Health Beneficial Food Emulsifier Produced from Fishery Byproducts

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Abstract: The bioavailability of DHA-bound phospholipids, especially the DHA-bound lysophospholipid (DHA-LPL) could be considered the most effective DHA chemical forms for DHA accretion in the brain. Such a DHA-LPL should also have very high emulsifying stability performance based on its analogy with conventional soy LPL. Therefore, in this study, we describe two fishery byproducts, rich in DHA-bound phospholipids, to derive DHA-LPL via sn-1 positional specific lipase partial hydrolysis of the phospholipids. Through this reaction, the DHA composition increased to 43.8 % from 29.1 % in the salmon head phospholipid-derived DHA-LPL, and to 84.0 % from 47.4 % in the squid meal phospholipid-derived DHA-LPL. In fact, these obtained DHA-LPLs exhibited far higher emulsifying stability than the conventional food emulsifiers in the market. For example, the prepared high-purity squid meal phospholipid-derived LPL sustained an emulsion form for a week even under 80°C. Thus, food emulsifiers produced from fishery byproducts are considered to exhibit very high values of both in a sense of outstandingly high health benefits and sustaining emulsions even under very high temperatures.

Key words: DHA, lysophospholipids, emulsifier, omega 3, lipase

1 Introduction

Soybean-derived phospholipids have been generally used as food emulsifiers. However, they are rich in linoleic acid, which has been suggested to consume with moderation due to an increased risk of inflammation, as well as colon, breast, and prostate cancer. If we could substitute linoleic acids in phospholipid-based food emulsifiers with n-3 fatty acid that might at least in part help to decrease such risks. Moreover, n-3 fatty acid-bound phospholipids have been recently described as highly-bioavailable n-3 lipids compared to n-3 fatty acid-bound triacylglycerins (TAGs) such as fish oils. For example, Ulven et al. demonstrated that the supplementing healthy individuals with krill oil rich in n-3 fatty acid-bound phospholipids containing 543 mg of DHA + EPA results in significantly higher DHA + EPA levels in their plasma than the supplementation of n-3 fatty acid-bound TAG containing 864 mg of DHA + EPA. The same results have been obtained for patients with obesity.

In addition to the high bioavailability of DHA + EPA-bound phospholipids, animal experiments indicated that DHA-bound phospholipid administration is twice as effective in brain DHA accretion as DHA-bound TAG is. In rats, the DHA-bound phospholipid was twice more effective in DHA accretion than the DHA-bound TAG not only in the brain but also in the liver and kidney. Higher DHA incorporations were also reported in the adrenal gland, brown fat, thymus, and uveal tract/retina. A study in obese Zucker rats, which compared n-3 fatty acids either given in the form of TAG-fish oil or phospholipid-based krill oil, showed that krill oil supplementation led to significantly higher incorporation of DHA and EPA into the tissue phospholipids. In the heart, there was 96% and 42% higher incorporations of DHA and EPA, respectively, into the phospholipids after phospholipid-based krill oil administration compared to the TAG-based fish oil supplementation. Similar observations were made in the case of the liver. The EPA and DHA levels were 47% and 13% higher after phospholipid-based krill oil administration. Several other papers are pertaining to the high bioavailability of n-3 bound phospholipids.

The endocannabinoid system is involved in various physiological processes. The more n-6 arachidonic acid is available in cell phospholipids, the more endocannabinoids i.e. N-arachidonoyl-ethanolamide and 2-arachinoylglycerol are produced as endocannabinoids are derived from n-6 arachidonic acid. When endocannabinoids are overproduced,
negative biomembrane signaling- and energy metabolism-related effects could occur. However, n-6 arachidonic acid substitution with n-3 DHA or EPA might help to counterbalance the disturbed n-3 to n-6 ratio and result in lower endocannabinoid levels that might positively affect the aforementioned processes\textsuperscript{15}. The DHA and EPA-derived phospholipids are significantly more effective in incorporating the n-3 fatty acids into the cells than the TAG form. Therefore, the phospholipid forms of n-3 DHA or EPA are considered to be significantly easier to substitute with n-6 arachidonic acid. As a result, the endocannabinoid overproduction must be avoided. Two studies\textsuperscript{14, 19} showed that the phospholipid forms of dietary DHA and EPA are superior to TAG forms regarding glucose homeostasis maintenance and the reversal of hepatic steatosis, adipocyte hypertrophy, and low-grade inflammation.

