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Effects of Phospholipid Extract from Squid Viscera on Lipid Oxidation of Fish Oil

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Abstract

Phospholipid (PL), phosphatidylcholine (PC) and phosphatidylcholine free PL (PCF) were extracted from squid viscera and the antioxidant effects of each fraction on the oxidation of refined fish oil were evaluated. Polyunsaturated fatty acid contents were the highest in PC (46.7%) followed by PL (44.8%) and PCF (40.9%). The effects of each phospholipid fraction on stabilizing fish oil were compared by incubating at 40° C for 10 days. At the initial period (2 days), changes in peroxide value did not show any significant difference ; however, as incubation time was extended, PC fraction showed the strongest antioxidant activity. PL and PCF added to fish oils also resulted in increased stability against oxidation. Antioxidative effect of PC at the 5% level was equivalent to 0.05% BHT, 1% catechin and 1% tocopherol.

Key words : phospholipid (PL), phosphatidylcholine (PC), phosphatidylcholine free PL (PC free PL, PCF)

INTRODUCTION

Unlike most vegetable oils, fish oils are easily deteriorated by the oxidative reaction with atmospheric oxygen because of their high contents of polyunsaturated fatty acids (PUFA). Omega-3 PUFA in fish oils reportedly play an important role in improving thrombosis, arteriosclerosis, and inflammation, as well as aging and immunological process (1-4). Thus, use of fish oil as a raw material or phamaceutical product is of interest. However, the proper and safe antioxidant should be employed to prevent lipid oxidation.

Although many synthetic antioxidants presently used by the food industry are effective in preventing rancidity (5,6), their safety has recently been questioned in marine products, especially fish oil. Thus interest is increasing in utilizing natural food constituents with antioxidative properties. Despite a number of studies carried out on antioxidative properties of natural compounds, no significant antioxidant effects have not been reported so far.

Phospholipids have been studied as potential antioxidants because many crude oils containing them are more stable than refined oils (7,8). King *et al.* (9) reported that phosphatidylcholine appeared to be more effective antioxidant than total phospholipid, phosphatidylethanolamine, and phosphatidylinositol. The concentration of phospholipid varied to achieve the same magnitude of antioxidative effect when using different types of oils. The addition of 0.1% (by wt) soybean lecithin to lard enhanced the storage stability by 50% ; whereas the addition of 1.0% was required to obtain an equivalent stability in sunflower oil (10).

To employ phospholipids as antioxidants, the cost of the starting material should be very low. Thus, squid was selected as a starting material because the amount of annual harvest exceeded 7,000tons (11) and the viscera of squid has been almost entirely wasted causing environmental problems ; therefore, the cost of phospholipids from squid viscera would be negligible compared to tocopherol or other antioxidants from plants. The main objective of this study lies in the utilization of squid viscera phospholipids for low cost and compatible antioxidants.

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MATERIALS AND METHODS

Preparation of squid viscera and phospholipid fractionation

Fresh squid (*Todarodes pacificus*) was purchased from a local fish market (Chungmu-dong, Pusan) and used immediately. Viscera of squid was prepared according to the method of Jeong *et al.* (12) and total lipid (TL) was extracted by silicic acid column chromatography (13). Phospholipid was separated from TL using mixture of chloroform and methanol (1 : 5). Phospholipids fraction was further fractionated by silicic acid column chromatography. Different ratios of methanol to chloroform (C/M) were employed to obtain PCF ; elution was carried out with 400ml of C/M (95 : 5, v/v), and C/M (4 : 1, v/v), 800ml of C/M (3 : 2, v/v), and C/M (1 : 4, v/v) in that order. PC fraction was obtained with C/M (3 : 2, v/v) and the remaining fractions were combined and designated as PCF fractions. PCF fractions were composed of cardiolipin, phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin.

