A Study of the Antioxidative Substance(s) in Squid Mantle Muscle

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The lipid peroxidation suppressing ability of whole lipid extracted from commercially purchased squid mantle muscle of three common squids, *i. e.*, Japanese common squid (*Todarodes pacificus*), flying squid (*Ommastrephes bartrami*) and short-finned squid (*Illex argentinus*), was evaluated by the progress of peroxide value (PV) and carbonyl value (CV) of purified sardine oil with or without addition of whole lipid fraction at 10% level. Every whole lipid extracted from the three species of squid markedly suppressed the peroxidation of purified sardine oil. The whole lipid of *I. argentinus* showed the strongest suppressive activity of the three. Neither differences in compositions of lipid and fatty acids nor content and composition of tocopherols in the mantle muscle could sufficiently account for their antioxidative ability. The addition of purified phospholipid fraction, fractionated from the whole lipid of *I. argentinus*, to purified sardine oil at the concentration of 5% (w/w) markedly suppressed the peroxidation of sardine oil.

With increasing demand for natural antioxidants to replace artificial ones such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), which in recent times the food industry has been reluctant to use because of their questionable safety, efforts to replace them by novel natural antioxidants are being made¹⁾⁻³⁾. As a result, antioxidants in marine organisms have become a subject of great interest⁴⁾⁵⁾.

In Japan, a large quantity of squid is consumed raw and/or as a dry processed food. In spite of the fact that these processed squid have a large content of polyunsaturated fatty acids that are susceptible to lipid peroxidation, squid products usually do not pose any problem of rancidity during the processing such as air drying⁶⁾⁷⁾. This study examined whether or not lipids in squid mantle muscle have lipid peroxidation suppressing ability.

Materials and Methods

Materials

Samples used in the present study were Japanese common squid (*Todarodes pacificus*) (average mantle length, 230 mm ; average body weight, 250 g), flying squid (*Ommastrephes bartrami*) (average mantle length, 290 mm ; average body weight, 370 g) and short-finned squid (*Illex argentinus*) (average mantle length, 250 mm ; average body weight, 370 g). All samples were provided in the frozen state. Purified sardine oil, used as the specimen for determination of PV and CV as indices of lipid peroxidation, was kindly supplied by Nippon Kagaku Shiryo Co., Ltd. (Hakodate, Japan).

Evaluation of antioxidative activity of squid lipids

The lipid peroxidation suppressing ability of squid lipids was evaluated by comparing PV

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and CV as follows: one gram each of the whole lipid extracted from each of the three different squid mantle muscles according to the method of FOLCH *et al.*⁸⁾ were separately mixed with 9 g of a purified sardine oil. The mixtures were placed in petri dishes (70 mm in diameter) and incubated at 37°C in the dark. PV⁵⁾ and CV⁹⁾ were periodically measured and compared between the three different squid lipids. As a control, purified sardine oil was incubated under the same condition. For determining lipid peroxidation suppression ability for the phospholipid fraction, 150 mg of phospholipid fraction, fractionated by gel permeation chromatography according to the method of TIPTON *et al.*¹⁰⁾, from the whole lipid fraction of *I. argentinus* was added to purified sardine oil (3 g). PV and CV of the mixture was measured periodically during incubation as described above, but in this case the incubation temperature was set at 50°C.

Analysis of lipid composition of squid mantle muscle

In order to make it clear whether or not lipid oxidation suppression ability differs with changes in whole lipid composition and/or phospholipid composition, whole lipid and phospholipid compositions were analyzed by thin-layer chromatography on a Silica Gel 70 plate (0.25 mm thick; Wako Pure Chemical Ind. Ltd., Osaka). As the first step, each plate was developed with a solvent system (*n*-hexane : diethyl ether : acetic acid = 80 : 20 : 1, by vol.). For the second step¹¹⁾, this was followed by developing with another solvent system (chloroform : methanol : acetic acid : water = 25 : 15 : 4 : 2, by vol.). Phospholipid was further analyzed by a two single dimensional thin-layer chromatography; *i. e.*, for the first step development acetone : diethyl ether = 1 : 1, by vol., and the second step development chloroform : methanol : acetic acid : water = 25 : 1 : 4 : 2, by vol. were used¹¹⁾. The proportion of individual lipid component was determined densitometrically with a Cosmo Model F-808 densitometer (Cosmo Co. Ltd., Tokyo)¹²⁾. The total phospholipid content was determined by Fiske-Subbarow's method¹³⁾.

