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Antioxidative Activity of Phospholipid from Squid Mantle Muscle

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Abstract

An attempt was made to examine if phospholipid from squid takes part in the suppression of lipid peroxidation. Phospholipid fraction prepared from short-finned squid (*Ilex argentinus*) significantly suppressed peroxidation of purified sardine oil. The mechanism of antioxidative activity of squid phospholipid has not yet been known, however, results in the present study strongly suggest that phospholipid from squid suppressed lipid peroxidation not in a manner of a synergist to tocopherols but by an unknown mechanism, because contents of tocopherol in both purified sardine oil and phospholipid fraction from the squid were negligible.

Introduction

With the increase of demand for natural antioxidants over artificial antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), which the food industry has been reluctant to use for safety reason, efforts to substitute them with novel natural antioxidants are being made (Dugan, 1980).

Squid is a major marine resource in Japan, and large quantities of squid mantle muscle are consumed mostly as a raw food and/or a dry processed food. In spite of high levels of polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, squid products usually do not have the troubles accompanied with rancidity during food processing such as air drying. That is, squids are slow to form the so-called "rancid odor" during the making of dried squid mantle muscle, ("surume" in Japanese). The major reason may largely be because of low lipid content, however, some substances which suppress lipid peroxidation and subsequent formation of secondary carbonyl compounds may also contribute to this.

In this study an attempt was made to see if any other antioxidative substance besides known substances is present in the squid mantle muscle by comparing parameters of lipid peroxidation. The antioxidative activity of lipids in squid mantle muscle was examined by *in vitro* experiment using purified sardine oil and linoleic acid, and was found that phospholipid fraction prepared from squid mantle muscle showed significant antioxidative activity.

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Materials and Mathods

Materials

Sample used in the present study was short-finned squid (*Illex argentinus*) caught off-shore of New Zealand (average mantle length, 250 mm; average body weight, 370 g) and kept at −20°C until analyses. Purified sardine oil used as the substrate for determination of peroxide value (POV), carbonyl value (COV) and weight gain for the estimation of lipid peroxidation was kindly supplied by Nippon Kagaku Shiryo Co. Ltd. (Hakodate, Japan).

Linoleic acid and its methylester used as substrates for estimation of lipid peroxidation were purchased from Wako Chemical Co., Osaka, as guaranteed reagent grade.

Extraction of total lipids and preparation of phospholipid fraction

After thawing squids at room temperature, surface skin was peeled off and matnles were provided for total lipid extraction with a mixture of chloroform : methanol = 2 : 1 (v/v) according to Folch et al. (1957). Phospholipid fraction used for estimation of antioxidant activity was prepared from the total lipid fraction on a gel permeation column using a Bio-Beads S-X2 (Bio-Rad, Richmond, CA) column (51 × 3 cm *i.d.*) (Tipton, 1964). Phospholipid was eluted with benzene. Purity of the eluted phospholipid was confirmed on a Silica Gel 70 TLC plate using n-hexane : diethyl ether : acetic acid = 80 : 20 : 1, by vol. as a developing solvent.

Analysis of lipid composition of squid mantle muscle

Total lipid and phospholipid compositions were determined by thin-layer chromatography on a Silica Gel 70 plate (0.25 mm thick; Wako Pure Chemical Ind. Ltd., Osaka) using n-hexane : diethyl ether : acetic acid = 80 : 20 : 1, by vol. as the development solvent (Skipski, 1964). Phospholipid was further analyzed by two steps single dimensional development; *i.e.*, acetone : diethyl ether = 1 : 1, by vol. for the first step development, followed by chloroform : methanol : acetic acid : water = 25 : 15 : 4 : 2, by vol. for the second step development (Skipski, 1964). The proportion of individual lipid component was determined densitometrically using a Cosmo Model F-808 densitometer (Cosmo Co. Ltd., Tokyo) (Fewster, 1969). Total phospholipid contents were determined colorimetrically using Fiske-Subbarow's method (Fiske-Subbarow, 1925). The purity of phospholipid fraction thus obtained was confirmed to be almost tocopherol-free (Table 3).

