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Antioxidant Activity of Different Lipid Extracts from Squid Viscera

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To utilize waste of squid effectively, antioxidant properities of squid viscera were elucidated. Major fatty acids of total lipid, neutral lipid and phospholipid were $C_{16:0}$, $C_{18:1}$ and $C_{20:5}$, $C_{22:6}$ and consisted 63~71% of total fatty acids. Total lipid did not show significant antioxidant activity when added to the fish oil at the concentration below 5.0%, Antioxidant activity of hexane extract was lower than total lipid or tetra carbon chloride-methanol(CCl_4-MeOH) extract. Extracts with CCl_4-MeOH exerted higher antioxidant activity as the methanol ratio was increased, suggesting that polar lipid plays and important role.

Introduction

Marine products are easily deteriorated by the oxidative reaction with atmospheric oxygen because of their high content of unstable polyunsaturated fatty acids (PUFA). n-3 PUFA in fish oil reportedly plays an important role in improving thrombosis, arteriosclerosis, and inflammation, as well as aging and immunological process (Lands *et al.*, 1977; Terano *et al.*, 1986; Singh and Chandra, 1988; Fernandes and Venkatraman, 1993). Thus, fish oil is expected to broaden its usefulness in the near future. However, n-3 PUFA is very susceptible to oxidation, especially during the process of related products. The resultant oxidation products can cause cancer, aging and deleterious effects on various tissues of human body (Vergroeson, 1977; Saito, 1988).

Consequently, prevention measures for oxidation should be emphasized to utilize fish oil as a raw material of food or pharmaceutical product. Various kinds of synthetic antioxidants have been explored and used in many seafoods to prevent lipid oxidation but the use of these synthetic antioxidants for foodstuffs must be restricted from the stand point of food safety. To avoid this safety problem, many researchers investigated the possible use of various natural antioxidants such as tocopherol, selenium, carotenoids, ascorbic acid (Schwartz, 1975), and flavonoids (Oota, 1985). Except for tocopherol the effectiveness of these compounds are not so significant. Even tocopherol is not so effective as synthetic antioxidants and also involve high manufacturing cost. Especially in case of fish oil and its related products, there is an urgent need for effective and safe natural antioxidants.

Therefore, this study was focused on the search for natural antioxidants with low cost. Squid was selected as a starting material considering the amount of annual harvest exceeded 70,000 tons (Fisheries Statistics, 1990). The viscera of squid has been almost entirely wasted resulting in environmental problems; thereby, cost for raw material would be negligible compared to tocopherol or other antioxidants from plant. The main objective of this study lies in the utilization of squid viscera extracts for low cost and compatible antioxidant.

Materials and Methods

Materials

Fresh squid (*Todarodes pacificus*) was purchased from a local fish market (Chungmu-dong, Pusan) and used immediately. Viscera was removed from squid and homogenized with Waring blender. Homogenate was treated with 10 volumes of chloroform : methanol (2 : 1) and stored in the refrigerator overnight.

Refined fish oil (EPA : 28%, DHA : 12.4%) was donated from Central Research Institute of Japan Fisheries Co. Ltd. Antioxidant was not originally added to this fish oil.

Lipid extraction and fractionation

Total lipid (TL) was extracted by the method of Folch (1956). Pretreated viscera homogenate mixture was occasionally stirred to facilitate diffusion of solvent mixture. After filtration (Whatman No.2), lower part was concentrated using rotary evaporator with vacuum at 40℃.

TL was calculated from the dried sample weight substrated by the container weight. To evaluate appropriate solvents for extraction, hexane and carbon tetrachloride-methanol (1 : 1, 1 : 3, or 1 : 9) were employed. For column chromatography, activated silica gel (120*g*) was packed in glass column (3.0×70.0*cm*). TL was dissolved in chloroform and loaded on the top surface of the column. Using each solvent or solvent mixture, TL was fractionated. To separate phospholipid(PL), TL was eluted with hexane-benzene-chlololform (6 : 9 : 10), (chloroform-acetone=2 : 1)-acetone (1 : 1), and (chloroform-methanol=1 : 1)-methanol (1 : 2) successively (Folch, 1956). Neutral lipid (NL) and phospholipid (PL) were obtained by hexane-benzene-chloroform (6 : 9 : 10) and (chloroform-methanol =1 : 1)-methanol (1 : 2) extract, respectively.