To examine the possibility that the lipid peroxidation suppression is due to tocopherols, the whole squid lipid, tocopherol content and composition of the whole lipid was analyzed. The whole lipid extract (3 ~ 4 g) from the skinned mantle muscle was mixed with 9 ml of a mixture of 1% pyrogallol, 60% methanol and 1.5 ml of 50% KOH. Saponification was carried out at 90°C for 45 min. A small volume of water was added to the saponified alkaline whole lipid solution, and tocopherols were extracted by *n*-hexane. The extract was concentrated to 4.5 ml under vacuo. The obtained tocopherol fraction was subjected to a quantitative analysis of isomers by high-performance liquid chromatography using a Cosmosil 5 SL

Fig. 1 Time course of suppression of peroxidation of purified sardine oil by whole lipid fraction extracted from mantle muscle of three different squid species

To 9 g of the purified sardine oil, 1 g of whole lipid fraction extracted from mantle muscle of three different squid species was separately added and the mixture was incubated at 37°C for the indicated time. Experimental details are described in Materials and Methods.

○, purified sardine oil alone; ■, *T. pacificus*; ▲, *O. bartrami*; ●, *I. argentinus*. The change of PV (meq/kg) with time (day) is represented in the top panel, and that of CV (meq/kg) is in the bottom panel.

column (150 × 4.6 mm i. d. ; Nacalai Tesque Inc., Kyoto) on a Shimadzu LC-5 A high performance liquid chromatograph equipped with a 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.). The flow rate was kept at 1.0 ml/min.

Results

Antioxidative activity of whole lipid extracted from squid

The effect on changes of PV and CV in sardine oil as a result of adding total lipid extract obtained from three different species of squid mantle muscle was examined. In comparison to purified sardine oil alone, the peroxidation of purified sardine oil was markedly suppressed by the addition of total lipid extract obtained from each of the three different species of squid mantle muscle down to the level of 10% (Fig. 1). The length of time taken to reach rancid state, *i. e.*, 100 meq/kg as PV and CV, was 51~75 days when whole lipid of squid was added to sardine oil. However, it took only 4 days for sardine oil alone to reach the same level. The total lipids extracted from mantle muscle of the three squid species

obviously showed antioxidative activity. In particular, extract from *I. argentinus* displayed the most potent effect of the squid species examined. Similar results were obtained by an incubation system using methyl linoleate or linoleic acid¹⁴. The antioxidative stability of squid lipid themselves, however, was not examined.

Comparison between lipid composition of three different squid mantle muscles

To examine where the antioxidative effect originated, lipid compositions of the three squid mantle muscle were compared (Table 1). Table 1 shows that phospholipid was the major constituent of the extracted lipids. Phospholipid contents in the total lipid from *T. pacificus*, *O. bartrami* and *I. argentinus* were 71.7%, 61.8% and 73.4%, respectively. As to the phospholipid composition, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in *T. pacificus*, and *I. argentinus* were the major components, occupying 38.9~54.7% of the total phospholipids. However, the contents of PC and PE in *O. bartrami* were lower than those of the other two squid species.

Cholesterol was the second major component in the whole lipid fraction. However, triacylglycerol was present only in a small amount.

Table 1 Compositions of whole lipid and phospholipid obtained from the mantle muscle of three different squid species

(A) Whole lipid composition

	PL*	S	FFA	TG	Others
<i>T. pacificus</i>	71.7	19.9	6.5	0.8	1.1
<i>O. bartrami</i>	61.8	15.8	21.6	0.1	0.7
<i>I. argentinus</i>	73.1	15.4	6.8	3.2	1.5

(B) Phospholipid composition comprising whole lipid

	LPC/SPM	PC	PS	PE	Unidentified
<i>T. pacificus</i>	7.5	31.5	8.9	19.7	4.1
<i>O. bartrami</i>	12.4	22.1	7.8	16.8	2.7
<i>I. argentinus</i>	9.6	31.5	5.4	23.2	3.4

Values are expressed as relative weight percent. *.

Individual component of total phospholipid are given in Table 1-(B).