Measurement of fish oil oxidation

The antioxidant properties of phospholipids from squid viscera were determined by the method of Jeong *et al.* (12). Briefly, all phospholipids were dissolved in 2ml of chloroform, mixed with refined fish oil (20ml) using a vortex mixer at medium speed for 1min., placed in 100ml beaker with four-fold gauze lid, and stored at 40° C. The control fish oil was prepared by adding 2ml of chloroform. Oxidative stability was determined by changes in peroxide value (POV) (14) and fatty acid composition. POV of refined fish oil was 4.4meq/kg. Refined fish oil from sardine was donated by Dr. K. Hada of Japan Fisheries Co., Ltd.

Determination of fatty acid composition

Fatty acid composition was determined by the method of Metealfea and Schmitz (15). Sample was methylated by 10% boron trifluoride in methanol at 100 ° C and analyzed using the Perkin Elmer 8700 gas chromatograph equipped with a DB-225 capillary column (0.248mm i.d. × 30m, England) and a flame ionization detector. The column oven, injection port and detector were held at 200, 230, and 250° C, respectively. Helium gas was used as a carrier gas with a flow rate of 30ml/min.

RESULTS AND DISCUSSION

Fatty acid composition of phospholipids from squid viscera

The fatty acid composition of PL, PC and PC-free PL (PCF) from squid viscera are summarized in Table 1. PC showed the lowest contents in saturated fatty acids and monoene but the highest in polyene ; coincidentally, phospholipid fractions from mackerel viscera showed the same pattern (16). Fatty acids consisted mainly of $C_{16:0}$ (21.1% for PL, 18.89% for PCF, 19.62% for PC), $C_{20:5}$ (16.01% for PL, 11.77% for PCF, 7.58% for PC) and $C_{22:6}$ (20.21% for PL, 23.13 % for PCF, 30.23% for PC). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were major polyunsaturated fatty acids (PUFA) in squid viscera. EPA and DHA contents were the highest in PC, followed by PL, PCF in that order. The major fatty acids in PC descended in the following order : $C_{22:6}$ (30.23%) > $C_{16:0}$ (19.62%) > $C_{20:5}$ (7.58%) > $C_{20:3}$ (4.97%). PL and PCF showed similar patterns to PC except for $C_{18:1}$ which was considerably low as com-

Table 1. Fatty acid composition of phospholipids from squid viscera (area %)

Fatty acid	PL[a]	PC[b]	PCF[c]
14 : 0	3.53	1.25	1.86
16 : 0	21.01	19.62	18.89
18 : 0	4.88	3.63	7.67
S.F.A.	29.42	24.50	28.42
16 : 1	4.08	0.73	0.98
18 : 1	12.35	4.49	6.92
20 : 1	4.86	6.54	7.87
Monoene	21.29	11.76	15.77
18 : 2	1.60	1.15	1.06
18 : 3	1.70	2.81	0.78
20 : 3	2.97	4.97	4.15
20 : 5	16.01	7.58	11.77
22 : 6	22.20	30.23	23.13
Polyene	44.48	46.74	40.89

[a]Phospholipid [b]Phosphatidylcholine [c]PC-free PL (PL-PC)

pared to PC.

Antioxidant activity of PL from squid viscera

To evaluate the antioxidant activity of PL, PL was added to the fish oil at three different levels (0.5, 1 and 5%) and incubated at 40° C. Addition of phospholipids at all levels improved the overall oxidative stability of fish oil over the control (Fig. 1). Addition of PL at the 5% level was the most effective in stabilizing fish oil, whereas additions at the 0.5% or 1% level did not show as much antioxidant activity as 5% level. Thus, addition of 5% level was selected for the further study.

So far, the role of PL in oxidation has not been elucidated conclusively. Many investigators reported that phospholipids showed antioxidant effect on the autoxidation of fat and oils (17,18); in addition, at elevated temperatures some of the phospholipids, particularly PE, greatly enhanced the activity of primary antioxidants in edible oils. PC and PS are aslo reported to be effective (19,20); in contrast several studies have reported that hydrolysis of PL fatty acid results in increased lipid oxidation (21-23). However, the varing conditions of the those studies make it impossible to compare each other and to draw any conclusion about the role of PC, PE and PS on the oxidation of edible oil.