Analysis of fatty acid composition

Fatty acid composition of total lipid was determined by gas-liquid chromatography of fatty acid methyl esters prepared by interesterification using 2N HCl-methanol (Prevot, 1976). Fatty acid methyl esters were analyzed by a Hitachi gas chromatograph Model 263-30 equipped with a flame ionization detector using a G-300 capillary column of 40 m × 1.2 mm *i.d.* coated with polyethylene glycol of 0.5 μm thick (Chemicals Inspection & Testing Institute, Tokyo). Column temperature was raised from 160 to 200°C at the rate of 2°C/min, injector and detector temperatures were 270°C, and carrier gas (He) flow rate was kept at 30 ml/min.

Analysis of tocopherols in the squid phospholipid and sardine oil used as substrate for lipid peroxidation

The amount of tocopherol in the total phospholipid fraction and in the purified sardine oil used as substrate for the evaluation of lipid peroxidation was analyzed by high-performance liquid chromatography using Cosmosil 5SL column (150×4.6 mm *i.d.*; nacalai tesque Inc., Kyoto) on a Shimadzu LC-5A high performance liquid chromatograph equipped with Shimadzu Fluorescence Monitor RF-530 (Excitation 298 nm; Emission 325 nm) as a detector. The mobile phase used was n-hexane: ethanol: dioxane = 95.8 : 0.2 : 4.0, by vol. and the flow rate was kept at 1.0 ml/min.

Evaluation of antioxidative activity of squid phospholipid fraction

Evaluation of antioxidative activity of squid phospholipid fraction was carried out by comparing POV, COV and weight gain. One gram of total lipid prepared from squid mantle muscles was mixed with 9 g of a purified sardine oil, and the mixture was incubated at 50°C in petri dishes (70 mm *i.d.*) to compare peroxide values (POV) (Bilinski, 1978), carbonyl values (COV) (Kumazawa and Oyama, 1965) and weight gain. Purified sardine oil alone was incubated at 37°C in a petri dish as control.

The phospholipid fraction and the non-phospholipid fraction prepared from the squid total lipid fraction by gel permeation chromatography were further used for the determination of their antioxidative potentials by mixing them separately with the purified sardine oil (3 g) at the concentration of 5% of sardine oil, and POV and COV values were compared after the *in vitro* incubation as described above.

Linoleic acid (gas chromatographically pure reagent grade; Wako Pure Chemicals, Osaka, Japan) was also used as substrate for determination of POV (Mitsuda, 1966) and weight gain.

Results and Discussion

Lipid composition of squid mantle muscle

Table 1 shows that the major component of mantle muscle of short-finned squid was phospholipid being 73.1%. Phosphatidylcholine and phosphatidyl ethanolamine made up 75.7% of the total phospholipid (data not shown). Besides phospholipid, cholesterol was the second major component, and triacylglycerol was found only in a small amount. Tocopherol content was only 0.9 μg/g of total lipid (Table 3).

Table 1. Lipid compositions of total lipid prepared from short-finned squid mantle muscles

Lipid species	%
Triacylglycerol	3.5
Free fatty acid	7.6
Sterols	15.8
Phospholipid	73.1

Table 2. Fatty acid compositions of total lipid and the phospholipid fraction of short-finned squid mantle muscle

	TL (%)	PL (%)
Satd. FA		
14 : 00	2.4	2.4
16 : 0	35.8	35.1
18 : 0	3.8	4.4
others	1.5	1.4
Sum of satd. FA	43.5	43.3
Monoenoic FA		
16 : 1	1.6	1.6
18 : 1	5.3	5.3
20 : 1	4.6	5.5
others	0.7	0.6
Sum of monoenoic FA	12.2	13.0
Polyenoic FA		
20 : 4	1.3	1.3
20 : 5	13.2	12.6
22 : 6	28.7	28.0
others	1.0	1.5
Sum of polyenoic FA	44.2	43.4

Abbr.: FA, fatty acid; TL, total lipid fraction; PL, phospholipid fraction.

Concerning the fatty acid composition, considerably high proportion of polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid were found in the phospholipid fraction (Table 2).

Table 3. Tocopherol Content in Phospholipid Fraction and in Purified Sardine Oil

Tocopherols	Squid Phospholipid [ng/g][a]	Purified Sardine Oil [ng/g][a]
α-Tocopherol	800	1900
β-Tocopherol	ND[b]	60
γ-Tocopherol	40	70
δ-Tocopherol	ND	ND
Total	840	2030

[a] Values are expressed as ng/g of 1 g of phospholipid and of purified sardine oil. [b] Not detected.