The lowering effect of serum TAG and cholesterol was significantly higher in the case of DHA and EPA-bound phospholipid-based krill oil than TAG-based fish oil\textsuperscript{16}. The enzymatic activities involved fatty acid synthesis, i.e., acetyl-CoA carboxylase and fatty acid synthetase, significantly decreased. In contrast, hepatic fatty acid oxidation increased simultaneously, indicated by the increased activity of carnitine palmitoyltransferase I and carnitine levels. High-fat-diet-fed mice supplemented with DHA and EPA-bound phospholipid-based krill oil had significantly lower fasting serum glucose and higher serum adiponectin levels compared to the high-fat-fed mice not receiving krill oil\textsuperscript{17}. High-fat-fed mice supplementation with DHA and EPA-bound phospholipid-based krill oil (1.25–5%\textsuperscript{19}) significantly reduced the amount of the produced hepatic TNF-α protein\textsuperscript{18}.

A notable feature is that DHA and EPA bound phospholipid-based krill oil supplementation enhanced both the short- and long-term memories in rats. Moreover, lipid peroxidation in the plasma and brain decreased, cell generation in the dentate gyrus was enhanced, and the level of the reactive oxygen species in the cerebral cortex and hippocampus were lowered\textsuperscript{19}.

The ability of the DHA and EPA-bound phospholipid-based krill oil to influence gene expression by binding to specific gene transcription factors was explored in a 12-week study on healthy CBA/J mice\textsuperscript{20}. Even though the DHA and EPA-bound phospholipid-based krill oil diet and the DHA and EPA-bound TAG-based fish oil diet contained approximately 0.3% of EPA and DHA in similar ratios, the DHA and EPA-bound phospholipid-based krill oil modulated 52 molecular pathways while the DHA and EPA-bound TAG-based fish oil only affected 4 pathways.

The DHA-bound phospholipid administration is twice more effective in brain DHA accretion than the DHA-bound TAG\textsuperscript{3–7} as aforementioned. DHA transport into the brain has also been identified and it was shown that DHA is transported in a lysophospholipid form via the Mfsd2a transporter and not as unesterified fatty DHA\textsuperscript{21}. In this study, we carried out the sn-1 positional specific lipase-mediated partial hydrolyses on marine phospholipids rich in DHA. Through this approach, we obtained a DHA extremely rich in DHA-bound lysophospholipid (DHA-LPL) both from salmon head and squid meal phospholipids. Although the transport behavior to the blood-brain barrier (BBB) is not clear for the DHA-LPL after the incorporation, the prepared lysophospholipid via partial hydrolysis must be highly beneficial in a sense that it must be very effective in reaching to BBB. In addition to this, soy lysophospholipids are known to have extremely high emulsifying properties, especially the emulsifying stability under acidic or high-temperature conditions. Therefore, DHA-LPLs must also have extremely high emulsifying activities.

Thus, the purpose of the present study was to propose a highly health-beneficial excel performance food emulsifier using fishery byproducts.

2 Experimental Procedures
2.1 Materials

Chum salmon (Oncorhynchus keta) landed on the eastern coast of Hokkaido was decapitated and the heads were transferred to Kurosu Inc. (Ishinomaki Miyagi, Japan). Then, salmon head cartilage was removed by a rotating machine shown in Fig. 1 after protein denaturation under 60°C hot water. The remaining was subjected to 95% iso-propanol (IPA) lipid extraction. Squid meal was obtained from the Iwasaki Suisan Inc. (Hakodate Hokkaido, Japan). The monoolein (Sun soft 8070) was purchased from the Taiyo Kagaku Co., Ltd. (Yokkaichi city, Japan) and the sucrose fatty acid ester with di- and triester (DK ester F-10) from the DKS Co. Ltd. (Kyoto, Japan). We obtained the 99% purity monooester sucrose palmitate from The Nissin OilIiO Group, Ltd. (Tokyo, Japan) and sucrose fatty

Fig. 1 Salmon head cartilage removing machine run by rotating.
2.2 Lipid extraction
2.2.1 Lipid extraction of salmon head phospholipids and preparation of salad dressing model oils

To the cartilage removed salmon head tissues, a two-fold weight of 60°C 0.9% NaCl solution was added and stirred under 60°C for 0.5 h to float out simple lipids. Then it was centrifuged for 10 min at 5,000×g. The obtained precipitate was supplemented with 5-times 95% IPA, then stirred under 60°C to recover complex lipids, i.e., mainly phospholipids.

For the salad dressing model oils used for the evaluation of emulsion stability, chum salmon oil which was extruded from the head cartilage at Kurosu Inc. (Ishinomaki Miyagi, Japan) or deacldized silver salmon (Oncorhynchus kisutch) oil obtained by n-hexane:IPA(2:1, v/v) extraction after freeze-drying were used. The silver salmon used was cultured in Peru.

