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ORIGINAL ARTICLE

Characteristics and emulsifying properties of structured phospholipids from palm pressed fiber and omega-3 fatty acid concentrates from by-products of fish processing by enzymatic acidolysis

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Abstract The synthesis of structured phospholipids (SPLs) containing ω-3 fatty acids was carried out through enzymatic acidolysis reactions using lipase from *Rhizomucor miehei* between palm pressed fiber phospholipids (PPF PL) with four ω-3 fatty acid concentrates as different acyl sources. The purity of SPLs increased compared to original PPF PLs. The degree of ω-3 fatty acid incorporation to the SPLs was different that depended on the sources of acyl. The highest degree of incorporation was in PE (phosphatidylethanolamine). The phenomenon of acyl migration of EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) was found from the sn-1 to the sn-2 position of the PE. This acyl migration occurred at all four SPLs. Modification of PLs made better emulsifying properties for oil in water emulsion system, compared to PPF PL. The increase in the HLB (hydrophilic-lipophilic balance) value, EAI (emulsifying activity index), and ESI (emulsion stability index) of SPLs compared to PPF PL was supposed to relate to an increase in polarity. The composition of more polar PLs (PC/phosphatidylcholine, PE, PG/phosphatidylglycerol, and PA/phosphatidic acid) in the four SPLs is higher than that of PPF PL. The sources of acyl also affected the emulsifying properties of four SPLs.

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Introduction

One of the significant roles in the country's growth is industrialization (Ahmad et al. 2019). Food processing industries have grown tremendously, but they also generate a significant quantity of wastes. There is a need to valorize waste into various value-added products to reduce environmental contamination from the waste (Saini et al. 2019).

Oil palm is a valuable source of edible oil produced from the fruits of *Elaeis guineensis* and *Elaeis oleifera*. During processing of the fresh fruit bunch (FFB) into crude palm oil, several by-products are produced, including empty fruit bunch, palm kernel shell, palm oil mill effluent, and palm pressed fiber (PPF). Therefore, the valorization of waste materials is interesting in mitigating some damage to the environment while increasing the profits of palm industries. PPF is a by-product produced during crude palm oil extraction from FFB in the palm oil milling process (Chang et al. 2018). PPF is a biomass with a considerable quantity that represents approximately 15% of fresh fruit bunch (Husain et al. 2002). PPF entraps 5–7% of the residual oil within its fibrous structure (Ooi et al. 2019) with excellent oxidative stability that might be contributed by the occurrence of phospholipids (PLs) (Lau et al. 2006).

Phospholipids are used extensively in food industries as emulsifiers to stabilize emulsion systems, antioxidants, and stabilizers (Hama et al. 2015). Phospholipids are also found in residual oils of PPF. Choo et al. (2004) extracted PPF with ethanol and 46,800 ppm of phospholipids were obtained. The fatty acids composition of PL from PPF is dominated by palmitic acid, oleic acid, and linoleic acid,

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and to some extent also presents stearic acid and arachidic acid. Due to a considerable quantity of PPF, Choo et al. (2004) estimated that PPF could provide 21,645 tons of PPF lecithin based on the world production of FFB. Thus, it is an alternative source of lecithin, which is usually provided by soy lecithin. PPF PL is still limitedly explored, and so far, it is separated by solvent extraction (Choo et al. 2004).

One factor that affected PL properties is fatty acid composition. Fatty acid in PL might be modified to be more valuable, such as higher oxidative stability, better emulsifying properties, and higher nutritional value. Modification of PL is achieved by exchanging the fatty acids of PL with the intended one, mainly by the enzymatic approach to have better characteristics.

Other food industrial waste that is not yet extensively used is liquid waste from fish canning or meal processing. This liquid waste still contains an appreciable amount of fish oil that can be separated by decantation. These oils contain a significant level of ω-3 fatty acids, mainly EPA and DHA (Estiasih et al. 2008). A combination of EPA/DHA from fish oils and PPF PL is expected to provide greater health benefits of modified PL. ω-3-Fatty acid-binding phospholipids are known to be a potent carrier of ω-3 fatty acids and have better bioavailability than triglycerides (Chen et al. 2019).

Hachem et al. (2016) showed that DHA containing PC is efficiently targeted DHA to the brain. LysoPC containing DHA could be used for therapy against neurodegenerative diseases, stroke (Van et al. 2017), and diabetes (Chen et al. 2019). DHA/EPA-rich LPC (lysophosphatidylcholine) was reported to increase EPA and DHA in the brain and retina higher than free EPA and DHA (Yalagala et al. 2019).

The fatty acid composition of PL might affect their functions in food processing. Gan et al. (2014) modified lecithin with various degrees of saturation and length of fatty acids, and the emulsifying properties of this structured lipids also increased. Besides fatty acid composition, phospholipid species also influence the emulsifying properties. The study of Guiotto et al. (2016) showed that PC-enriched lecithin exhibited better emulsion stability. Different fatty acid compositions in different phospholipid fractions might also affect the ability of phospholipids as emulsifiers. The amphiphilic nature of phospholipids is governed by esterification of phosphate group with other groups and the esterified fatty acid. Therefore, this surfactant has different solubility and interfacial properties (Arnold et al. 2014).

In this study, PLs from PPF is modified by incorporating EPA and DHA into their structure to obtain better functional properties. We examined the characteristics of SPLs produced by acidolysis of phospholipids from PPF and ω-3 fatty acid concentrate prepared from fish oil from by-products of fish processing. We used different fish oil sources from by-products of tuna (*Thunnus* sp) and sardine (*Sardinella longiceps*) canning and meal processing as the sources of EPA and DHA.

Materials and methods

Materials

Palm pressed fiber as a waste was kindly obtained from Surya Dumai Inc., Riau Province, Indonesia. Fish oil from by-products of tuna and sardine canning and meal processing were obtained from Maya Muncar Inc. at Muncar, Banyuwangi, Indonesia. All the wastes were taken once for each location. All chemicals used for ω-3 fatty acid concentrate preparation, phospholipids extraction from PPF, and acidolysis reaction and characterization were analytical grade (Merck, Germany). Standards that consisted of PC (purity $\geq$ 99% by TLC), PE (purity $\geq$ 98% by TLC), PI (purity $\geq$ 98% by TLC), PG (purity $\geq$ 99% by TLC), DPG (purity $\geq$ 97% by TLC), and PA (purity $\geq$ 98%), fatty acid methyl ester mixture, and TLC Silica Gel G60 plates were obtained from Sigma Aldrich (Singapore) and LPE (lysophosphatidylethanolamine) (purity $\geq$ 99%), and LPC (purity $\geq$ 98%) from Nacalai (Japan).

