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PHOSPHOLIPIDS FROM PUMPKIN (*Cucurbita moschata* (Duch.) Poir) SEED KERNEL OIL AND THEIR FATTY ACID COMPOSITION

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ABSTRACT

The phospholipids (PL) of pumpkin (Cucurbita moschata (Duch) Poir) seed kernel and their fatty acid composition were investigated. The crude oil was obtained by maceration with isopropanol followed by steps of extraction yielded polar lipids. The quantitative determination of PLs content of the dried pumpkin seed kernel and their polar lipids were calculated based on the elemental phosphorus (P) contents which was determined by means of spectrophotometric methods. PL classes were separated from polar lipids via column chromatography. The fatty acid composition of individual PL was identified by gas chromatography-mass spectrometry (GC-MS). The total of PL in the pumpkin seed kernels was 1.27% which consisted of phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidyletanolamine (PE). The predominant fatty acids of PL were oleic and palmitic acid in PC and PE while PS's fatty acid were dominantly consisted of oleic acid and linoleic acid.

Keywords: *(Cucurbita moschata (Duch.) Poir); Pumpkin; Fatty acid; Phospholipids*

INTRODUCTION

Food by-product exploitation recently gets more attention in order to add value of food material and to avoid problems of food industrial disposal. Pumpkin (*Cucurbita moschata* (Duch.) Poir) seed, a food by-product of pumpkin processing, potentially could be used as source of fine chemical, simply because its contents of phospholipid (PL) [1]. PL is one of natural raw material of which widely used as base component of drug, cosmetic and emulsifier. PL has been formulated as anti hepatotoxic due its capability to repair of damaged membrane as result redundant drug usage as well as infection diseases such as hepatitis. PL could also be constructed as liposome to be used as drug transporter having low dissolve in water and decreasing side effect of the drugs [2]. Cosmetic industries apply PL as base component of lotion as spherical vesicle acts as delivery active compounds (i.e vitamin E) which mostly insoluble in water [3]. In food industry, PL has been used as emulsifier. In chocolate candy, PLs assist in stabilizing mixture of chocolate with margarine [4].

The main source of PLs is food material such as soy, canary seed, pig oil as well as serum. PL originated from these materials available only in limited amount, due to low content in the sources leading to high price of PLs. Nevertheless, fast development in pharmacy, cosmetic and food industries need more PLs. Additional issue such as Halalness concern regarding material originated from pig has been also catalyzed effort to search alternative source of PLs. Therefore, exploiting industrial by-product could be one interesting option. In case of pumpkin seed kernels, many researchers report the present of PLs in the seed [5-7]. Therefore it is possible to make pumpkin seed as a new source of PLs. However detailed information regarding type of PLs present in the seed as well as fatty acid composition of the PLs remain little known. This information is essentially needed if the PLs to be applied for certain application since requirements for PLs as antihepatotoxic, emulsifier, liposome need different type of PLs and its fatty acid. This paper reports study on type and fatty acid composition of PLs isolated from pumpkin seed.

EXPERIMENTAL SECTION

Materials

Pumpkin seeds were purchased from local market in Yogyakarta then dried under sunshine. The chemicals of primuline, BF_3-methanol (10%) and ninhydrin are all from Sigma-Aldrich, while all solvent for TLC and column chromatography are from Merck as well as silica gel 60 (230-400 mesh) and silica TLC plate.

Instrumentation

The main instruments used in the research were column chromatography (diameter = 1 cm and length = 30 cm), rotary evaporator (Buchii R-124),

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Ultrasonic bath (Julabo USR3), spectrophotometer UV-Vis (Milton Roy Spectronic 3000), ^{1}HNMR (JEOL60) and gas chromatography-mass spectrometer (GC-MS) (Shimadzu QP-2010S).

Procedure

Determination of total phosphorus

The determination of total phosphorus was obtained by spectrophotometric methods according to procedure AOAC *Official Method 970.39* [8] with slight modification. The dried pumpkin seed kernels and their polar lipids were dissolved in HNO_3 65%, and $HClO_4$ 70%, complexed by ammonium molybdovanadate incubated for 30 min at room temperature and measured the absorbance at λ = 430 nm. For quantification, various concentration of KH_2PO_4 prepared with the same way with the sample, were used as standards.