2.2.2 Lipid extraction of squid meal phospholipids at a semi-plant scale

The 191 kg of dried comminuted squid meal was supplemented with 900 L of 95% IPA. The lipid extraction was performed for 4 h at 55°C. After the IPA removal, the sample was supplemented with another 200 L of 95% IPA and rinsed. Both 95% IPA solutions were combined and concentrated under vacuum to become 160.1 kg. After draining this extract, the vessel was rinsed with 6 L of water, and the extract and the rinsed water were transferred to another vessel and vacuum concentrated at 1.3 kPa and 60°C until it became 50 L.

2.3 Lipid class separation

Complex lipids (mainly phospholipids) were separated from the salmon head-derived IPA-extracted lipid using a Sep-Pak Plus silica cartridge (Waters Corporation, Milford, MA, USA). The IPA-extracted 200 mg lipid was loaded onto the top of the cartridge, then simple lipids and phospholipids were eluted using chloroform and methanol, respectively, in sequential order. In this study, phospholipids were also separated using the acetone precipitation method. Briefly, more than a 20-fold volume of ambient temperature acetone was added to the IPA extracted lipid, then dissolved, was kept overnight in a freezer (−20°C), then the phospholipid concentrated precipitate was recovered.

The salmon head phosphatidylcholine (PC) was obtained as follows. The crude salmon head phospholipid fraction obtained through the acetone precipitation method was subjected to sphere silica gel PSQ 60B (Fuji Silysia Chemical Ltd., Kasugai city, Japan) packed column chromatography. First, the column was eluted thoroughly with chloroform to remove the remaining simple lipids, then chloroform:methanol:water (60:40:5, v/v/v) was eluted using a fraction collector to obtain the PC by complex lipid monitoring thin-layer chromatography (TLC) systems, which were performed as follows (squid meal PC was obtained similarly).

The lipid compositions were determined in two ways. First, by using a silica gel 60F254 (Merck, Darmstadt, Germany) TLC plate with solvent systems consisted of n-hexane-diethyl ether:acetic acid (80:30:1, v/v/v) for simple lipid fraction, and chloroform:methanol:water (60:40:5, v/v/v) for complex lipid fraction. The plates were sprayed with 8% phosphoric acid containing 3% cupric acetate and heated at 150-160°C for 15 min. Each appearing spots were identified by authentic lipid standards, then the lipid compositions were analyzed using a scanner and TLC analysis software (JustTLC version 4.0.3, Sweden, Lund, Sweden). Second, by using a TLC-flame ionization detector (FID) (LATROSCAN, LSI Medience Corporation, Tokyo, Japan). Briefly, sample lipids were diluted in 1% chloroform:methanol (2:1, v/v) and the individual 5 μL were spotted on SIII chromarods (LSI Medience Corporation), then developed twice with chloroform:methanol:water (45:20:2, v/v/v) and detected using FID. Since the FID response is proportional to the amount of the carbon with C-H bonds. The calibration was done by taking into consideration the mean molecular weight of the individual lipid classes.

2.4 Fatty acid composition analysis

The lipids were converted into methyl ester derivatives following the method of Prevot and Morde (20) with modifications. Briefly, the dried samples were dissolved in 1.0 mL of n-hexane and were supplemented with 0.2 mL of 2 normal-methanolic-NaOH. Then, the mixture was shaken, kept at 50°C for 30 seconds, supplemented with 0.2 mL of 2 normal-methanolic-HCl, and shaken until neutralization. The n-hexane layer was collected, concentrated using nitrogen gas flow, and subjected to gas chromatographic analysis using a 0.5 µm PEG-20M liquid phase-coated 40 m × 1.2 mm diameter G-300 column (Chemicals Evaluation and Research Institute, Saitama, Japan) connected to a GC353 gas chromatograph (GL Sciences Inc., Tokyo, Japan) that was equipped with FID. The temperatures of the column, detector, and injection port were 190, 240, and 250°C, respectively. The fatty acids were identified by comparing the peak retention times to authentic standards (GL Sciences Inc., Tokyo, Japan) and by following the theory of the linear relationship between the carbon number units or the number of double bonds of fatty acids.
2.5 Partial hydrolysis of phospholipids

2.5.1 Partial hydrolysis of salmon head phospholipids

The partial hydrolysis of the salmon head phospholipid was carried out on the lab-scale as follows. The 50 mg IPA-extracted crude phospholipid sample was supplemented with 5 mL of water and dispersed thoroughly until becoming homogeneous. Next, it was supplemented with 10 mg of powdered Li-lipase A-10D to initiate the partial hydrolytic reaction, then the reaction was carried out at 40°C after filling the headspace of the reaction vial with argon gas. Stirring was provided using a magnetic stirrer. The reaction rates were monitored at 1, 2, 4, 8, 12, 24, and 48 h, by adding chloroform:methanol:water (2:1:0.5, v/v/v) every time to the individual vials, and recovering the chloroform layer lipids, then applying those to Sep-Pak silica cartridges (Waters Corporation, Milford, MA, USA) under the same conditions as described above for removing the released free fatty acids during the partial hydrolytic reactions. The lipid and fatty acid compositions of the obtained phospholipid fraction were analyzed as described above in subsections 2.3 and 2.4.