Extraction of crude phospholipids from palm pressed fiber

The extraction of PL from PPF was started by total lipid extraction by the method of Yunoki et al. (2008). A total of 100 g of fine and dry palm fiber was extracted twice with 100 mL chloroform:methanol (2:1 v/v) and once with 100 mL chloroform:methanol (1:2 v/v) for one h at room temperature. After filtering by coarse filter paper, each extract was concentrated with a rotary evaporator, and both lipid extracts were mixed. Crude phospholipid was then extracted by the method of Palacios and Wang (2005) with a slight modification. Every 10 g of crude lipid was extracted with 40 mL chloroform with maceration for 10 min. The mixture was then centrifuged at 1542 *g* for 10 min to separate the chloroform-soluble and insoluble fractions. Chloroform was removed by rotary evaporation from the chloroform-soluble fraction, and the residue was extracted again with 30 mL of chloroform. The chloroform-insoluble fraction from the first extraction was mixed with chloroform-insoluble fraction from the second extraction. After removing the residual chloroform, the chloroform-insoluble fraction was extracted by methanol 20 mL to obtain methanol-soluble fraction. Methanol was removed from this fraction, and crude phospholipid was obtained.

Preparation of ω-3 fatty acid concentrate

This preparation was referred to the method of Haagsma et al. (1982) with different urea crystallization times and the ratio of fatty acid to urea of each source of fatty acid. The differences were based on the best ratio and crystallization time from our previous study (Estiasih et al. 2008). The ratio of fatty acid to urea was 1:2.99; 1:3.07; 1:2.06; 1:2.69 and crystallization time of 23 h 35 min; 24 h 18 min; 23 h 38 min; 25 h 6 min for fish oils from by-products of tuna canning processing (TC); tuna meal processing (TM), sardine canning processing (SC), and sardine meal processing (SM), respectively.

Synthesis of structured phospholipids by enzymatic acidolysis

The synthesis of SPLs was referred to the method of Haraldsson and Thorarensen (1999) with a slight modification. As much as 80 mg of crude phospholipid was put in a test tube and 10% of water was added and then homogenized. The ω-3 fatty acid concentrates were added with a ratio to phospholipids that was based on our previous studies (Estiasih et al. 2009) which were 3.72:1; 3.56:1; 3.66:1; 3.66:1 for SPLs with ω-3 fatty acid concentrates from fish oil from by-products of tuna canning (SPL TC), tuna meal processing (SPL TM), sardine canning (SPL SC) and sardine meal processing (SPL SM), respectively. *Rhizomucor miehei* lipase was added in the amount of 20% (w/w) of the substrate weight or the ratio of enzyme to substrate was 1:5. As much as 3 mL of hexane was added, and then the mixture was incubated in a shaker water bath with agitation of 150 rpm, temperature of 40 °C, and the incubation time was varied depending on the sources of fatty acid, which were 24 h 28 min; 23 h 38 min; 24 h 57 min; and 26 h 33 min for SPL TC, SPL TM, SPL SC, and SPL SM, respectively. After incubation, the solvent was removed, and the SPLs were washed once with 1 mL of hexane. The ratio of ω-3 fatty acid concentrate to phospholipids and incubation time were selected on th basis of the optimum condition in obtaining structured phospholipids with the highest EPA and DHA incorporation for each ω-3 fatty acid concentrate (Estiasih et al. 2009).

Analysis of structured phospholipids

Identification and quantification of phospholipids

Each standard phospholipid was dissolved in a chloroform:methanol solution (95:5 v/v) with a concentration of 1 mg/mL. Silica gel G60 plates were developed with a chloroform:methanol:water (75:25:3 v/v/v) solution according to the Nzai and Proctor (1998) method. The plates were dried for 10 min and activated for 20 min in an oven at 100 °C before use. In the same volume (10 µL) the phospholipid extracts and the standard phospholipid were spotted on the plate. Plates containing spots were placed in a development chamber for 40 min and then dried at room temperature for 10 min, and placed in a 90 °C oven for 10 min. Quantification of the types of phospholipids were determined with the TLC scanner (CAMAG TLC Scanner 3). The concentration of phospholipids in the samples was calculated by comparing the standard area curves of each phospholipid.

Analysis of fatty acid profile of each phospholipid

The identification of the types of fatty acids in each phospholipid was carried out by gas chromatography. The separated phospholipids from TLC plates and each phospholipid fraction were extracted using a mixture of chloroform:methanol:water (5:5:1 v/v/v) according to the method of Christie (1982). The extracted lipids were then methylated by the Park and Goins method (1994) that converted fatty acids into their corresponding methyl esters. Fatty acid composition was analyzed using gas chromatography (Shimadzu GC 8A) with a capillary column (CBP20 M25-025 with 0.25 µm bonded silica column and column dimension was 50 mm in length, i.d. 0.22 mm, and o.d. 0.33 mm). Nitrogen was a gas carrier with a 200 kg/m^2 pressure and the flowrate was 0.3 mL/min. The temperature of the injector, column, and detector was 230 °C, 250 °C, and 230 °C, respectively. As much as 2 µL samples and standard were injected. Quantification was conducted by using C17:0 as an internal standard.

Stereospecific analysis

Stereospecific analysis was carried out on SPLs produced by enzymatic acidolysis reaction to determine the position of ω-3 fatty acids in each type of phospholipid according to the method of Vikbjerg et al. (2005). Each phospholipid fraction was separated in silica gel 60 TLC plates by the method of Nzai and Proctor (1998). Each fraction was extracted 4 times with 10 mL of chloroform:methanol:water (65:35:5 v/v/v). After the solvent was evaporated, each fraction was hydrolyzed with the enzyme phospholipase A2 (PLA2) to remove fatty acids from the sn-2 position. A total of 2.5 mg of the phospholipid fraction was dissolved in 2 mL of diethyl ether and incubated with 10 µL of the PLA2 enzyme dissolved in 100 µL of water. After stirring for 5 min, the residual solvent was evaporated with nitrogen. The hydrolyzed phospholipid was then dissolved again with chloroform and spotted on TLC for separation. The lysophospholipid band was scraped off and then methylated by the Park and Goin method (1994) before gas chromatography analysis.