Effect of viscera extracts on the lipid oxidation

The antioxidant properties of viscera extracts were determined by their addition to the refined fish oil. Peroxide value of refined fish oil is 4.4 *meq/kg*. All extracts were dissolved in 2*ml* of chloroform, mixed with refined fish oil(20*ml*) on a vortex mixer at medium speed for 1*min*. placed in 100*ml* beaker with four-fold gauze lid, and stored at 40℃. Chloroform (2*ml*) was added to fish oil as control. Samples were taken for peroxide value (POV) analyses at the predetermined time. Oxidative stability was determined by changes in POV (AOCS, 1971) and fatty acid composition.

Determination of fatty acid composition

Fatty acid composition was determined by the method of Metealfea and Schmitz (1949). Sample was methylated by 10% boron trifluoride in methanol at 100℃. Perkin Elmer 8700 gas chromatograph equipped with a DB-225 capillary column (0.248*mm* i.d.×30*m*, England) and a flame-ionization detector. The column oven, injection port and detector were held at 200, 230, and 250℃, respectively (Kwon *et al.*, 1993). Helium gas was used as a carrier gas and flow rate was 30*mL/min*.

Determination of tocopherol content

Saponified sample was injected to HPLC column (Ultrapac TSK NH_2, Toyosoda, Japan). Mobile phase and flow rate were n-hexane-isopropyl alcohol (98 : 2, v/v) and 0.5*ml/min*, respectively. Tocopherol peaks were detected by fluorescence detector (Ex 298*nm*; Em 330*nm*).

Results and Discussion

Fatty acid composition of lipids from squid viscera

Considerable variation was detected in the PUFA composition of TL, NL, PL fractions from squid viscera (Table 1). The NL fraction contained only 22~68% polyene compared to 39.06% and 44.48% for TL and PL, respectively. EPA and DHA were major PUFA in squid viscera. EPA and DHA contents were highest in PL, followed by TL and NL, in that order. Saturated and monoenoic fatty acid con-

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

Fatty acid	Squid		
	TL	NL	PL
14 : 0	3.44	3.78	3.53
16 : 0	17.94	28.27	21.01
18 : 0	3.99	6.69	4.88
Saturated	25.37	39.01	29.42
16 : 1	2.87	3.72	4.08
18 : 1	16.40	17.57	12.35
20 : 1	5.54	6.53	4.86
Monoene	24.18	27.97	21.29
18 : 2	1. 8	1.31	1. 6
18 : 3	1.77	1.51	1. 7
20 : 3	2.13	1.49	2.97
20 : 5	13.91	7. 2	16.01
22 : 6	19.45	11.35	22.20
Polyene	39.06	22.68	44.48

TL : Total lipid; NL : Neutral lipid; PL : Phospholipid

sisted mainly of $C_{16:0}$(17.94% for TL; 28.27% for NL; 21.01% for PL) and $C_{18:1}$(16.40% for TL; 17. 57% for NL; 12.35% for PL), respectively. The major fatty acids in TL descended in the following order : $C_{22:6}$(19.45%) > $C_{16:0}$(17.94%) > $C_{18:0}$(16. 40%) > $C_{20:5}$(13.91%). The PL fraction showed similar patterns except content of $C_{20:5}$(16.01%) was slightly higher than $C_{18:1}$(12.35%). In contrast, the content of $C_{16:0}$ in NL was the highest, followed by $C_{18:1}$, $C_{22:6}$, and $C_{20:5}$, in that order. These four fatty acids consisted 64~72% of the total fatty acids.

Fatty acid composition of various extract from total lipid.

Total lipid was extracted with hexane. In addition, mixture of carbon tetrachloride : methanol (C-M) with different mixing ratio were employed. Nonpolar lipids was extracted with hexane, whereas different ratio of C-M were employed to separate lipids of different polar characteristics. Fatty acid composition for each extract was similar to TL or PL, in that $C_{16:0}$, $C_{20:5}$, and $C_{22:6}$ consisted more than 65% of total fatty acids. Monoenoic acid contents were increased as the ratio of methanol increased but hexane extract contained the highest in that content. Highest contents in saturated fatty and monoenoic acid were observed in hexane extract; in contrast, the lowest polyenoic acid was resulted in the same extract