2.5.2 Partial hydrolysis of squid meal phospholipids

The partial hydrolysis on the semi-plant scale was performed using 95% IPA extracted squid meal lipid as follows. The 2/3 of the 50 L squid meal lipid concentrated solution, described above in section 2.2.2, was supplemented with 90 L of water and enzyme solution, consisting of 3 kg of Li-lipase A-10D powder dissolved in 10 L water, to initiate the partial hydrolytic reaction for overnight at 30°C. This reaction mixture was supplemented with 39.8 kg of n-butanol, mixed for 30 min, then sat still for another 30 min. The reaction was quenched by separating the aqueous enzyme solution. This vacuum-dried n-butanol layer, obtained at 52°C, 2.7 kPa in 6.5 h, was supplemented with 40 kg 95% IPA and 10 L of water and the liquid portion was evaporated under 1.3 kPa, 60°C for 4 h to obtain the crude squid meal lysophospholipid (LPL). After freeze-drying, it was subjected to a Japanese Unexamined Patent purification method based on a combination of activated carbon, and reusable iron exchange resin/silica gel column chromatography. From the hydrolysate with LPL 20-30% lipid, more than 60% purity LPL was easily prepared using this method.

The purification extent of the squid meal LPL was varied to investigate the relationship between the purity of the obtained LPL and the increase in DHA content, as well as lysophosphatidylcholine (LPC) ratio. Various purity squid meal LPLs were prepared following the solvent partitioning procedure performed as follows. The 335 mg crude squid meal LPL sample, corresponding to the hydrolysate obtained above, was supplemented with 8 mL of n-hexane: ethanol:water (10:1:1, v/v), then shaken and the n-hexane layer was removed. The remaining sample was supplemented with 3.5 mL of n-hexane, then shaken and the n-hexane layer appeared after standing was recovered again. It was similarly supplemented with 3 mL, 2.5 mL, and 2 mL of n-hexane, then removed individually. When the phase separation did not occur, a small volume of ethanol was added. The obtained lower layers were evaporated and the semi-purified squid meal LPLs were prepared. These various purity squid meal LPLs were subjected to the following emulsifying performance analyses.

2.6 Determination of the emulsifying properties

We determined the emulsifying properties, such as surface tension and critical micelle concentration (CMC) of the obtained purified LPCs from salmon and squid. To isolate the LPCs, PCs were first obtained by silica gel column chromatography (Fuji Silysia Chemical Ltd. PSQ60B, Kasugai city, Japan) using chloroform as an eluent to remove simple lipids, then using chloroform:methanol:water (65:40:5, v/v/v) as an eluent to obtain purified PCs. These PCs were then subjected to Li-lipase-mediated partial hydrolysies as described above. The pure LPCs were isolated by silica gel column chromatography (Fuji Silysia Chemical Ltd. PSQ60B, Kasugai city, Japan) using chloroform:methanol (1:2, v/v) as an eluent. The elution monitors were performed on a silica gel 60F254 (Merck, Darmstadt, Germany) TLC plate using solvent systems consisting of chloroform:methanol:water (60:40:5, v/v/v).

The surface tensions of the suspensions prepared with phospholipids, LPLs, purified LPCs, and the emulsifiers of the market were measured using a CBVP-Z tensiometer (Kyowa Interface Science Co. Ltd., Saitama, Japan) by employing the Wilhelmy plate technique. The measurements were performed at 25°C in an equilibrium state, i.e., the surface tension was measured after the change in the observed value was less than 0.1 mN/m for 1 h. The CMC was determined from the plots of the surface tension against the logarithm of the concentration (log C).

The emulsifying stability was determined through visual judgment of phase separation imitating a salad dressing emulsion system. Briefly, 0.4 g of the salmon head LPC sample, and 0.4 g of monoolein or soy PC as emulsifier references were mixed with 0.9 mL water and incubated overnight at 5°C, headspace filled with argon gas. Then the samples were homogenized at 11,400 rpm using a VH-10 homogenizer equipped with an S10N-10G shaft generator (AS ONE Corporation, Osaka, Japan). The sample was homogenized again at 11,400 rpm after adding 1.5 g of rice vinegar, and another homogenization step was performed at 11,400 rpm after adding 0.2 g salt. Finally, the sample was supplemented with 7 g of salmon TAG, homogenized at 11,400 rpm, then homogenized for another 30 s at 20,450 rpm. This freshly prepared emulsion system was considered as 0 min. We performed the visual judgment of
the phase separation an hour after, then the temperature was increased to 80°C and the phase separations were judged every 1, 2, 6, 24, 48, and 72 h.