Extraction of the seed oil

Dried pumpkin seeds were unshelled by cracking with a small iron rod and manually peeled to separate the kernel. The kernel were crushed, then soaked in isopropanol and stirred at 30 °C for 2 h using ultrasonic wave [9]. Centrifugation of the mixture results in residue and filtrate. The residue was extracted again with chloroform/isopropanol (1/1, v/v). The filtrates were combined and dried in rotary vacuum evaporator at 40 °C. The crude oils were extracted using partition with water saturated ethanol/n-hexane (1/1, v/v) result in upper phase and lower phase. The upper phase was extracted again and the lower phases were then combined and concentrated by means of vacuum evaporator at 40 °C result in polar lipids which contain PLs [10].

Identification and separation of PL classes

Identification of PL classes was performed by TLC according to the procedure reported by Dreyfus [11]. Silica gel plates was activated by spraying with solution 2.3% H_3BO_3 in ethanol followed by heating at 110 °C for 15 min. Approximately 15 µg of lipid polar were spotted to the plate and the plate was the developed using chloroform/ethanol/H_2O/triethylamine (30:35:7:35, v/v/v/v) as migration solvent. The plate was then sprayed with primuline (0.05% in acetone/H_2O, 4/1, v/v). The fluorescent spots of PLs were viewed under UV light (λ = 340 nm). Phosphatidylcholine (PC) was also visualized specifically by spraying using dragendroff reagent ((0.5 g [$Bi(NO_3)_3.5H_2O$] in 20% acetic acid), 5 mL (40% KI in H_2O) and 70 mL H_2O)). Meanwhile PL that contain free amine group, phosphatidylserine (PS) and phosphatidyletanolamine (PE) was also specifically detected using ninhydrin reagent (0.2 g of ninhydrin 100 mL of water saturated butanol) [9].

The polar lipid was separated into neutral lipid (NL), glycolipids (GL) and PL by passing through column chromatography silica gel (230-400 mesh packed in column with 1 cm diameter and 30 cm long. Separation was performed according to the procedure reported by Ramadlan and Morsel [12]. The gradient eluting solvents for NL, GL and PL were hexane, ethyl acetate, ethyl acetate/methanol (1/1, v/v) and methanol, respectively. Solvents in each fraction were evaporated by using N_2. Each fraction was identified for the present of PLs. The isolated PLs were analyzed using H-NMR and its fatty acid profile by means of GC-MS.

Analysis of fatty acid compositions of individual PLs

Fatty acids of the PLs were converted to methyl esters (FAME) by derivatization using BF_3-methanol at 60 °C for 60 min according to the procedure reported by Yoshida [13]. The FAME was recovered using n-hexane followed by GC-MS analysis. The condition for GC analysis: capillary column Rtx-5MS 30 m x 0.25 mm was set at 280 °C, injector temperature was 300 °C and detector temperature was 250 °C.

RESULT AND DISCUSSION

Total P and PL Content of Pumpkin Seed Kernel

The total P content in dried pumpkin seed kernel and polar lipid was determined using spectrophotometric methods and the results listed in Table 1. Total PLs contents of the polar lipids were calculated based on the elemental P contents. The ratio between PL content (%PL) and %P can be expressed by the following equation [14]

$$\%PL = \%P\frac{MW_{PL}}{MW_P} = \%Px\ 26$$

where : MW_{PL}, MW_P are molecular weight of PL and P, respectively; factor 26 is used for conversion of P to %PL.

The calculation of total P in dried pumpkin seed kernel result in 0.99% of P content. This result is in line with previous result reported by El-Adawy and Taha [1]. Meanwhile, the total P present in polar lipid, which directly correlates to PLs content, is only 0.049%. This significant differences mainly caused of the present of many others compound that containing element P besides PL, such as phytin; phosphate sugar; nucleoprotein; ATP; ADP; AMP; NAD; NADP and FAD present in seed kernels which functioned later during germination. Although the polar lipid has P contents lower than pumpkin seed, the P contents only contributed by PL content. Therefore calculation of PLs

Table 1. Measured PO_4 and calculated P present in pumpkin seed kernel

Sample	Weight (g)	PO_4 content (%, w/w)	P content (%, w/w)
Seed kernels	0.8872	3.04	0.99
Polar lipids	0.4248	7.99	0.049*

*P content in 100 g dry weight pumpkin seed

Table 2. Composition of fatty acid attached to pumpkin seed PLs

Fatty acid	Content in every PL (%)		
	PC	PE	PS
Palmitic acid ($C_{16:0}$)	24.10	18.14	30.17
Stearic acid ($C_{18:0}$)	7.30	4.5	1.58
Oleic acid ($C_{18:1}$)	38.21	45.23	28.83
Linoleic acid ($C_{18:2}$)	19.15	30.44	28.22