Antioxidant effects of total lipid from squid viscera

Fig. 1 shows the changes in POV of fish oil supplemented with three different levels of TL. The TL contents of the squid visera were 24*g/100g* sample (24%). Endogenous α-tocopherol, β-tocopherol and γ-tocopherol levels were detected relatively very low concentrations in the TL, hexane extract and carbon tetrachloride extract (data not shown). Addition of TL at all levels improved the overall oxidative stability of fish oil over the control. Addition of TL at the 5% level was the most effective in stabilizing fish oil, whereas additions at the 0.05 % and 0.1% level did not differ from each other. However, 5% TL resulted in significantly greater stability of the oil than non- supplemented control oil. In addition, fractions extracted with hexane

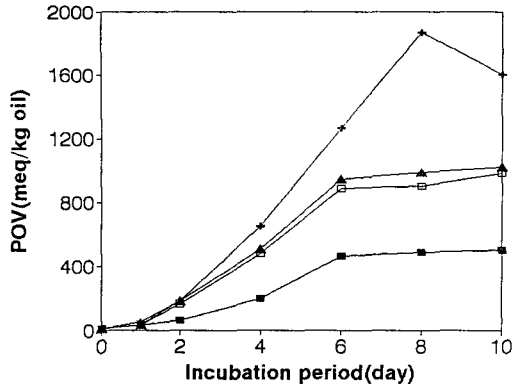

Fig. 1. Antioxidant effects of total lipid (TL) from squid viscera.
+, control; ■, 5% TL; □, 1% TL; ▲, 0.5% TL

and C-M also exhibited remarkable effect in stabilizing fish oil at the 5% level compared to the 0.1 and 1% level (data not shown). Therefore, addition of 5% level was selected for further study.

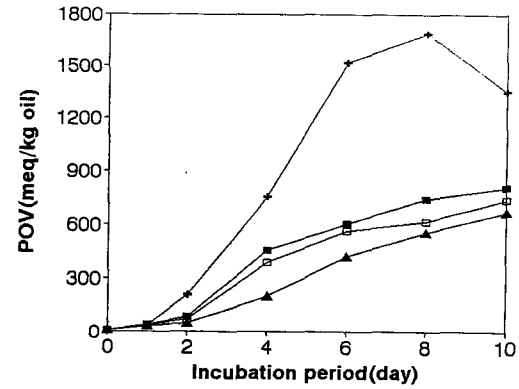

Fig. 2. Antioxidant effects of solvent extract from total lipid(TL) of squid viscera.
+, control Fish oil; ■, 5%, carbon tetrachloride-methanol (1 : 1, v/v) extract; □, 5% carbon tetrachloride-methanol(1 : 9, v/v) extract; ▲, 5% carbon tetrachloride-methanol(1 : 9, v/v) extract

Antioxidant effect of solvent extracts from TL.

Polar characteristics of extraction solvent mixture (C-M) were varied to evaluate the antioxidative effects of resultant extracts. Significant antioxidant effects were observed upon addition of all C-M extracts of different mixing ratios (Fig. 2). Increasing methanol concentration resulted in a proportional increase in antioxidant activity. Especially on the day 4, the difference in POV became significant. The antioxidative effects were the largest on the day 8. As compared to the control, POV of C-M (1 : 9), C-M (1 : 3), and C-M (1 : 1) extract additions were 33, 37, and 44%, respectively. This implies that polar lipid plays an important role in retarding oxidation of fish oil. Lee *et al* (1984) also reported the similar result; that is, the highest antioxidative effect was observed with methanol extract of TL compared to varing ratios of chloroform and methanol. Therefore, extract of C-M (1 : 9) was selected for further study.

Antioxidant activity of different lipid extracts

To compare the stability effect of different lipid extracts, 5% level of each extract was added to the fish oil and incubated at 40℃(Fig. 3). Hexane extract shows significant effect but did not retard oxidation of fish oil remarkably compared to other extracts. On the day 2, all the extracts exhibited antioxidant effects. TL and C-M (1 : 9) extract showed similar pattern of POV changes : however,

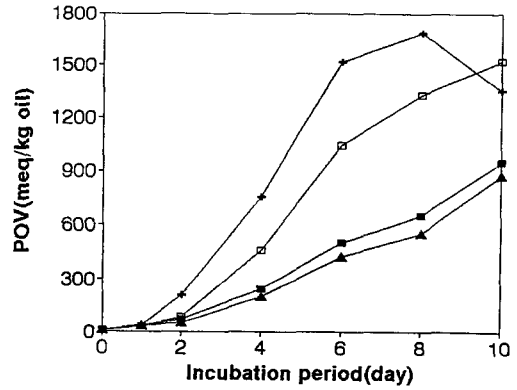

Fig. 3. Antioxidant effects of different lipid extract from total lipid(TL) extract from squid viscera.
+, control; ■, 5% TL; □, 5% hexane extract; ▲, 5% carbon tetrachloride-methanol(1 : 9, v/v) extract

C-M (1 : 9) extract was slightly more effective than hexane one. In all, antioxidant activity is likely to be exerted by polar lipids.