The squid meal LPL salad dressing imitation emulsion systems were prepared as follows. We mixed 0.25 g of the individual emulsifiers, shown in Table 1, with water to obtain the whole weight of 1.3 g and incubated overnight. This process was followed to achieve complete dissolution. The types of emulsifiers are shown in Table 1. The emulsifiers from Table 1 were supplemented with 1.5 g of rice vinegar, 0.2 g of salt, and 7 g of salmon oil in three homogenization steps using a VH-10 homogenizer equipped with an S10N-10G shaft generator (AS ONE Corporation, Osaka, Japan) at 11,400 rpm, then 20,450 rpm for 30 s. The head-space gas of the containers was replaced with nitrogen gas and the samples were incubated for 2 h at room temperature, then at 80°C for a maximum of 14 days. The time when the emulsions began to collapse and when they completely collapsed were recorded. The temperature was set at 80°C for shortening the stable emulsion time.

3 Results and Discussion

3.1 Partially hydrolyzed phospholipid compositions

3.1.1 Partially hydrolyzed salmon head phospholipid compositions

As shown in Table 2, the maximum LPC amount was obtained at 12 h while the maximum DHA amount in crude phospholipid was obtained at 8 h reaching up to 43.8%, as showed in Table 3, after the partial hydrolysis of the prepared salmon head crude phospholipid. The initial DHA amount in the substrate salmon head crude phospholipid, i.e., the 0 h reaction time, was 29.1%, meaning that the

Table 1 The individual contained emulsifier used for the evaluation of salad dressing model emulsifying stability.

| Water only                                      |
|-----------------------------------------------|
| Monoolein (82 % as monoolein molecular species) |
| Sucrose fatty acid ester with di and triester (99% purity) |
| Monoester sucrose palmitate (99% purity)       |
| LPL derived from 38.8 % soy phospholipid       |
| Before partial hydrolysis (66.7 % phospholipid) |
| Partial hydrolysate itself (45.7 % phospholipid) |
| First solvent partitioning (74.7 % phospholipid) |
| Third solvent partitioning (88.4 % phospholipid) |
| Fifth solvent partitioning (92.0 % phospholipid) |
| Unexamined Patent purification method* (96.4 % phospholipid) |

*Method based on a combination of activated carbon, reusable iron exchange resin/silica gel column chromatography.

Table 2 Lipid composition of the partial hydrolytic reaction products of the salmon head polar lipid catalyzed with Li-lipase A-10D in aqueous media*. (%)  

| Lipid class | Reaction time (hour) |
|-------------|---------------------|
|             | 0 h     | 1 h     | 2 h     | 4 h     | 8 h     | 12 h    | 24 h    | 48 h    |
| PE          | 26.9    | 19.8    | 16.3    | 16.1    | 12.7    | 14.0    | 14.8    | 14.9    |
| PC          | 52.0    | 30.6    | 31.0    | 30.7    | 28.6    | 28.1    | 26.2    | 25.4    |
| SPM         | 6.7     | 11.0    | 11.9    | 10.7    | 13.5    | 12.6    | 13.0    | 13.1    |
| LPC         | 2.5     | 21.6    | 21.9    | 23.6    | 26.6    | 26.8    | 26.3    | 22.2    |

* Determined by thin layer chromatography and small amounts are omitted.
Refer to "2.5.1 Partial hydrolysis of salmon head phospholipids" in the text for the reaction condition.
PE, Phosphatidylethanolamine; PC, Phosphatidylcholine; SPM, Sphingomyelin; LPC, Lysophosphatidylcholine
h: hours (reaction time)
DHA exhibited a 1.5-fold ratio increase after the 8 h reaction. We also carried out a partial hydrolytic reaction in IPA-containing media and observed that the more IPA was in the media, the less LPL was produced (data not shown). This was also true for the reaction mediated by immobilized phospholipase A2 (Aspergillus oryzae, a gift from Mitsubishi Chemical Foods Co. Ltd., Tokyo, Japan, data also not shown). We thus considered that IPA in the media deprived the essential water for the activation of the Li-lipase A-10D or the immobilized phospholipase A2.

