish the method of recovering PS from the reaction mixture. As mentioned before, PS was adsorbed on $CaSO_4$ powders. The author and his coworkers used the following procedure: (i) reaction mixture was centrifuged, (ii) precipitates were washed with water first, then (iii) $CaSO_4$ particles were extracted with a mixed solvent composed of hexane, ethyl alcohol, water and 1N HCl., then (iv) the system was centrifuged, resulting in three parts, namely: the precipitate, the lower liquid phase and the upper liquid phase. By the analyses of three parts, it was found that PS was exclusively in the upper liquid phase. The total recovery yield that was calculated by this recovery experiment was ca. 80 %.

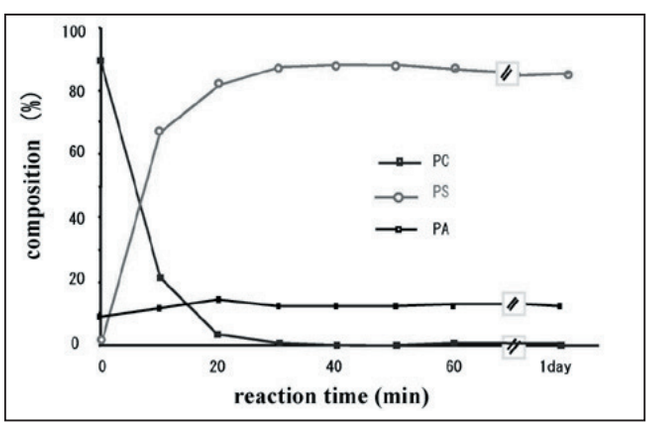

Fig 4. High Yield of PS Production from Lecithin By Adding Fine Powders of CaSO4 in Aqueous System

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