To resolve the argument, the effect of individual PL component need to be evaluated. Thus, phospholipids were fractionated into PC, PC free PL, PCF because PL was consisted of mainly PC (more than 70%) and the rest-PE, PI (phosphatidylinositol), PS etc. The PC fraction did not contain any significant amount of other components other than phosphatidyl choline ; in contrast, PCF was mainly composed of PE, PI, and PS. Since PC was the major component of PL, our study was focused on the effect of PC on stabilizing the fish oil. In addition, 5% level was selected to compare the antioxidant effects of different fraction.

Fig. 2 shows changes in POV of fish oil incubated at 40° C in which fish oil was supplemented with PL, PC, or PCF. All fractions exhibited strong antioxidative activity on the day 2. Both PL and PCF exhibited less antioxidant activity than PC. This result contradicts the report by Kashima *et al.* (24). They found that the oxidative stability of perrila oil was increased significantly by additions of PE and PS, but PC scarcely showed an antioxidant effect. However, their claim cannot be justified because the perilla oil contained substantial amount of tocopherol. In this study, the fish oil employed did not contain any significant amount of tocopherol ; therefore, the antioxidant effect was attributed to the phospholipid fractions only. The strong oxidative stability of fish oil supplemented with PC during the extended incubation time implies that PC plays major role in antioxidant activity. This result can also be confirmed by the antioxidant effect of PC from mackerel viscera (16).

It should be noted that the PE, PI, and PS also exert significant antioxidant activity, if not stronger than PC since PCF fraction retarded the rate of oxidation remarkably. However PE, PI and PS were not likely to have synergistic effect with PC because PL which con-

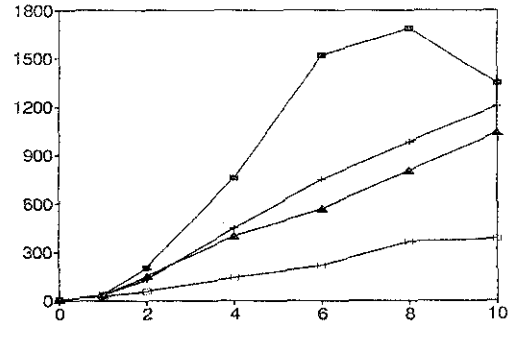

Fig. 1. Antioxidant effects of phospholipid (PL) from squid viscera.
(■, control ; +, 0.5% PL ; ▲, 1% PL ; □, 5% PL)

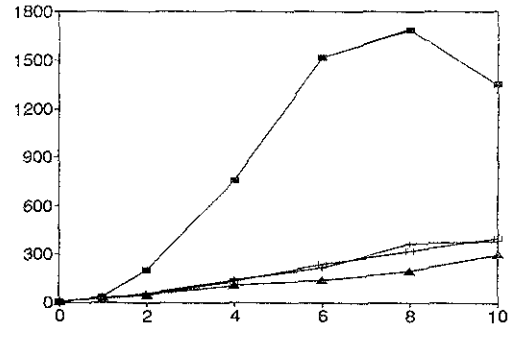

Fig. 2. Antioxidant effects of phospholipid (PL), phosphatidylcholine (PC) and PC-free PL (PCF) extract from squid viscera.
(■, control ; +, 5% PL ; ▲, 5% PC ; □, 5% PCF)

tained PC over 70% show less antioxidant activity than 100% PC. If PE, PI and PS have synergistic effect with PC, the change in POV upon incubation are likely to show the same magnitude as PC added oil, if not better.

Now that the antioxidant activity of PC was found to be strong enough to be utilized as natural antioxidant, it was compared with other known antioxidants of strong activity : butylated hydroxy toluene (BHT), tocopherol and flavonoids-(+)catechin (Fig. 3). The concentration of each antioxidant was adjusted to produce the same effect in stabilizing the fish oil for 2 days of incubation at 40° C. During the initial 4 days of incubation, all the antioxidants retarded the oxidation rate considerably with the same magnitude. However, from day 6, addition of PC was the most effective in stabilizing the fish oil. Other antioxidants also resulted in significantly greater stability than the non supplemented control oil.