### 3.1.2 Partially hydrolyzed squid meal phospholipid compositions

As shown in Table 4, we could detect a negligible LPC amount before the partial hydrolysis of the squid meal phospholipid, which increased to 30.0, 48.3, 59.1, and 62.0% upon performing the partial hydrolysis, then the first, third and fifth solvent partitioning, respectively, using n-hexane:ethanol:water. When we carried out the Japanese Unexamined Patent purification method based on a combination of activated carbon, reusable iron exchange resin/silica gel column chromatography, the LPC purity reached 80.6% with no remaining phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE).

Table 5 shows the fatty acid composition of phospholipids with various purities before and after the partial squid meal phospholipid hydrolyses. The PC itself contained 47.4% DHA in the squid meal 95% IPA extract, while the LPC itself contained 84.0% DHA after the partial hydrolytic reaction, as indicated in the right end column in Table 5.

By viewing the DHA% through the solvent partitioning procedures, the DHA% increased from 39.0% in the partial hydrolysate itself to 61.7% after the first solvent partitioning step. However, the increase in DHA% after the third and fifth solvent partitioning steps were small. When

### Table 3

| Fatty acid | Reaction time (hour) |
|-----------|----------------------|
|           | 0 h | 1 h | 2 h | 4 h | 8 h | 12 h | 24 h | 48 h |
| C16:0     | 22.4| 7.3 | 5.6 | 6.0 | 5.8 | 6.3  | 6.4  | 6.1  |
| C18:0     | 7.0 | 4.8 | 2.1 | 1.9 | 1.7 | 1.9  | 1.6  | 1.7  |
| C18:1     | 21.2| 24.2| 19.2| 21.3| 19.6| 20.2 | 21.9 | 20.3 |
| C20:5     | 4.4 | 6.6 | 8.5 | 8.5 | 8.6 | 8.2  | 8.4  | 8.1  |
| C22:6     | 29.1| 37.5| 43.0| 42.9| 43.8| 43.2 | 41.1 | 40.1 |

Refer to “2.5.1 Partial hydrolysis of salmon head phospholipids” in the text for the reaction condition.

### Table 4

| Before partial hydrolysis (66.7 % phospholipid) | Partial hydrolysate itself (45.7 % phospholipid) | First solvent partitioning (74.7 % phospholipid) | Third solvent partitioning (88.4 % phospholipid) | Fifth solvent partitioning (92.0 % phospholipid) | Unexamined Patent purification method (96.4 % phospholipid) |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Cholesterol + Free fatty acids                | 33.4                                          | 54.3                                          | 25.3                                          | 11.6                                          | 8.0                                          | 3.7                                           |
| PE                                            | 7.6                                           | 1.8                                           | 4.5                                           | 5.1                                           | 3.9                                          | N.D.                                          |
| LPE                                           | N.D.                                          | 2.2                                           | 4.3                                           | 2.1                                           | 4.8                                          | N.D.                                          |
| PC                                            | 48.6                                          | 7.9                                           | 10.0                                          | 12.5                                          | 13.8                                         | 14.8                                          |
| LPC                                           | N.D.                                          | 30.0                                          | 48.3                                          | 59.1                                          | 62.0                                         | 80.6                                          |
| others                                        | 10.5                                          | 3.9                                           | 7.6                                           | 9.7                                           | 7.4                                          | 0.9                                           |

*Method based on a combination of activated carbon, reusable iron exchange resin/silica gel column chromatography.

Refer to “2.5.2 Partial hydrolysis of squid meal phospholipids” in the text for the reaction condition.

h: hour (reaction time)

For the abbreviations, See Table 2.
the above-mentioned Japanese Unexamined Patent purification method was carried out, the DHA% increased to 76.7%.

3.2 Emulsifying properties

Concerning the emulsifying property evaluation of various surfactants, we considered surface tension, critical micelle concentration, hydrophile-lipophile balance, bubble force, and emulsification stability. Among these indices, we chose critical micelle concentration (CMC) and emulsification stability.

3.2.1 The critical micelle concentration (CMC)s of the salmon head PC- and squid meal PC-derived purified LPCs

CMC is defined as a minimum concentration of surfactants that could form a micelle. The smaller the CMC, the higher the performance of the surfactant. By measuring the surface tensions of a surfactant under various concentrations, following the hanging plate method, then by plotting the relationship between surface tension and logarithm of the concentration of the surfactant, the bending point on the plotted line highlights the CMC as illustrated in Fig. 2. In usual cases, the bending point of widely used surfactants results in one which means CMC is only one. However, in some rare cases, two bending points could also occur, resulting in two CMCs. As indicated in Fig. 2, the high-purity squid meal LPC showed the first CMC at 0.30 × 10⁻³ weight%. Moreover, the second CMC was 1.2 × 10⁻³ weight%. Similarly, as shown in Fig. 2, various surfactant CMCs were determined, and the results are shown in Table 6. Salmon head phospholipid-derived crude LPL contained almost the same amounts of LPC and LPE. The first CMC