Abbr. : PL, phospholipid; S, sterol; FFA, free fatty acid; TG, triacylglycerol; others, sterol esters and unidentified compound(s); LPC/SPM, lysophosphatidylcholine and/or sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine

Free fatty acid content in *O. bartrami* was considerably high. This suggests that hydrolysis of phospholipid took place during storage of the squid. Lipids listed as unidentified in Table 1 may include sterol esters and melanoidine-like browning substance(s).

The peroxidation suppression effect of whole squid lipid against purified sardine oil was thought to be attributed to tocopherols in the whole lipid fraction. Total tocopherol contents in the whole lipid fraction from mantle muscle of the three different squid are shown in Fig. 2. Total tocopherol content in *O. bartrami* was the highest of the three squid species. However, the total squid lipid which showed the most potent peroxidation suppres-

Fig. 2 Tocopherol contents in the whole lipid fraction extracted from the mantle muscle of three different squid species

Values are expressed as $\mu\text{g/g}$ of whole lipid.

, total tocopherol content; , α -tocopherol content; ■, γ -tocopherol
 Since the contents of γ -tocopherol and δ -tocopherol were too low in comparison to those of total tocopherol and α -tocopherol, columns are not drawn in the graph except for γ -tocopherol in *O. bartrami*. β -Tocopherol was not detected from any samples examined. The contents of γ -tocopherol and δ -tocopherol in *T. pacificus* were 0.30 and 0.08 $\mu\text{g/g}$ of whole lipid, respectively, and those in *O. bartrami* were 1.78 and 0.21 $\mu\text{g/g}$ of whole lipid. The content of δ -tocopherol in *I. argentinus* was 0.89 $\mu\text{g/g}$ of whole lipid, but γ -tocopherol was not detected.

sion effect was not that from *O. bartrami* but from *I. argentinus*. On the other hand, of the three squid species, the content of δ -tocopherol, known to have the most potent antioxidative activity, was the highest in the total lipid of *I. argentinus*. The peroxidation suppression effect of the total lipid from squid mantle muscle could be explained by the contribution of tocopherols.

On the other hand, several papers have reported that phospholipid prevents lipid peroxidation¹⁵⁾. Although the antioxidative effect of phospholipids has been denied by OLCOTT and van DEEN¹⁶⁾, several studies suggest the antioxidative potential of phospholipid¹⁷⁻¹⁹⁾. Therefore, we tried to examine whether phospholipid fractionated from the total lipid of short-finned squid (*I. argentinus*) could show any suppressive effect on lipid peroxidation of purified sardine.

Phospholipid fraction from the total lipid of *I. argentinus* was added to purified sardine oil at a level of 5%. The loading test result is shown in Fig. 3. Purified phospholipid fraction from *I. argentinus* showed evidence of antioxidative activity. The addition of purified phospholipid fraction to the sardine oil definitely delayed the time required to reach rancid state (PV : 100 meq/kg ; CV : 100 meq/kg). It required approximately 5.3 times as long as that of sardine oil alone.

Discussion

Concerning the mechanism of antioxidative activity of squid mantle lipid, several possibilities can be considered ; (1) antioxidative effect of tocopherols, (2) synergistic effect of phospholipid and tocopherols, (3) the presence of novel unknown antioxidative substance(s) in the lipid fraction, and (4) antioxidative effect of phospholipid.

Accounting the result to the antioxidant effect arisen from tocopherols is not enough. The contents and compositions of tocopherols in the squid mantle muscles did not account for the difference in the antioxidant activity. Little δ -tocopherol content having antioxidative effect was found in squid mantle muscle ;

Fig. 3 Comparison of antioxidative activity of phospholipid fraction and non-phospholipid fraction prepared from squid whole lipid fraction against purified sardine oil

To 3 g of purified sardine oil 150 mg of each fraction prepared from *I. argentinus* was separately added, and the mixture was incubated at 50°C in the dark.

○, purified sardine oil alone; ■, mixture with non-phospholipid fraction; ▲, mixture with phospholipid fraction; ●, mixture with whole lipid from *I. argentinus*

The changes of PV (meq/kg) with time elapsed (day) are given in the top panel, and those of CV (meq/kg) are given in the bottom panel.

i.e., the content of tocopherols other than α -tocopherol did not seem high enough to act as antioxidant. In addition, there was no accounting for the difference in antioxidative activity of the whole lipid fraction from the squid mantle muscles. Furthermore, the difference in antioxidative effect could not be explained by the δ -tocopherol content, either.