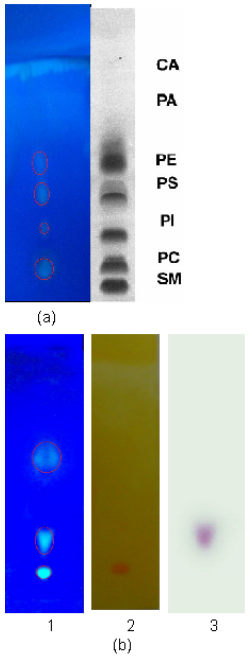

Fig 1(a). TLC plate of PL isolated from pumpkin seed kernel (lane 1) and PL standards by Dreyfus et al. (1997) (lane 2). (b). TLC plate separated using ethyl acetate:methanol (1:1) then visualized by 1) primulin spray, 2) dragendorff and 3) ninhydrin reagents

content using Szydlowska-Czerniak and Szlyk equation was carried using data of total P present in polar lipid. In this experiment the phospholipid contents (%PL) present in pumpkin seed kernel equal to 1.27%. This result is slightly higher than previous report by El-Adawy and Taha [1] which was 1.09%. The difference most probably due to the difference of the geographical origin of the seed and isolation methods applied to take pumpkin seed extract. This argument support by report by Yoshida et al. [13] who observed variability of PLs content among soy bean seed (*Glycine max* L.) from three different area of Mikawajima, Fuki and Shishou.

PL classes and their fatty acid composition

PLs classes can be simply determined by its retardation factor (R*f*) on TLC plates by specific techniques such as dragendroff, ninhydrin and primuline spray [9]. It could be done since the reagents are specified to the PLs by previous isolation of the PLs (only PLs, the group of compounds present in the lipid polar, which gives respond to the reagents). Examination of individual PL species (Fig. 1a) using premulin reagent with Drayfus TLC system (chloroform/methanol 1/1) show that pumpkin seed kernel oil consist of four spots with R*f* value are 0.13, 0.25, 0.36 and 0.53. These spots have the same R*f* value with same separation methods of phospholipids standard reported by Dreyfus [11] and identified as phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserin (PS) and phosphatidylethanolamine (PE), respectively.

Other TLC system was also developed in order to separate more PLs and also to be used as elution system for preparative column chromatography. The optimized TLC system consists of ethyl acetate/methanol (1/1) as mobile phase result in separation shown at Fig. 1b. Comparison of primuline spray, dragendorff and ninhydrin, showed existence of three spot, with R*f* value 0.04, 0.28, and 0.68. Spot with R*f* 0.04 gives positive sign to reagent Dragendorff which has orange color (Fig. 1b.2), leading to conclusion that compound at this spot contains quaternary ammonium salt. Fig. 1b.3 indicates spot risided in middle with Rf 0.28 showing purple after sprayed with ninhydrin reagent indicating that spot contains primary amine moieties or secondary amine. If it is compared to dissociation of PL types at Dreyfus report [11], hence it can be told that three spot started from lowest R*f* value are PC, PS and PE, respectively. While for visible previous PI as spot with TLC as according to Dreyfus [12], simply at TLC with ethyl acetate:methanol (1:1) system could not observe. This is possibly to be caused by very low PI amounts present in the sample.

Three identified PLs (PC, PS and PE) of pumpkin seed kernel have been successfully isolated using silica column chromatography. The solvent involved in the isolation were n-hexane, ethyl acetate, ethyl acetate/methanol (1/1) and methanol. The n-hexane was used to elute remaining non polar lipid (triacylglicerol), while ethyl acetate played role in washing glycolipid. The isolation was performed using ethyl acetate/methanol (1/1) and methanol where the sequence of PLs elution is started with PC followed by PS and PE. The isolated PLs are identified by TLC and visualized with primulin spray. All of the three PLs are confirmed by their R*f*. The fatty acid of PLs of pumpkin