Butylated hydroxytoluene, tocopherol, and flavonoids are used most often as antioxidants and hydroxyl groups of phenol ring act as functional groups (Torel *et al.*, 1986). Antioxidant activity increases as the increase in hydroxyl groups (Saito and Nakamura, 1990). Furthermore, high antioxidant activity of sesamol and its derivatives was reportedly derived from the presence of hydroxyl groups (Osawa *et al.*, 1985). Therefore, the antioxidant effect of TL might be the outcome of polar lipids in it.

Animal and plant tissue contains a certain amount of tocopherol (Kim, 1994). Sometimes high tocopherol contents in edible oils misleads stability test of lipid oxidation. Besides, tocopherol exhibited synergism with other antioxidants (Oshima *et al.*, 1993). However, fish oil contained insignificant amount of α-tocopherol (*7.7ppm*) and mixtures of fish oil and extract also showed negligible amount of α-tocopherol. This implies the antioxidant effect of extract is mainly derived from extract itself, not the effect of α-tocopherol. Kashima *et al.*(1991) claimed that the stabilizing effect of phospholipids in perilla oil might be related to the antioxidant activity of coexisting α-tocopherol. Komatsu *et al.* (1991) believed, however that the inhibitory effect of the phospholipids fraction prepared from squid mantle muscle on peroxidation of sardine oil might not be due to synergism between the phospholipids and α-tocopherol because the lipids contained α-tocopherol in an extremely small quality.

Changes in fatty acid compositions affected by the addition of various extracts

The polyene indices showed different trend that observed for POV changes; that is, control was the most effective in preventing the loss of PUFA, whereas 5% hexane extract was the least effective (Fig. 4). This contracts the result of King *et al* (1992) indicating the polyene index cannot be effective criteria to determine antioxidant activity. Changes in fatty acid composition also supported

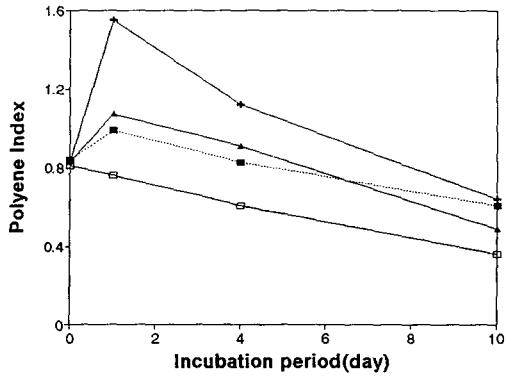

Fig. 4. Effects of addition of different extracts on polyene index of fish oil incubated at 40℃.
+, control(fish oil); ■, 5% TL; □, 5% hexane extract; ▲, 5% carbon tetrachloride-methanol(1 : 9, v/v) extract

the ineffectiveness of polyene index (Table 3). On the day 10, polyene contents were 31.36, 30.58, and 23.18, 31.88% for control, TL, and Hexane, C-M (1 : 9) treatments, respectively. Except for hexane extract, other treatments did not show any significant difference in polyene content. Conversely, monoene contents were changed significantly. Compared to control (33.34%) and 5% hexane extract (35.31%), monoene contents of TL and C-M (1 : 9) treatments 22.95% and 25.99%, respectively. In this case changes in monoene contents might be more effective criteria in evaluating antioxidant activity.

When stabilizing effects of fish oil by addition of squid viscera extract were compared with changes in fatty acid composition, hexane extract showed the lowest antioxidant effect. At the same time, the decreases in EPA and DHA were the largest. In addition, C-M extract resulted in considerable or insignificant decrease in EPA and DHA. Therefore, antioxidant activity is not likely to be directly related to the changes in fatty acid composition or PUFA in particular. Sardine oil model system of King *et al.* (1992) also confirmed our results.