Table 1 Crude phopsholipid (PL) composition (%) of palm pressed fiber (PPF) and structured phospholipids (SPL) produced with ω-3 fatty acid concentrate from various sources of fish oils

Phospholipids	PPF PL	SPL-TC	SPL-TM	SPL-SC	SPL-SM
Phosphatidylinositol (PI)	4.72 ± 0.21	3.30 ± 0.09	3.43 ± 0.23	3.36 ± 0.09	4.29 ± 0.25
Phosphatidylcholine (PC)	6.63 ± 0.32	6.55 ± 0.21	5.59 ± 0.12	6.24 ± 0.46	4.45 ± 0.36
Phosphatidylethanolamine (PE)	9.56 ± 0.37	9.66 ± 0.33	8.39 ± 0.34	9.13 ± 0.32	7.42 ± 0.42
Phosphatidylglycerol (PG)	17.14 ± 0.98	20.85 ± 0.89	19.73 ± 0.54	20.57 ± 0.67	17.44 ± 0.44
Phosphatidic acid (PA)	13.08 ± 0.88	19.05 ± 0.97	18.98 ± 0.87	18.32 ± 0.76	16.04 ± 0.76
Diphosphatidylglycerol (DPG)	10.85 ± 0.37	13.88 ± 0.78	15.28 ± 0.97	13.88 ± 0.64	13.43 ± 0.43
Free fatty acids (FFA)	12.30 ± 0.51	17.96 ± 0.85	19.15 ± 0.76	17.33 ± 0.97	18.29 ± 0.78
Neutral lipid	25.72 ± 0.76	8.75 ± 0.34	9.45 ± 0.45	11.17 ± 0.11	18.64 ± 0.95
Purity	61.97 ± 0.78	73.29 ± 1.23	71.40 ± 0.65	71.50 ± 1.03	63.07 ± 0.75

Structured phospholipids produced with ω-3 fatty acid concentrate from by-product of:

SPL-TC, una canning processing; SPL-TM, tuna meal processing; SPL-SC, sardine canning processing; SPL-SM, sardine meal processing

Hydrophilic lipophilic balance (HLB) analysis

HLB value represents the ability of an emulsifier to bind water and oil simultaneously. HLB values can be calculated from the saponification numbers (AOAC 920.160, 1998) and acid numbers (AOAC 969.17, 1996) according to the Griffin equation (İbanoğlu et al. 2004), as follows:

$$HLB = 20 \times \left[1 - \frac{S}{A}\right]$$

S = saponification number, A = acid number.

Emulsifying activity index and emulsion stability index

Emulsifying properties are measured by the emulsifying activity index (EAI), which is the surface area that can be stabilized by phospholipids, and the emulsion stability index (ESI), a parameter that shows the ability of phospholipids to stabilize the emulsion. Both parameters were analyzed using Pearce and Kinsella's (1978) turbidity method. Emulsions were made by mixing 0.5 g of soybean oil with 9.5 mL of distilled water to obtain a 0.5% oil fraction, then adding 0.05 g of crude PPF PL or SPLs and homogenized with ultra turax (12,500 rpm, 1 min). Emulsion analysis was conducted by taking the emulsion to have a 6×10^{-5} oil fraction, or 0.12 mL of emulsion was taken and dissolved in a phosphate buffer (0.005 M pH 7) containing 0.3% SDS (sodium dodecyl sulfate), and the volume was adjusted to 10 mL. The time it was first dissolved was calculated as t_0 with an absorbance at λ 500 nm was A_0 or A_{500}. The mixture was then allowed to stand for 10 min, and the absorbance was measured at λ 500 nm and expressed as A_{10}. EAI (m^2/g) and ESI (min) were calculated as follows:

$$\text{EAI} = \frac{2 \times 2.303}{C \times (1 - \emptyset) \times 10^4} \times A_{500} \times Dilution$$

A_{500} was the absorbance value at a wavelength of 500 nm, C was the SPL concentration (g/mL) before emulsification, and Ø was the volume of oil fraction (v/v) of the emulsion.

$$ESI = A_0 \times \frac{10}{A_0 - A_{10}}$$

A_{10} and A_0 were absorbance values of 500 nm at 10 and 0 min after emulsification.

Data analysis

Data of HLB value, EAI, and ESI were analyzed using the ANOVA of the Randomized Complete Design method by using SPSS software. LSD was further used if the treatment indicated a significant difference. The experiment was replicated three times.

Results and discussions

Characteristics of phospholipids from palm pressed fiber

Phospholipids from PPF contains PG, PA, DPG, PC, PE, and PI with the predominant phospholipid is PG (Table 1). The purity of this PL is 61.97% (Table 1). The study of Choo et al. (2004) only found four phospholipids: PC, PE, PG, and PA. In this study, phospholipids were extracted by chloroform to obtain total lipids and then separated by extraction with methanol. Phospholipids are soluble in methanol because they are from PPF PL polar lipids. Choo

et al. (2004) used hexane and ethanol to extract phospholipids. Hexane is less polar than chloroform and ethanol is less polar than methanol. Thus, in this study more polar phospholipids such as PI are also extracted.

The PI in PPF PL was found to be 4.72% (Table 1) and was not found in the study of Choo et al. (2004). The study results of Goh et al. (1982) found a PI fraction in palm oil phospholipids. It is suspected that the PI fraction is more easily extracted in the chloroform:methanol solvent system compared with ethanol. According to Cabezas et al. (2009), PI is contained in an insoluble fraction of lecithin fractionation with ethanol. Yunoki et al. (2008), Wang et al. (2009), and Rao et al. (2009) used different samples, and all showed the presence of a PI fraction from crude phospholipids extracted by a chlorofom:methanol solvent system.