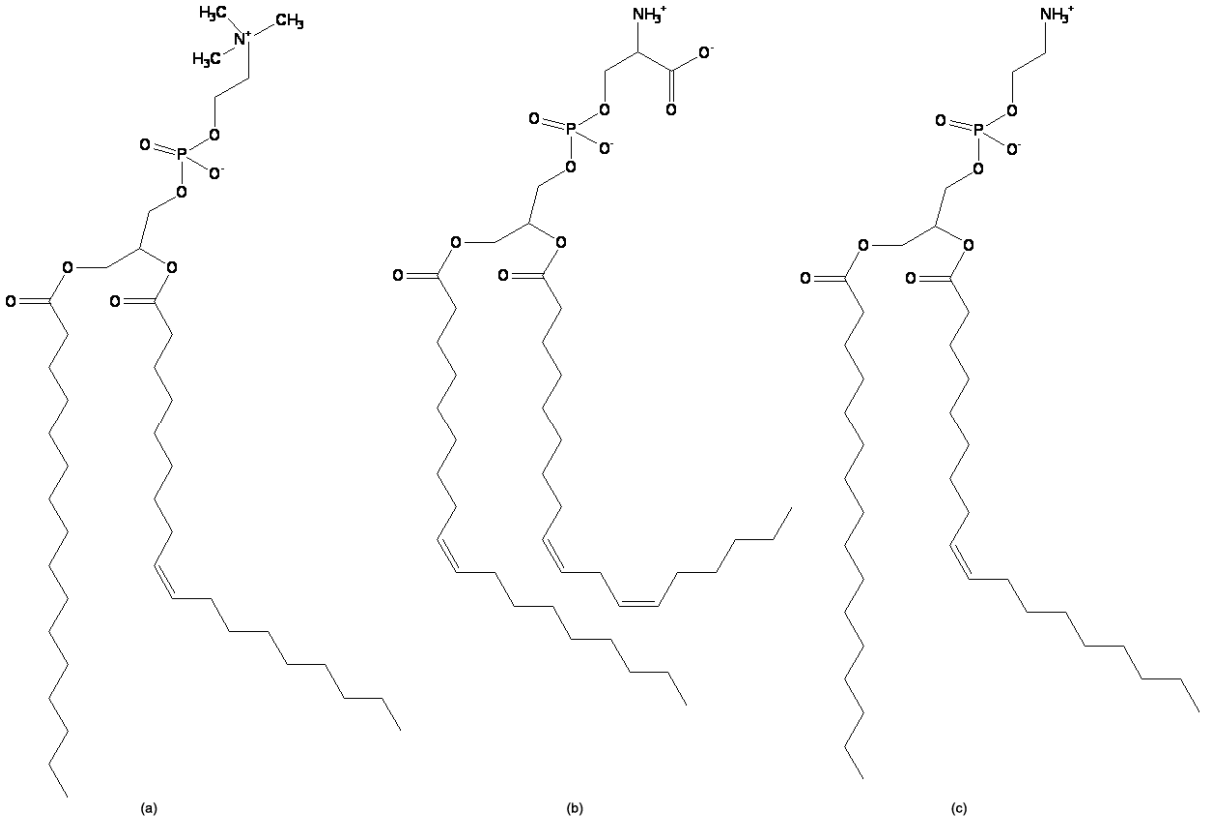

Fig 2. Proposed structutres of Phospholipids (PLs) present in Pumpkin Seed : a) phosphatidylcholine (PC); b) phosphatidylserin (PS); c) phosphatidylethanolamine (PE)

seed have different composition summarized at Table 2. Fatty acids of all PLs are dominated by four fatty acid (palmitic, stearic, oleic and linoleic acid). Fatty acid attached PC and PS are dominated by oleic acid while in PE, palmitic acid is the main faty acid attach to. This result is in line with report by Yoshida [15] who reported fatty acid of phospholipid in nut oil (*Arachis hypogaea* L.) as well as in red pumpkin [16]. Fatty acid differences of each PLs also found which PE and PC were dominantly by oleic acid attachment, while fatty acid of PI was mainly palmitic acid. Early NMR studies show that pumpkin seed PLs have fatty acid esterification at carbon atoms number one and two at glycerol moieties with pattern that unsaturated fatty acid usually bind at sn_2 (closer to phosphate group) while saturated fatty acid bind at sn_1 position. According to NMR data PC has oleic acid and palmitic acid at position sn_2 and sn_1 while PS has linoleic and oleic acid at sn_2 and sn_1 and PE has oleic and palmitic acid at sn_2 and sn_1. The proposed structure of pumpkin seed PLs are presented at Fig. 2.

CONCLUSION

The PLs present at pumpkin seed kernel are 1.03% dry-weights, which consist of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserin (PS) and phosphatidylethanolamine (PE). The predominant fatty acids present at PC are oleic (attached at sn_2) and palmitic acid (sn_1), whereas fatty acids of PS are dominated by oleic acid (sn_1), linoleic acid (sn_2), while fatty acids of PE are mainly oleic (sn_2) and palmitic acid (sn_1).

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