In conclusion, squid viscera extract proved to be utilized as low cost natural antioxidant rather than treated as waste material. Further research is in progress to elucidate the major components of antioxidant activity by squid viscera extract.

Table 3. Changes in fatty acid composition as affected by various lipid extract from squid viscera (area%)

Fatty acid	Control[a]				Ex[b]			Hex[c]			C-M(1 : 9)[d]		
	0 day	1 day	4 day	10 day	1 day	4 day	10 day	1 day	4 day	10 day	1 day	4 day	10 day
14 : 0	6.92	6.05	6.85	9.27	7.32	5.79	6.15	9.81	6.17	10.45	5.66	5.86	7.71
16 : 0	13. 5	8.59	12. 3	18. 5	15.27	14.79	17.70	18.83	21.56	20.73	11.76	13. 6	15.23
18 : 0	3.02	3. 4	3.45	3.45	4.24	2.81	2. 6	3.32	1.93	3.36	2.08	1.84	2.87
Saturated	23.44	18.04	22.66	31.22	26.83	23.39	26.45	31.96	29.66	34.54	19.41	21. 3	25.81
16 : 1	8.19	7.23	9.66	12. 1	9.47	6.87	6.65	10.21	8.18	13.18	7.88	12.14	9.75
18 : 1	10. 9	10. 2	13.88	19. 3	14.73	14. 8	15.20	6.32	14.61	22.13	13.46	12.43	16.24
20 : 1	1.32	1.52	2.28	1.94	2.22	3.62	0. 9	0. 9	2.11		2.21	2.65	
Monoene	20.41	18.95	25.82	33.34	26.42	25.29	22.75	17.34	24.90	35.31	23.55	28.22	25.99
18 : 2	2.41	1.23	2.36	2. 0	1. 2	1.12	1. 3	1. 3	1.27	trace	0.67	1.33	2.15
18 : 3	1.76	1.03	3.26	2.00	1.51	1.51	1.07	2.69	2.03	trace	2.18	1.82	1.83
20 : 3	2.78	3.25	4.28	1.81	1.81	2.42	1.69	1.93	2.83	2.33	2.73	3.02	2.64
20 : 5	24.14	27.67	16.15	13. 5	22.58	20.20	15.61	28.74	26.90	16.13	27.58	6.37	18.30
22 : 6	11.18	13.43	13.88	11. 9	15. 9	12.74	10.91	14.53	13.07	7.05	12.89	12.48	6.96
Polyene	42.29	49.57	39.93	31.36	39.84	37.99	30.58	49.09	46. 1	23.18	46.05	44.28	31.88
PUFA/Saturated	1.80	2.75	1.76	1.00	1.48	1.62	1.16	1.54	1.55	0.67	2.37	2.08	1.24
DHA/PA	0.83	1.56	1.13	0.64	1.04	0.86	0.62	0.77	0.61	0.34	1.20	0.91	0.46

*a : fish oil; b : Total lipid; c : Hexane extract; d : carbon tetrachloride-methanol(1 : 9, v/v) extract
PA : palmitic acid

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Antioxidant Activity of Different Lipid Extracts from Squid Viscera

오징어 내장으로부터 추출한 지질성분의 항산화효과

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요 약

수산 폐기물의 효과적인 이용을 위하여 오징어 내장의 지질을 추출하여 용매를 달리하여 분리한 후 어유에 첨가하여 산화효과를 검토하였다.

오징어 내장의 주요 지방산은 팔미틴산($C_{16:0}$), 올레산($C_{18:1}$), 아이코산펜타엔산($C_{20:5}$), 그리고 도코사헥사엔산($C_{22:6}$)으로 추출용매의 종류에 따라 64~71%를 차지하였으며 그 중 오메가-3 고도불포화지방산(n-3 PUFA)의 함량이 41% 이상이었다. 조지질을 정제어유에 각각 5%, 1%, 0.5% 첨가하여 40℃에서 저장하면서 산화억제정도를 검토한 결과 적정농도 (5%) 이하로 첨가할 경우의 효과는 미약한 것으로 나타났다. 헥산 추출물은 조지질이나 사염화탄소-메탄올추출물 보다 산화억제효과가 낮았으며 사염화탄소-메탄올(1 : 1, 1 : 3, 1 : 9 v/v)추출물은 극성이 높을 수록 효과가 큰 것으로 미루어 볼 때 극성 지질이 산화억제에 중요한 역할을 하는 것으로 추정할 수 있었다.