Table 5 Fatty acid composition of the partial hydrolytic reaction products of the squid meal crude phospholipid mediated by Li-lipase A-10D in aqueous media. (%)

| Fatty acid composition | Before the partial hydrolytic reaction (All the fatty acids which are esterified on individual lipid classes) | After the partial hydrolytic reaction (All the fatty acids which are esterified on all the phospholipid classes and free fatty acids) | PC or LPC scraped off from the TLC plate |
|-----------------------|--------------------------------------------------|------------------------------------------------------------------------------------------------------------------|----------------------------------------|
|                       | Partial hydrolysate itself | First solvent partitioning | Third solvent partitioning | Fifth solvent partitioning | Unexamined Patent purification method | PC substrate before the partial hydrolytic reaction | LPC after the partial hydrolytic reaction |
| C16:0                 | 30.0                          | 28.9                                 | 4.7                          | 3.2                          | 4.4                          | 2.0                          | 32.1                          | 0.8                          |
| C18:1                 | 3.1                           | 3.2                                 | 1.5                          | 1.4                          | 1.6                          | 1.2                          | 1.2                           | 3.0                          |
| C20:5 (EPA)           | 13.6                          | 16.1                                 | 25.1                         | 26.0                         | 23.0                         | 14.6                         | 8.2                           | 11.9                         |
| C22:6 (DHA)           | 40.3                          | 39.0                                 | 61.7                         | 62.7                         | 64.4                         | 76.7                         | 47.4                          | 84.0                         |

*1 TLC: Thin layer chromatography (chloroform:methanol:water (60:40:5, v/v/v))  
*2 Method based on a combination of activated carbon, reusable iron exchange resin/silica gel column chromatography.  
Refer to "2.5.2 Partial hydrolysis of squid meal phospholipids" in the text for the reaction condition.

h: hour (reaction time)
of this crude salmon head phospholipid-derived LPL was more than 20 times larger than the first CMC of high-purity salmon head phospholipid-derived LPC. This suggests that LPE extremely spoils the emulsifying potential of LPC. From this perspective, we could consider that the contaminated LPE from LPL removal is important when salmon heads are used. The first CMCs of the high-purity salmon head phospholipid-derived LPC and the high-purity squid meal phospholipid-derived LPC were comparable to the sucrose fatty acid ester with monoester sucrose palmitate, which is widely used in the market. For this reason, these two fishery byproduct-derived LPCs stand in comparison with the emulsifying performance of the widely used emulsifier and have a significant benefit in intaking DHA at the highest bioavailability.

The emulsifying stability determined by the visual judgment of phase separation, imitating a salad dressing emulsion system, is shown in Fig. 3 and Table 7. As illustrated in Fig. 3, salmon head phospholipid-derived high-purity LPC showed far higher emulsifying stability than soy PC and monoolein. It is important to note that, as showed in Table 7, even the partial hydrolysate of the squid meal 95% IPA extract with 74.7% phospholipid purity crude LPL, the salad dressing emulsion system was stable even under 80°C. When the LPC content was increased to 96.4% by employing the unexamined patent purification method based on a combination of activated carbon, reusable iron exchange resin/silica gel column chromatography, we observed the notable feature of the salad dressing emulsion system lasting for a week under 80°C, although allowing a partial emulsion collapse, and lasting at least for 2 weeks under 80°C until the emulsion completely collapsed. The LPE removal could be responsible for the increased emulsifying stability.

Thus, we could conclude that fishery byproduct phospholipid-derived LPCs, such as squid skin meal- and salmon head-derived LPCs, are highly valuable concerning their outstandingly high emulsifying stability performance and prominent DHA composition in the form of the most effective lipid chemical form in passing through the blood-brain barrier.

Table 6 The CMCs of LPC derived from salmon head PC and LPC derived from squid meal in comparison to various surfactant CMCs.

| Surfactant                                      | CMC (weight %, 25°C) |
|------------------------------------------------|----------------------|
| Monoester sucrose palmitate (99% purity)       | 0.29 × 10⁻³          |
| Sucrose fatty acid ester with 70% monoester and 30% di and triester (99% purity) | 0.21 × 10⁻³          |
| Soy PC (99%)                                    | ND                   |
| LPL derived from 38.8 % soy phospholipid        | 1.5 × 10⁻³           |
| Crude lysophospholipid derived from salmon head phospholipid (LPC+LPE) | 10 × 10⁻³, 70 × 10⁻³ |
| High purity LPC derived from salmon head phospholipid (99% purity) | 0.50 × 10⁻³, 6.0 × 10⁻³ |
| High purity LPE derived from salmon head phospholipid (99% purity) | 0.36 × 10⁻³, 1.7 × 10⁻³ |
| High purity LPC derived from squid meal phospholipid (99% purity) | 0.30 × 10⁻³, 1.2 × 10⁻³ |

Fig. 3 Emulsion stability of 99% purity LPC derived from salmon head phospholipid. Filled bars illustrate complete emulsions. Gray bars and open bars illustrate partial separation and complete separation of oil and water, respectively.