Prolonged incubation of fish oil indicated that 5% PC was the most effective. BHT (0.05%) and catechin (1%) showed the same antioxidative effect followed by 1% tocopherol. PC from mackerel viscera also showed the same effect when compared with BHT, tocopherol, and cathechin except that BHT exerted stronger antioxidant activity than tocopherol (16). This result was in agreement with several other studies (9,25) which showed that addition of small quantities of PL to refined vegetable and animal fats improved their stability.

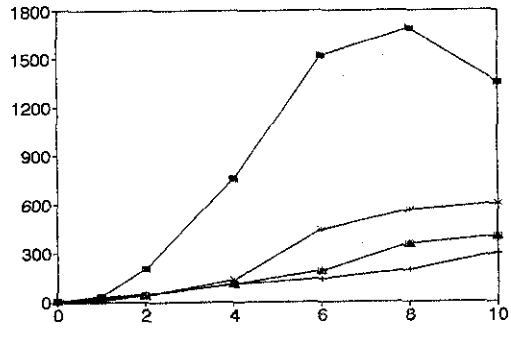

Fig. 3. **Antioxidant effects of phosphatidylcholine compared to other antioxidants.**
(■, control ; +, 5% PC ; ▲, 0.05% BHT ; □, 1% catechin ; ✱, 1% tocopherol)

Changes in fatty acid compositions affected by the addition of PLs

To compare the effect of PL, PC and PCF on the fatty acid compositions of fish oil during oxidation, each fraction was added to the fish oil and incubated at 40° C. Table 2 summarizes the changes in fatty acid composition of treatments at different periods of incubation at 40° C. The percentage of monoenes ($C_{16:1}$, $C_{18:1}$) and saturates ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$) for each treatment increased with the reduction in n-3 fatty acid. $C_{16:0}$ and $C_{18:1}$, in particular, showed significant increase ; on the contrary, EPA and DHA decreased significantly. This result was also coincided with that of phospholipid fractions from mackerel viscera (16).

On the day 10, polyene index ($C_{22:6}/C_{16:0}$) were 0.64, 0.42, 0.5 and 0.66% for control, PL, PC and PCF treatment, respectively. This confirms the result of King *et al.* (25) who claimed that PC was effective in preventing the loss of PUFA. However, it cannot be compared with our results because they heated the oil at 180° C which underwent thermal oxidation, not autoxidation. We conclude that polyene index cannot be used as the criteria for antioxidant activity. As fish oil was oxidized, monoene contents were significantly increased ; compared to control (33.34%), monoene contents of PL, PC and PCF treatments were 29.27, 24.49 and 28.8%, respectively. This result coincides with that of Fig. 2, which showed the strong antioxidant activity of PC compared with PL or PCF. Therefore, changes in monoene contents might be more effective criteria in evaluating antioxidative activity than polyene index ; in addition, the fatty acid composition of each phospholipid fraction appeared to play little or no role in the antioxidant activity.

The antioxidant activity of phospholipid appeared to be more related to functional group than their fatty acid composition. In some studies, it has been demonstrated that phospholipids could act as synergists with endogenous α-tocopherol, a primary antioxidant, to protect lipid from oxidation (25,26). Although phospholipids contained higher concentration PUFA, Komatsu *et al.* (27) claimed that the inhibitory effect of the phospholipid fraction prepared from squid mantle muscle in peroxidation of sardine oil might not be

Table 2. Effects of phospholipid extract from squid viscera on the fatty acid composition of fish oil incubated at 40° C