The purity of phospholipids in this study was based on a comparison of all phospholipid fractions to total lipids in PPF. The purity of PPF PL was 61.97%. The crude phospholipids produced still contain much free fatty acid (FFA) and neutral lipids of 12.30% and 25.72%, respectively. According to Palacios and Wang (2005), the phospholipid separation by solvent extraction also extracts other components as impurities. Soy lecithin in hexane extract had 79.9% phospholipids (PC 14.5%; PE 20.1%; PS/phosphatidylserine 3.2%; PI 11.9%; and PA 30.2%) whereas in chloroform–methanol extract contained 82.8% phospholipids (PC 36.7%; PE 13.3%; PS 2.9%; PI 19.3%; and PA 10.6%) after purification by column chromatography (Sikorski and Kolakowska 2003).

The data in Table 2 shows that the dominant fatty acids of PPF PL are palmitic acid (C16:0) and oleic acid (C18:1), which are 39.61 and 49.79%, respectively. This result is consistent with the study of Choo et al. (2004) that palmitic acid and oleic acid in ethanol extract of PPF PL were the most dominant, respectively, 34.58 and 33.75%. PPF PL also contained linoleic acid of 30.05%, however, this fatty acid is found in trace amount in this recent study. This difference might be affected by the type of PPF, the method, and the type of solvent used for extraction.

Each phospholipid identified in the PPF PL was analyzed for its fatty acid composition. The dominant fatty acid was C18:1 in the PC, PE, PI, and DPG, and C16:0 in the PA and DPG (Table 3). According to Sikorski and Kolakowska (2003) differences in the types and composition of fatty acids might occur between different phospholipids from the same sources.

ω-3-Fatty acid concentrate

Comparison of the composition and types of fatty acids from fish oils from by-products of tuna and sardine canning and meal processing with ω-3 fatty acid concentrates is shown in Table 2. The concentration of total saturated fatty acids of the original fish oils was significantly higher than in ω-3 fatty acid concentrates. Saturated fatty acids will form an inclusion complex with urea. ω-3-Fatty acids (EPA and DHA) in the four concentrates significantly increased in the range of 1.5–1.8 times (Table 2) compared to original fish oils. According to Haagsma et al. (1982), the formation of fatty acid-urea complexes is based on the degree of unsaturation of fatty acids. The more unsaturated fatty acids, the less likely it is to be trapped in urea crystals. EPA (C20:5) and DHA (C22:6) have a high degree of unsaturation, thus the possibility of forming an inclusion complex with urea is less than saturated fatty acids.

Degree of ω-3 fatty acids incorporation into structured phospholipids

SPLs are phospholipids with modification of fatty acid composition to increase nutritional and physicochemical properties. SPLs could be used as carriers to increase functional fatty acid absorption in the body (Ang et al. 2019). The degree of EPA and DHA incorporation from each SPL (Table 2) shows that EPA incorporation is higher than DHA for SPL-SM and SPL SC. Conversely, SPL TC and SPL TM have higher DHA incorporation than EPA. The preference of EPA and DHA incorporation into PPF PL structure is highly affected by the concentration of EPA and DHA (Table 2) as the substrate and sources of fatty acids. ω-3-Fatty acid concentrate from fish oils from by-products of tuna canning and meal processing had higher DHA than EPA. Thus, the SPLs had a higher incorporation degree of DHA than EPA. The opposite was for SPLs from by-product of sardine canning and meal processing.

Higher EPA incorporation than DHA in SPL SC and SPL SM (Table 2) is affected by the abundance of this fatty acid. It might also be affected by *R. miehei* lipase specificity. The study of Lee and Parkin (2000) showed that the *R. miehei* lipase specificity during esterification decreased with increasing chain length of fatty acids. Therefore EPA, which has 20 carbon atoms, will be more easily esterified or hydrolyzed than DHA, which has 22 carbon atoms. A similar phenomenon is shown by the results of Haraldsson and Thorarensen (1999) in which the degree of EPA incorporation was higher than DHA in PC molecules with 32% EPA and 16% DHA. Li et al. (2014) reviewed EPA/DHA incorporation into SPLs by using various enzymes, including PLA1, PLA2, and lipase, and the degree of incorporation ranged from 18 to 43% by acidolysis reaction. According to Ang et al. (2019), there are two general methods for modifying PL, a one-step acidolysis (direct transesterification) and a two-step acidolysis. The first step includes the hydrolysis of PL into lysophospholipids (LPL), followed by the esterification of LPL as the second

Table 2 Fatty acid composition (%) of palm pressed fiber phospholipids (PPF-PL) and structured phospholipids (SPL) produced with ω-3 fatty acid concentrate from various sources of fish oils