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| Emulsifier                  | Time when the emulsion completely collapses | Time when the emulsion begins to collapse | References                                                                 |
|----------------------------|---------------------------------------------|------------------------------------------|-----------------------------------------------------------------------------|
| Water only                  |                                             |                                          |                                                                             |
| Monoolein (82% as monoolein molecular species) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 1) Ulven, S.M.; Kirkhus, B.; Langlait, A.; Basu, S.; Elind, E.; Haider, T.; Berge, K. et al. Metabolic effects of krill oil are essentially similar to those of fish oil but at lower dose of EPA and DHA, in healthy volunteers. *Lipids* **46**, 37-46 (2011). |
| Sucrose fatty acid ester with di and triester (99% purity) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 2) Maki, K.C.; Reeves, M.S.; Farmer, M.; Kevin, C.; Griniari, M.; Berge, K.; Vik, H. et al. Krill oil supplementation increases plasma concentrations of eicosapentaenoic and docosahexaenoic acids in overweight and obese men and women. *Nutr. Res.* **29**, 609-615 (2009). |
| Monoester sucrose palmitate (99% purity) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 3) Lagarde, M.; Bernoud, N.; Brossard, N.; Lemaitre-Delannay, D.; Thiès, F.; Croset, M. et al. *Lyso*phosphati-dylcholine as a preferred carrier form of docosahexaenoic acid to the brain. *J. Mol. Neurosci.* **16**, 201-204 (2001). |
| LPL derived from 38.8% soy phospholipid | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 4) Picq, M.; Chen, P.; Perez, M.; Perez, M.; Michaud, M.; Vérecil, E. et al. *DHA* Metabolism: Targeting the brain and lipoxygenation. *Mol. Neurobiol.* **42**, 48-51 (2010). |
| Before partial hydrolysis (66.7% phospholipid) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 5) Graf, B.A.; Duchateau, G.S.; Patterson, A.B.; Mitchell, E.S.; van Bruggen, P.; Koek, J.H. et al. Age dependent incorporation of 13C-DHA into rat brain and body tissues after dosing various 13C-DHA-esters. *Prostaglan-dins Leukot. Essent. Fatty Acids* **83**, 89-96 (2010). |
| Partial hydrolysis (45.7% phospholipid) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 6) Liu, L.; Bartke, N.; van Daal, H.; Lawrence, P.; Qin, X.; Park, H.G. et al. Higher efficacy of dietary DHA provided as a phospholipid than as a triglyceride for brain DHA accretion in neonatal piglets. *J. Lipid Res.* **55**, 531-539 (2014). |
| First solvent partitioning (74.7% phospholipid) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 7) Batetta, B.; Griniari, M.; Carta, G.; Murr, E.; Ligresti, A.; Cordeddu, L. et al. Endocannabinoids May Mediate the Ability of (n-3) Fatty Acids to Reduce Ectopic Fat and Inflammatory Mediators in Obese Zucker Rats *J. Nutr.* **139**, 1495-1501 (2009). |
| Third solvent partitioning (88.4% phospholipid) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 8) Schuchardt, J.P.; Schneider, I.; Meyer, H.; Neubronner, J.; von Schacky, C.; Hahn, A. Incorporation of EPA and DHA into plasma phospholipids in response to different omega-3 fatty acid formulations - a comparative bioavailability study of fish oil vs. krill oil. *Lipids Health Dis.* **10**, 145 (2011). |
| Fifth solvent partitioning (92.0% phospholipid) | An hour under ambient+80°C, 2 hours under ambient | 6 hours under ambient+80°C, 6 hours under ambient | 9) Ulven, S.M.; Kirkhus, B.; Langlait, A.; Basu, S.; Elind, and Managing director Sadahiro Suzuki as well as Corporate Adviser Mr. Shigenori Nagao of the Kurosu Inc. (Ishinomaki Miyagi, Japan) for providing salmon head tissues, and chum salmon oil exuded from salmon head cartilage. Thank are due to Mr. Masashi Kanoh for his experimental help. This work was partially supported by “A Scheme to Revitalize Agriculture and Fisheries in Disaster Area through Deploying Highly Advanced Technology” from the Ministry of Agriculture, Forestry and Fisheries of Japan. |
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