The antioxidative activity of whole lipid from the mantle muscles of the three species of squid examined showed different extent of antioxidant activity. The lipid composition of *T. pacificus* was similar to that of *I. argentinus*, but antioxidative effect of total lipids from those two species differed from each other. It is, therefore, difficult to explain the difference in the antioxidant effect by their lipid compositions. Furthermore, any novel substances which may contribute to an anti-

oxidative effect were not detected by thin-layer chromatography. Though there were some differences in the composition of the whole lipid fraction among these three squid species, each whole lipid fraction was considerably rich in highly polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid. Major fatty acids in the fraction were: $C_{16:0}$ (33.6~37.7%), $C_{20:5}$ (8.2~13.2%), and $C_{22:6}$ (28.4~28.7%). The content of mono-unsaturated fatty acids was relatively low (10.2~12.8%).

Phospholipid, the major constituent of the squid mantle lipid, suppressed peroxidation of purified sardine oil. The possibility can be excluded that traces of tocopherol, present in either the squid phospholipid fraction or purified sardine oil, exhibited antioxidative effect in cooperation with phospholipid as synergist. This is because the contents of total tocopherol in squid phospholipid (0.84 μ g/g) from *I. argentinus* and in purified sardine oil (2.03 μ g/g) were considered to be too low to act as primary antioxidant. This point will be discussed in detail elsewhere¹⁴.

Similar facts dealing with the antioxidative activity of phospholipids and related compounds have been reported¹⁷⁾⁻¹⁹⁾.

YAMAGUCHI *et al.*²⁰⁾ reported that lipids extracted from plaice, stripped pigfish and several other fishes exhibited oxidative stability. The degree of stability of these lipids were correlated with their total phospholipid contents and composition. Further, they¹⁵⁾ attempted to make clear the mechanism of antioxidative action of phospholipids by using isolated phospholipid from total lipid of the ordinary muscle of plaice with methyl linoleate as the critical standard material. They suggested that phospholipid *per se* could not play a role as a primary antioxidant but would act as a synergist against α -tocopherol.

Studies on the participation of phospholipid base in the suppression of lipid peroxidation were carried out by TAKAGI *et al.*²¹⁾, TERAO²²⁾, KANEDA and his colleagues^{23) 24)}. However, there have been no detailed studies at the molecular level on the antioxidative activity of

squid lipids.

Sardine oil often contains small amounts of tocopherols as a trace contaminant. These are only slightly removed by a conventional purification procedure. A possibility was inferred that the antioxidative effect of squid phospholipid might be due to the synergistic effect of tocopherols contained in purified sardine oil^{1*}. Similar antioxidative phenomenon has been observed by experiments using methyl linoleate as an evaluation standard material in place of sardine oil¹⁴⁾. This occurred where the content of tocopherols in the purified phospholipid was ca. 40 ng/g in the incubation mixture.

To explain the antioxidation mechanism of squid phospholipid, it is still necessary to make a detailed model experiment by using purified individual constituent of phospholipid fraction and a highly refined substrate. Detailed studies with purified squid phospholipid are now being undertaken.

An interesting and necessary point is the oxidative stability of the components comprising the whole lipid fraction of the squid. However, in the present study we did not evaluate the oxidative stability of lipid and/or lipid soluble components nor did we examine distribution of the antioxidative activity in the body of squid. It is under investigation.

Although the antioxidative ability of squid lipid is far weaker than that of BHT and BHA when compared by weight (1/37 of that of BHT, data not shown), it may be advantageous in practical usage. This is because its use is not restricted by food hygiene law. When rat was fed squid phospholipid, carbon tetrachloride-induced liver injuries of the rat was prevented. This will be reported elsewhere²⁵⁾.