Fatty acid	By-product of Sardine Meal Processing		By-product of Tuna Canning Processing		By-product of Tuna Meal Processing		By-product of Sardine Canning Processing	
	Fish oil	Concentrate	Fish oil	Concentrate	Fish oil	Concentrate	Fish oil	Concentrate
C8:0	nd	Nd	nd	nd	nd	nd	nd	nd
C10:0	nd	nd	nd	nd	nd	nd	nd	nd
C12:0	0.11 ± 0.00	0.14 ± 0.01	0.11 ± 0.00	0.59 ± 0.02	0.66 ± 0.01	0.09 ± 0.00	0.22 ± 0.00	0.38 ± 0.01
C14:0	11.57 ± 0.32	nd	4.29 ± 0.23	4.80 ± 0.34	4.36 ± 0.03	3.68 ± 0.43	12.88 ± 0.43	nd
C16:0	15.54 ± 0.65	20.67 ± 0.65	25.15 ± 2.25	5.47 ± 0.43	7.44 ± 0.15	10.62 ± 0,51	26.29 ± 0.43	nd
C16:1	24.62 ± 0.71	6.92 ± 0.21	7.21 ± 0.26	12.21 ± 0.67	24.64 ± 1.41	4.05 ± 0.03	15.04 ± 0.52	10.26 ± 0.52
C18:0	12.00 ± 0.08	15.97 ± 0.67	8.54 ± 0.32	0.14 ± 0.00	21.15 ± 1.06	26.35 ± 0.65	5.43 ± 0.02	22.44 ± 0.65
C18:1	3.42 ± 0.21	5.69 ± 0.12	21.7 ± 0.98	26.97 ± 0.87	8.50 ± 0.43	0.05 ± 0.00	5.43 ± 0.03	5.68 ± 0.23
C18:2	0.01 ± 0.00	0.05 ± 0.00	0.10 ± 0.00	0.14 ± 0.02	0.02 ± 0.00	0.15 ± 0.00	0.01 ± 0.00	0.03 ± 0.00
C18:3	nd	0.26 ± 0.00	0.04 ± 0.00	0.40 ± 0.01	0.20 ± 0.00	0.29 ± 0.00	0.17 ± 0.00	0.35 ± 0.00
C20:0	0.19 ± 0.00	0.11 ± 0.01	0.07 ± 0.00	0.19 ± 0.01	0.11 ± 0.00	0.13 ± 0.00	0.01 ± 0.00	0.08 ± 0.00
C20:2	0.18 ± 0.00	0.02 ± 0.00	0.13 ± 0.00	0.14 ± 0.00	0.11 ± 0.00	0.12 ± 0.00	0.21 ± 0.00	0.04 ± 0.00
C20:5 (EPA)	17.60 ± 0.24	27.81 ± 0.32	6.48 ± 0.31	12.23 ± 0.96	7.24 ± 0.43	11.63 ± 046	19.11 ± 0.32	34.08 ± 0.27
C22:2	3.21 ± 0.23	3.85 ± 0.32	2.19 ± 0.24	2.15 ± 0.25	0.47 ± 0.09	1.15 ± 0.01	3.14 ± 0.08	4.63 ± 0.08
C22:3	0.7 ± 0.03	1.14 ± 0.08	0.25 ± 0.00	0.59 ± 0.02	0.26 ± 0.01	0.45 ± 0.00	0.72 ± 0.02	1.33 ± 0.02
C22:6 (DHA)	10.84 ± 0.35	17.30 ± 0.87	23.73 ± 1.21	33.84 ± 1.26	25.43 ± 0.96	41.17 ± 1.21	11.36 ± 0.12	20.58 ± 0.98
EPA + DHA	28.43 ± 0.27	45.12 ± 1.19	30.21 ± 1.52	46.07 ± 2.22	32.67 ± 1.93	52.80 ± 1.67	30.46 ± 0.44	54.66 ± 1.25
Saturated Fatty Acid	39.41 ± 1.05	36.89 ± 1.34	38.16 ± 2.8	11.19 ± 0.80	33.72 ± 1.75	40.87 ± 1.08	44.83 ± 0.88	22.90 ± 0.66
Unsaturated Fatty Acid	60.40 ± 1.77	63.02 ± 1.92	61.70 ± 3.00	88.53 ± 4.06	66.76 ± 3.33	58.94 ± 1.71	54.98 ± 1.09	76.94 ± 2.10
EPA + DHA Enrichment	1.59		1.52		1.62		1.79	

Fatty acid	PPF PL	SPL-TC	SPL-TM	SPL-SC	SPL-SM
C8:0	0.21 ± 0.01	nd	nd	nd	nd
C10:0	0.36 ± 0.01	nd	nd	nd	nd
C12:0	3.34 ± 0.08	1.81 ± 0.01	2.22 ± 0.23	1.63 ± 0.04	2.28 ± 0.04
C14:0	2.25 ± 0.06	5.65 ± 0.08	5.53 ± 0.87	9.89 ± 0.52	9.57 ± 0.42
C16:0	39.61 ± 0.43	10.45 ± 0.42	13.28 ± 0.98	14.50 ± 0.16	18.42 ± 0.67
C16:1	0.64 ± 0.01	13.20 ± 0.54	9.20 ± 0.65	13.98 ± 0.43	12.26 ± 0.65
C18:0	2.93 ± 0.04	6.04 ± 0.05	13.47 ± 0.54	5.25 ± 0.04	4.17 ± 0.21
C18:1	49.79 ± 0.98	23.85 ± 0.98	16.73 ± 0.87	20.62 ± 0.97	25.85 ± 0.043
C18:2	0.30 ± 0.00	1.38 ± 0.02	0.56 ± 0.02	1.78 ± 0.01	2.68 ± 0.07
C18:3	0.21 ± 0.00	6.77 ± 0.08	3.03 ± 0.01	4.77 ± 0.06	3.49 ± 0.04
C20:0	0.36 ± 0.00	nd	nd	nd	nd
C20:2	nd	nd	nd	nd	nd
C20:5 (EPA)	nd	11.11 ± 0.11	10.94 ± 0.42	17.04 ± 0.08	14.71 ± 0.54
C22:2	nd	nd	nd	nd	nd
C22:3	nd	nd	nd	nd	nd
C22:6 (DHA)	nd	19.34 ± 0.65	25.03 ± 1.26	10.54 ± 0.99	6.58 ± 0.97
EPA + DHA	nd				
Saturated Fatty Acid	49.06 ± 0.63	23.95 ± 0.56	34.50 ± 2.82	31.27 ± 0.76	34.44 ± 1.34
Unsaturated Fatty Acid	50.94 ± 0.99	75.65 ± 2.38	65.49 ± 3.23	68.73 ± 2.54	65.57 ± 2.31
EPA + DHA Enrichment					

nd not detected

Structured phospholipids produced with ω-3 fatty acid concentrate from by-product of:

SPL-TC, tuna canning processing; SPL-TM, tuna meal processing; SPL-SC, sardine canning processing; SPL-SM, sardine meal processing

step. The advantage of two-steps acidolysis is the production of highly purified fatty acid compositions in SPLs.

The high degree of EPA and DHA incorporation into SPL structures during acidolysis reaction is accompanied by a decrease in the concentration of C12:0, C16:0, and C18:1. The position of saturated and monoenoic fatty acids in the PPF PL molecule is replaced by polyenoic acid, especially EPA and DHA. According to Lyberg et al. (2005), apart from *R. miehei* lipase's specificity to long-chain fatty acids, the exchange of these acyl groups with EPA and DHA was also made possible by higher amounts of EPA and DHA in the ω-3-fatty acid concentrates. Therefore, the chance of EPA and DHA to compete with other fatty acids in reaching the active site of the lipase enzyme is higher.