Antioxidative activity of total lipid prepared from squid mantle muscle

To determine if squid mantle muscle showed any significant antioxidative potential, the autoxidation levels in the mixture (1 : 9, w/w) of squid total lipid fraction and purified sardine oil were compared in terms of determination of POV, COV and weight gain as percentage weight increase to the sample at 0 day. Results showed that the autoxidation of purified sardine oil was significantly suppressed by the addition of total lipid prepared from squid mantle muscle at 1% level. The time length taken to reach apparent rancid levels, *i.e.*, 100 meq/kg as POV ; 100 meq/kg as COV ; and 1% weight gain were 51-75 days for total lipid. On the other hand, sardine oil without addition to squid total lipid reached 100 meq/kg POV and COV in 4 days, and took 11 days for 1% weight gain. The total lipids prepared from squid mantle muscle obviously showed antioxidative potential. Similar results were obtained from the incubation systems using methyl linoleate or linoleic

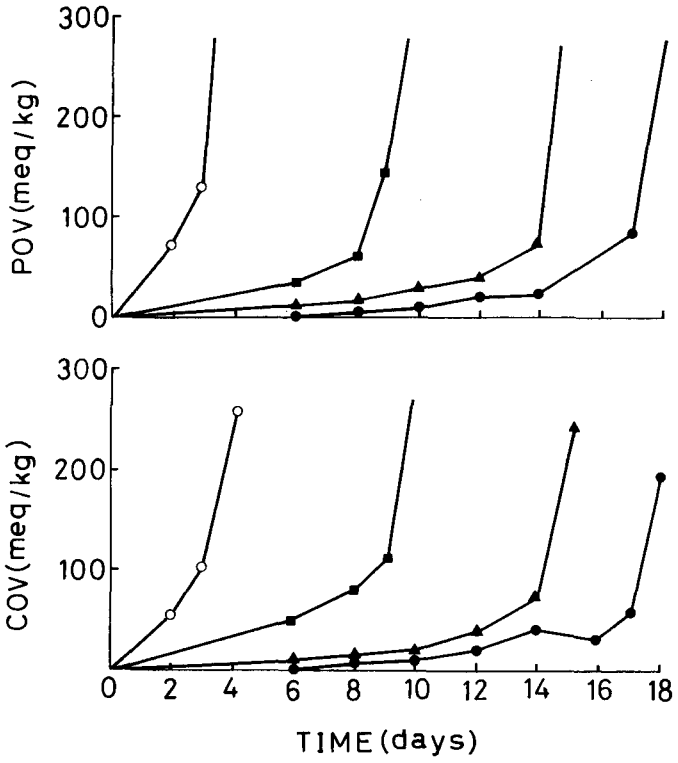

Fig. 1. Time course of peroxide value (POV) and carbonyl value (COV) of purified sardine oil with and without addition of squid mantle muscle phospholipid
To 3 g of purified sardine oil 150 mg of phospholipid fraction prepared from squid mantle muscle was added, and the mixture was incubated at 37°C.
○, purified sardine oil alone ; ■, mixture with phospholipid fraction ; ▲, mixture with non-phospholipid fraction ; ●, mixture with total lipid from short-finned squid mantle.

acid as substrates (data not shown).

Distribution of antioxidative activity in the squid total lipids

To examine whether the antioxidative effect was derived from phospholipids or not, phospholipid fraction was prepared from the total lipid of short-finned squid. The phospholipid fraction was added at a level corresponding to 5% of purified sardine oil. The result of antioxidative evaluation is shown in Fig. 1. It is obvious from the present study that purified phospholipid fraction from squids suppressed autoxidation of purified sardine oil. Purified phospholipid fraction exhibits antioxidative activity; *i.e.*, addition of purified phospholipid fraction to the sardine oil delayed the time required to reach rancid levels (POV, 100 meq/kg; COV, 100 meq/kg), about 5.3 times longer as compared to the sardine oil without