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References

- 1) RESURRECTION, A.V.A. and REYNOLDS, Jr., A.E.: *J. Food Sci.*, **55**, 627 (1990).
- 2) YAMAGUCHI, N., OKADA, Y. and NAITO, S.: *Nippon Shokuhin Kogyo Gakkaishi*, **37**, 676 (1990).
- 3) BARBUT, S., JOSEPHSON, D.B. and MAURER, A.J.: *J. Food Sci.*, **50**, 1357 (1985).
- 4) LEE, J.H., FUJIMOTO, K. and KANEDA, T.: *Nippon Suisan Gakkaishi*, **47**, 881 (1981).
- 5) BILINSKI, E., JONAS, R.E.E. and LAU, Y.C.: *J. Fish. Res. Board Can.*, **35**, 473 (1978).
- 6) YUKI, E.: *Nippon Shokuhin Kogyo Gakkaishi*, **9**, 149 (1962).
- 7) ISHIKAWA, Y. and YUKI, E.: *Agric. Biol. Chem.*, **38**, 1227 (1974).
- 8) FOLCH, J., LEES, M. and SLOANE STANLEY, G.A.: *J. Biol. Chem.*, **226**, 497 (1957).
- 9) KUMAZAWA, H. and OYAMA, T.: *Yukagaku*, **14**, 167 (1965).
- 10) TIPTON, C.L., PAULIS, J.W. and PIERSON, M.D.: *J. Chromatog.*, **14**, 486 (1964).
- 11) SKIPSKI, V.P., PETERSON, R.F. and BARCLAY, M.: *Biochem. J.*, **90**, 374 (1964).
- 12) FEWSTER, M.E., BURUS, B.J. and MEAD, J.F.: *J. Chromatog.*, **43**, 120 (1969).
- 13) FISKE, C.H. and SUBARROW, Y.: *J. Biol. Chem.*, **66**, 375 (1925).
- 14) KOMATSU, I., YASUDA, T., FUKUNAGA, K., SUZUKI, T., SUZUKI, S. and TAKAMA, K.: *Bull. Fac. Fish. Hokkaido Univ.*, **41**, 232 (1990).
- 15) YAMAGUCHI, K. and TOYOMIZU, M.: *Nippon Suisan Gakkaishi*, **50**, 1897 (1984).
- 16) OLCOTT, H.S. and VAN DER VEEN, J.: *J. Food Sci.*, **28**, 313 (1962).
- 17) HILDEBRAND, D.H., TERAŌ, J. and KITO, M.: *J. Am. Oil Chem. Soc.*, **61**, 552 (1984).
- 18) HUDSON, B.J.F., GHAVAMI, M.: *Lebensm. Wiss. Technol.*, **17**, 191 (1984).
- 19) KAJIMOTO, G., OONISHI, H., HIROMI, H. and SHIBAHARA, A.: *Nippon Nogeikagaku Kaishi*, **61**, 191 (1987).
- 20) YAMAGUCHI, K., TOYOMIZU, M. and NAKAMURA, T.: *Nippon Suisan Gakkaishi*, **50**, 1245 (1984).

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- 21) TAKAGI, T., ISHIZAWA, T. and IIDA, T. : *Yukagaku*, **28**, 548 (1979).
- 22) TERAOKA, J. : *Biochim. Biophys. Acta*, **1003**, 221 (1989).
- 23) MIYAZAWA, T., YAMAGUCHI, M., LEE, J.H., FUJIMOTO, K. and KANEDA, T. : *Agric. Biol. Chem.*, **8**, 1375 (1984).
- 24) LEE, J.H., FUJIMOTO, K. and KANEDA, T. : *Nippon Suisan Gakkaishi*, **50**, 1863 (1984).
- 25) TAKAMA, K., KOMATSU, I., SUZUKI, T., FUKUNAGA, K. and SUZUKI, S. : Proceedings of International Conference, Seafood 2000, Halifax, May 14-17, 1990 (Fishing News Books Co. Ltd.), in press.

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イカ外套筋の脂質画分に含まれる抗酸化性物質

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3種類のイカ(アカイカ [*Ommastrephes bartrami*], スルメイカ [*Todarodes pacificus*], アルゼンチンマツイカ [*Illex argentinus*]) の外套筋から脂質を抽出し、精製イワシ油に対する過酸化抑制能を過酸化価(PV), カルボニル価(CV)を指標に比較・検討した。その結果、いずれのイカ総脂質抽出物もイワシ油の過酸化を有意に抑制し、中でもアルゼンチンマツイカのそれが最も強い抗酸化能を示した。アルゼンチンマツイカから調製したリン脂質画分を用いて精製イワシ油に対する抗酸化性を調べた結果、明らかな抗酸化性を示した。その抗酸化能は脂質、脂肪酸の違い、あるいは単にトコフェロール組成の違いで説明出来るものではなく、リン脂質画分が深く関わっていることが示唆された。