Phospholipid composition and fatty acid profile

The data in Table 1 shows an increase in FFA accompanied by a decrease in neutral lipid from all SPLs compared to original PPF PL. Increasing FFA was supposed to be due to the neutral lipid hydrolysis by the *R. miehei* lipase. According to Ang et al. (2019), the first step in acidolysis is hydrolysis of PL to produce free fatty acids and lysophospholipids. *R. miehei* lipase hydrolyzed fatty acids in neutral lipids (TAG) in the sn-1 and sn-3 positions. Therefore, the neutral lipid content decreases, which concomitantly increases FFA content. According to Min and Akoh (2002), the *R. miehei* lipase enzyme is specific in the sn-1 and sn-3 positions on TAG and specific sn-1 in phospholipids. Increasing FFA level is also related to the use of ω-3 fatty acid concentrates as the acyl donor. Not all fatty acids in the concentrates reacted and incorporated into phospholipids. In general, EPA and DHA are more easily incorporated into neutral lipids than all other phospholipid fractions (Table 4) except in SPL-SM. The degree of EPA and DHA incorporation into neutral lipids is higher than into phospholipid because the neutral lipid molecule has an acyl group in the sn-1, 2, 3 positions. The acyl group in the phospholipid structure is only in the sn-1 and sn-2 positions. Lipase from *R. miehei* has positional specificity for fatty acids in the sn-1 and sn-3 positions of TAG. Thus, the chance of incorporating EPA and DHA into the TAG (neutral lipid) structure is greater than that of the phospholipids.

Analysis of EPA and DHA incorporation degree in each phospholipid in SPLs indicated that both fatty acids were more easily incorporated in the PE compared to others (Table 4). The phospholipid head group also influences lipase affinity in this case. According to Mutua and Akoh (1993), during the acidolysis reaction with the EPA as an acyl donor in the hexane solvent system, reactivity of *R. miehei* lipase to attach EPA and DHA to the phospholipids is PC > PI > PE > PS. Meanwhile, if the original donor is caprylic acid, the reactivity of lipase synthesis is PC > PE > PA > PI (Peng et al. 2002), which proves that the type of acyl donor group will affect lipase reactivity in the structuring of phospholipids.

The highest EPA and DHA concentrations are found in the PE for all types of SPLs, whereas the EPA and DHA incorporation to other phospholipids reveals different degrees. The source of the ω-3 fatty acid concentrate from the by-product of tuna canning processing has a degree of incorporation form the highest is PE > PA > DPG > PG > PC > PI. Meanwhile, for the concentrate of the by-product of tuna meal, sardine canning, and sardine meal processing respectively are as follows: PE > PA > DPG > PC > PG > PI; PE > PG > PI > DPG > PA > PC; PE > PI > PG > PA. According to Vikbjerg (2006) the type of phospholipid head groups also determined the reactivity of the lipase enzyme.

Based on the highest quantity (Table 1), PG should have the highest chance of incorporating the EPA and DHA. However, the preference of EPA and DHA incorporation is into PE. It shows that the abundance of phospholipids does not influence the degree of incorporation of EPA and DHA. Meanwhile, if based on the reactivity of *R. miehei* lipase to the phospholipid species, the highest degree of incorporation should be in the PC fraction (Mutua and Akoh 1993; Peng et al. 2002). In this study, the highest EPA and DHA incorporation is found in the PE. Another factor that causes PE to have the highest degree of incorporation is the difference in hydrolysis degree. According to Vikbjerg (2006), the order of hydrolysis of the *R. miehei* lipase enzyme on phospholipids was PG > PI > PC > PE. A higher degree of hydrolysis will make the hydrolysis reaction more dominant than the esterification. Furthermore, there is a possibility of re-hydrolysis of the EPA and DHA that has been incorporated. Therefore, with the lowest degree of hydrolysis, PE has a greater chance of maintaining the incorporated EPA and DHA.

Position of EPA and DHA in structured phospholipids

Stereospecific analysis was performed to determine the position of ω-3 fatty acids (EPA and DHA) in the SPLs with the highest degree of incorporation. The analysis was conducted in the PE using phospholipase A2 (PLA2) (Table 5). PLA2 will hydrolyze PE in SPLs in the sn-2 position, producing LPE with its native fatty acids in the sn-1 position and liberates free fatty acids from the sn-2 position. The hydrolysis is performed by using phospholipase A2 and then analyzed by TLC to separate the products of hydrolysis, and the composition of the fatty acids was analyzed by gas chromatography. Detected fatty acids in

Table 3 Fatty acid composition (%) in palm pressed fiber phospholipid

Fatty acid	PI	PC	PE	PG	PA	DPG	FFA	Neutral Lipid
C10:0	nd	nd	nd	nd	nd	nd	nd	nd
C12:0	nd	10.58 ± 0.76	0.91 ± 0.03	5.77 ± 0.76	3.65 ± 0.98	1.77 ± 0.05	6.92 ± 0.76	6.56 ± 0.65
C14:0	nd	5.66 ± 0.43	4.08 ± 0.12	12.88 ± 43	3.77 ± 0.75	3.19 ± 0.76	0.05 ± 0.00	3.40 ± 0.04
C16:1	18.58 ± 0.98	0.16 ± 0.01	1.49 ± 0.07	nd	1.96 ± 0.04	2.25 ± 0.23	nd	nd
C16:0	28.32 ± 1.23	1.21 ± 0.00	31.22 ± 2.34	32.02 ± 1.23	30.25 ± 1.28	23.55 ± 0.76	25.61 ± 0.97	38.16 ± 1.21
C18:1	29.32 ± 1.45	37.64 ± 0.65	46.09 ± 3.42	26.10 ± 0.98	26.67 ± 0.34	53.21 ± 2.34	41.18 ± 0.65	50.75 ± 1.98
C18:2	8.85 ± 0.42	19.85 ± 0.98	0.45 ± 0.00	nd	nd	nd	nd	nd
C18:0	15.93 ± 1.01	21.23 ± 0.65	15.75 ± 0.34	23.23 ± 1.24	15.39 ± 0.87	14.40 ± 0.07	0.17 ± 0.00	nd
C18:3	nd	nd	nd	nd	nd	nd	nd	nd
EPA	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
C20:0	nd	3.25 ± 0.03	nd	nd	1.33 ± 0.01	1.50 ± 0.01	nd	0.89 ± 0.00
DHA	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**
C22:0	nd	0.43 ± 0.01	nd	nd	3.12 ± 0.76	nd	3.72 ± 0.01	0.23 ± 0.00
C24:0	nd	nd	nd	nd	nd	nd	nd	nd
EPA + DHA	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**	**nd**