Fatty acid	Control[a]			PL[b]			PC[c]			PCF[d]		
	0day	1day	4day	10day	1day	4day	10day	1day	4day	10day	1day	4day	10day
14 : 0	6.92	6.05	6.85	9.27	7.68	5.44	9.19	6.14	4.99	7.15	5.84	6.62	4.30
16 : 0	13.50	8.59	12.30	18.50	12.82	10.96	19.62	11.73	11.81	14.28	11.34	13.25	16.30
18 : 0	3.02	3.40	3.45	3.45	2.16	1.72	2.16	1.73	1.82	1.96	1.88	2.21	2.90
S.F.A.	23.44	18.04	22.66	31.22	22.66	18.12	31.97	19.06	18.62	23.39	19.06	22.08	23.50
16 : 1	8.19	7.23	9.66	12.10	7.06	9.29	9.65	8.15	7.37	9.54	8.28	8.89	9.50
18 : 1	10.90	10.20	13.88	19.30	10.83	13.87	17.63	8.59	11.06	14.95	12.85	13.53	19.30
20 : 1	1.32	1.52	2.28	1.94	2.14	1.54	1.99	2.02	2.11	–[e]	1.99	1.87	–[e]
Monoene	20.41	18.95	25.82	33.34	20.13	24.7	29.27	18.76	19.54	24.49	23.12	24.29	28.80
18 : 2	2.41	1.23	2.36	2.00	0.97	0.86	1.06	1.02	1.02	1.62	0.94	1.26	1.17
18 : 3	1.76	1.03	3.26	2.00	2.55	1.87	1.66	1.99	1.96	1.88	2.02	1.45	1.20
20 : 3	2.78	3.25	4.28	1.81	2.67	2.35	2.49	2.63	1.11	1.92	2.59	2.78	1.82
20 : 5	24.14	27.67	16.15	13.50	27.37	20.04	17.08	27.55	21.53	13.80	27.70	24.17	9.00
20 : 6	11.18	13.43	13.88	11.90	15.79	11.11	8.22	11.52	10.93	7.180	12.40	12.17	10.70
Polyene	42.29	49.57	39.93	31.36	47.35	36.23	30.51	46.93	37.27	26.40	43.79	41.83	23.89
PUFA/SFA	1.80	2.75	1.76	1.00	2.18	1.20	0.95	2.46	2.00	1.13	2.29	1.89	0.94
DHA/$C_{16:0}$	0.83	1.56	1.13	0.64	1.23	1.01	0.42	0.98	0.93	0.50	1.11	0.92	0.66

[a] Fish oil [b] Phospholipid [c] Phosphatidylcholine [d] PC-free PL (PL-PC) [e] Not tested

due to synergism between the phospholipids and α-tocopherol. In this study, fish oil used contained insignificant amount of α-tocopherol and mixture of fish oil and each phospholipid fraction also showed negligible amount of α-tocopherol. This implies that the antioxidant effects of phospholipid treatment are mainly derived from phospholipid itself, not the effect of α-tocopherol. Furthermore, phosphatidylcoline, a major component of phospholipid from squid viscera, clearly showed strong antioxidant activity. In conclusion, the phospholipid fraction from squid viscera can be used as feasible natural antioxidant as long as the proper deodorization step is employed.

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오징어 내장으로 부터 추출한 인지질의 어유에 대한 항산화효과

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요 약

오징어 내장으로 부터 인지질(PL)을 추출하고 이것을 포스파티딜콜린(PC)과 나머지 부분(PCF)으로 나누어 각각의 항산화효과를 검토하였다. 추출획분의 고도불포화지방산(PUFA)의 함량은 각각 PC(46.7%), PL(44.8%), PCF(40.9%)의 순으로 PC 획분이 불포화도가 가장 높은 것으로 나타났다. 이들 각 획분을 정제어유에 중량당 5% 농도(w/w)로 첨가하여 40°C에서 10일간 저장하면서 정제어유의 산화안정성에 미치는 영향을 조사하였다. 과산화물가는 저장 2일째 까지는 비슷한 경향을 보였으나 그 이후에는 PC획분 첨가군이 가장 높은 산화안정성을 보였으며, PL과 PCF 첨가군에서도 대조군에 비해서 어유의 산화안정성에 있어서 효과가 대단히 높은 것으로 나타났다. PC의 산화안정성 효과는 정제어유에 BHT(0.05%), 카테킨(1%), 토코페롤(1%)을 첨가하였을 때 보다 높은 항산화효과를 보였다.