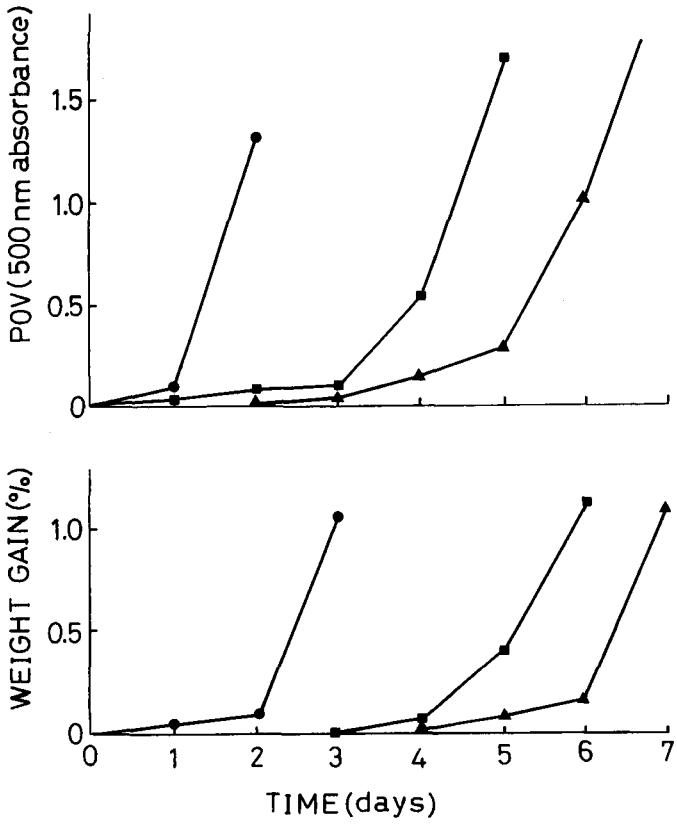

Fig. 2. Antioxidative activity of phospholipid fraction prepared from squid total lipid fraction on linoleic acid.
Time course of POV and weight gain of linoleic acid with and without addition of squid phospholipid.
●, linoleic acid alone; ■, with squid phospholipid fraction at 1%; ▲, with squid phospholipid fraction at 3%.

phospholipid addition. Similar results were obtained from the incubation systems using linoleic acid as substrate (Fig. 2).

To explain the antioxidative effect of squid phospholipid on purified sardine oil and linoleic acid, the commonest explanation is to ascribe to the synergistic effect of phospholipid to tocopherols (Hildebrand, 1984), however, contents and compositions of tocopherols either in the squid mantle muscles (0.9 μg/g of total lipid) and in the purified sardine oil (2 μg/g of sardine oil) does not give enough grounds for any reasonable explanation for the antioxidant activity of phospholipid fraction from the squid mantle muscles (Table 3). Data obtained from the system using linoleic acid suggests that the antioxidative activity of phospholipid from squid mantle muscle exerts its activity by a mechanism other than synergistic effect.

Our finding is not the first one to show that phospholipid exhibits antioxidative potential. Similar reports suggesting the possibility of antioxidative activity of phospholipid have been recognized (Olcott and Van der Veen, 1963; Hildebrand, 1984; Hudson and Ghavami, 1984; Yamaguchi, 1984a; Yamaguchi, 1984b; Kajimoto, 1987; Kashima, 1990; Takagi, 1979; Miyazawa, 1984; Lee, 1984; Lee, 1981). Olcott and Van der Veen (1963) studied antioxidant potential of phospholipid, but concluded that phospholipid itself did not have any antioxidant activity. Yamaguchi et al. (1984a) reported that lipids prepared from plaice, stripped pigfish and several other fishes implied oxidative stability and the stability of the lipids was correlated with their total phospholipid contents and composition. Further, they (Yamaguchi, 1984b) attempted to show the mechanism of antioxidative action by using methyl linoleate as substrate. They suggested that phospholipid could not play any role as primary antioxidant by itself but would act as a synergist for α-Toc. Our data strongly suggest the antioxidative activity of the phospholipid by itself.

Concerning the tendency in suppression of peroxidation of methyl linoleate as substrate, conclusion reached by Yamaguchi et al. (1984b) is apparently different from ours in the sense that in our experiment purified phospholipid fraction prepared from squid by itself suppressed the peroxidation of sardine oil. Since sardine oil often contains small amount of tocopherols as trace contaminants that are hardly removable by conventional purification procedure, a possibility was suggested that the antioxidative effect of squid phospholipid might be due to synergistic effect of phospholipid to tocopherols contaminanted in purified sardine oil (personal communication with Dr. Mamoru Sato, Kyoto University, Faculty of Agriculture). However, in our experiment the content of tocopherols both in the purified sardine oil and in the squid phospholipid can be negligible to consider phospholipid as synergist to tocopherols. Furthermore, clear antioxidative results were obtained from experiments using linoleic acid as substrate in place of the sardine oil.

The mechanism of antioxidative activity of squid phospholipid is yet unknown. Detailed studies with purified phospholipid species isolated from squid are now being undertaken.

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