Table 4 Degree of EPA and DHA incorporation into phospholipid fractions in structured phospholipids

Structured phospholipids	Degree of EPA and DHA Incorporation (%)							
	PI	PC	PE	PG	PA	DPG	FFA	Neutral Lipid
SPL-TC								
EPA	3.03 ± 0.12	3.14 ± 0.25	17.65 ± 0.43	4.49 ± 0.21	17.00 ± 0.76	16.06 ± 0.07	17.58 ± 0.45	19.380.65
DHA	6.18 ± 0.34	7.06 ± 0.21	33.54 ± 1.01	10.22 ± 0.54	21.12 ± 1.02	18.66 ± 0.11	20.02 ± 0.98	55.81 ± 2.34
SPL-TM								
EPA	7.34 ± 0.67	12.41 ± 0.87	28.56 ± 0.98	7.02 ± 0.05	25.72 ± 1.43	41.16 ± 1.34	12.62 ± 0.16	19.33 ± 0,87
DHA	5.91 ± 0.34	11.40 ± 0.54	28.83 ± 0.65	7.23 ± 0.09	25.51 ± 1.21	8.08 ± 0.43	9.81 ± 0.07	38.40 ± 1.65
SPL-SC								
EPA	10.43 ± 0.46	2.65 ± 0.05	15.70 ± 0.23	13.88 ± 0.77	1.94 ± .005	7.89 ± 0.65	13.15 ± 0.65	47.70 ± 2.12
DHA	16.60 ± 0.76	5.24 ± 0.23	20.30 ± 0.76	16.99 ± 1.12	3.78 ± 0.07	10.76 ± 0.44	11.98 ± 0.54	20.65 ± 1.27
SPL-SM								
EPA	12.06 ± 0.37	nd	18.61 ± 0.99	10.22 ± 0.51	12.70 ± 0.65	nd	11.57 ± 0.65	7.97 ± 0.98
DHA	10.81 ± 0.45	nd	19.63 ± 0.65	11.79 ± 0.53	2.27 ± 0.04	nd	13.39 ± 0.76	9.34 ± 0.45

Structured phospholipids produced with ω-3 fatty acid concentrate from by-product of:

SPL-TC, tuna canning processing; SPL-TM, tuna meal processing; SPL-SC, sardine canning processing; SPL-SM, sardine meal processing

the LPE were fatty acids in the sn-1 position of SPLs. FFA were derived from hydrolyzed fatty acids from the SPLs at the sn-2 position. *R. miehei* lipase incorporated ω-3 fatty acids into the sn-1 position. Thus the incorporated EPA and DHA would be detected in the LPE.

It seemed that there was acyl migration of EPA and DHA into sn-2 position that was identified by the existence of both fatty acids in FFA, especially in SPL-SC. Wijesundera et al. (2008) explained that PUFA (Polyunsaturated Fatty Acid), especially EPA-DHA, was more stable in

Table 5 Percentage of EPA and DHA at each fractions after enzymatic hydrolysis using phospholipase A2 at phosphatidylethanolamine (PE)

Structured Phospholipids	%				
	LPE (sn-1)	PE (sn-1.2)	Spot 3	Spot 4	FFA (sn-2)
SPL TC	2.49 ± 0.03	nd	1.95 ± 0.08	3.23 ± 0.12	11.60 ± 0.48
EPA	3.66 ± 0.65	4.16 ± 0.23	3.18 ± 0.12	2.78 ± 0.11	10.16 ± 0.43
DHA					
SPL TM	nd	7.41 ± 0.24	nd	nd	3.18 ± 0.12
EPA	nd	8.74 ± 0.37	nd	nd	4.33 ± 0.25
DHA					
SPL SC	8.80 ± 0.67	9.73 ± 0.54	nd	4.63 ± 0.25	22.14 ± 0.98
EPA	8.92 ± 0.76	10.28 ± 0.45	nd	11.64 ± 0.67	18.47 ± 0.87
DHA					
SPL SM	5.51 ± 0.45	2.12 ± 0.06	nd	11.85 ± 0.43	8.63 ± 0.23
EPA	5.81 ± 0.76	2.82 ± 0.11	bd	19.48 ± 0.66	24.06 ± 1.01
DHA					

Structured phospholipids produced with ω-3 fatty acid concentrate from by-product of:

SPL-TC, tuna canning processing; SPL-TM, tuna meal processing; SPL-SC, sardine canning processing; SPL-SM, sardine meal processing; LPE, lysophosphatidylethanolamine

Table 6 Hydrophilic lipophilic balance (HLB) value, emulsifying activity index (EAI_, and emulsion stability index (ESI) of structured phospholipids

Phospholipids	HLB	ESI (min)	EAI (m^2/g)
PPF PL	8.60 ± 0.32c	70.38 ± 1.23e	140.34 ± 2.87d
SPL TC	10.52 ± 0.44a	89.00 ± 2.12c	204.16 ± 4.99c
SPL TM	10.54 ± 0.41a	124.58 ± 3.21a	265.61 ± 5.65a
SPL SC	9.56 ± 0.23b	96.64 ± 2.26b	261.20 ± 2.78a
SPL SM	9.58 ± 0.49b	85.14 ± 1.98d	244.90 ± 3.69b

Note: Different letters following the values indicate a statistical difference at 5% probability level

PPF PL, palm pressed fiber phospholipid

Structured phospholipids produced with ω-3 fatty acid concentrate from by-product of:

SPL-TC, tuna canning processing; SPL-TM, tuna meal processing; SPL-SC, sardine canning processing; SPL-SM, sardine meal processing

the sn-2 position on TAG. It is suspected that after the acidolysis reaction, EPA and DHA incorporated in the sn-1 position migrate to the sn-2 position. The acyl migration from the sn-1 position to the sn-2 position occurs for all SPL products (Table 5). The phenomenon of acyl migration can be explained by LPC's formation, which is an intermediate product produced during the transesterification reaction using lipases (Haraldsson and Thorarensen 1999). The acyl migration from the sn-2 position to sn-1 or vice versa will continue until an equilibrium condition is reached. The phenomenon of EPA and DHA migration in this study was in accordance with the results of Haraldsson and Thorarensen (1999). Lipase will hydrolyze the PC in the sn-1 position to produce 1-LPC. The intermediate compounds can be re-esterified with EPA to form PC-EPA, or the acyl migrates to form 2-LPC. Lipase will very quickly hydrolyze these intermediate compounds to form GPC (Glycero-PC). Esterification with EPA at the sn-1 position of the GPC forms 2-LPC-EPA, followed by EPA migration to sn-2 position to form 1-LPC-EPA. In the end, if esterification occurs again, it forms PC-EPA with positions at sn-1 and 2. This phenomenon will continue to occur until equilibrium is reached.

Emulsifying properties

HLB value

There is a significant difference ($p < 0.05$) on HLB values between crude phospholipids and SPLs. Also, HLB values among SPLs are significantly different. Modification of phospholipid structure resulted in increasing HLB values (Table 6). The increase in the HLB value of SPLs compared to PPF PL was supposed to relate to an increase in polarity. PPF PL also still contained a lot of neutral lipids, thus made PPF PL more nonpolar. Reduction in neutral lipid caused more polar SPLs compared to PPF PL. More hydrophilic phospholipids have higher HLB values. The composition of the phospholipids of PPF PL and SPLs was also influenced by the level of polarity. The decreasing order of polarity of the phospholipid identified in PPF PL and SPLs is as follows: PI > PC > PE > PG > PA > DPG.

The composition of more polar phospholipids (PC, PE, PG, and PA) in the four SPLs is higher than that of PPF PL. Thus, SPLs are more polar compared to PPF PL, resulting in higher HLB values compared to PPF PL. All four SPLs have a different degree of ω-3 fatty acid incorporation (Table 4). The longer fatty acid chain is more nonpolar, whereas increasing degree of unsaturation results in increasing polarity. DHA is more polar than EPA. SPL TC and SPL TM have higher incorporated DHA than SPL SC and SPL SM (Table 2). Therefore, the HLB values of SPL TC and SPL TM were higher. HLB values in this study ranged from 8.6–10.54. The HLB value of 8–12 is suitable for oil in water emulsions (Colbert, 1998).

Emulsifying activity index and emulsion stability index

The emulsifying properties of an emulsifier can be analyzed on basis of the values of ESI and EAI. In this study, an oil-in-water emulsion system (5:95 v/v) with a phospholipid emulsifier (0.5%) was used. Phospholipids in oil-in-water (o/w) emulsions are absorbed on the surface of the oil droplet to form a layered lamellar structure. In the water in the oil system (w/o), phospholipids stabilize the emulsion through the formation of inverted micellar structures (Vikbjerg et al. 2006). Statistical analysis ($p < 0.05$) showed a significant difference in EAI and ESI between PPF PL and SPLs (Table 6). The increase in ESI and EAI values in the oil-in-water emulsion system for SPLs is in the following order: SPL TM > SPL SC > SPL SM > SPL TC. Increasing the emulsifying properties of SPLs is associated with an increase in the HLB value, which increases the stability of the oil in water emulsion. ESI value indicates the ability of emulsifiers to stabilize the emulsion system (McClements 2007). The highest ESI value is in SPL TM, which is 124 min.

Increasing emulsifying properties of SPLs are associated with the changes in fatty acid composition and composition of phospholipid species. The purity of SPLs is also higher, which increases their HLB values. Therefore, SPLs are better in stabilizing oil in water emulsion. SPL TM is more hydrophilic than others based on the degree of EPA and DHA incorporation, and the highest ESI and EAI. According to Li and Xiang (2019), the emulsifying properties of emulsifiers are related to the surface hydrophobicity, which determines the ability of emulsifiers to absorb at the interface of oil/water. The suitability between stabilized oil in the emulsion also determines the emulsifying properties of an emulsifier (Miura et al. 2006).

SPLs containing EPA and DHA from PPF PL and ω-3 fatty acid concentrate is suitable to use as an emulsifier for oil in water emulsion systems with advantages to have benefits on health. SPLs containing EPA and DHA also could be used for disease management and therapy or as food supplements. The utilization of by-products as raw material for these SPLs is the point for the food industries to have value-added products derived from their wastes.

Conclusion

Palm pressed fiber can be used as an alternative source of natural phospholipids. Extraction using chloroform and methanol produces crude phospholipids with a purity of 62% consisting of PI, PC, PE, PG, PA, and DPG with the dominant phospholipid is PG. The four SPLs have a similar phospholipid composition as PPF PL and had better purity (63–70%). Different compositions of acyl sources in the form of ω-3 fatty acid concentrate from by-products of fish processing affect the degree of EPA and DHA incorporation. The highest EPA and DHA incorporation is found in PE at sn-1 position, and there is an acyl migration to the sn-2 position. The modification of phospholipids results better emulsifying properties of o/w emulsion compared to palm pressed fiber phospholipids, which is indicating by the higher HLB, ESI, and EAI values of SPLs. The increase in the HLB value of SPLs compared to PPF PL was supposed to relate to an increase in polarity. Reduction in neutral lipid resulted in more polar SPLs compared to PPF PL. More hydrophilic phospholipids have higher HLB values. The composition of more polar phospholipids (PC, PE, PG, and PA) in the four SPLs is higher than that of PPF PL. An increase in ESI and EAI values in the oil-in-water emulsion system for SPLs is in the following order: SPL TM > SPL SC > SPL SM > SPL TC. Increasing the emulsifying properties of SPLs is associated with an increase in the HLB value, thus increases the stability of the oil in water emulsion. SPL TM is more hydrophilic than others based on the degree of EPA and DHA incorporation, and it possessed the highest ESI and EAI. SPLs containing EPA and DHA from PPF PL and ω-3 fatty acid concentrate is suitable to use as an emulsifier for oil in water emulsion systems with advantages to have benefits on health.

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