Improvement in Storage Stability of Fish Fillets Using Dietary Soybean Phosphatidylcholine and Phosphatidylethanolamine

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Received May 27, 2008; Accepted December 15, 2008

We previously reported that soybean phospholipids improved the storage stability of fish fillets. The object of this study was to identify the active component of soybean phospholipids and its mechanism. Rainbow trout were fed purified diets supplemented with no phospholipids (Control), 1% soybean phosphatidylcholine (S-PC), and 1% soybean phosphatidylethanolamine (S-PE) for 4 weeks. After the feeding period, fish fillets were subjected to an oxidation test. The storage stability of fish fillets was improved in the S-PC and S-PE groups compared to the Control group. The rate of phospholipid-bound docosahexaenoic acid (DHA) in the S-PC and S-PE groups was higher than that in the Control group. These results indicate that phosphatidylcholine and phosphatidylethanolamine in soybean phospholipids contributed to the improved storage stability of fish fillets. It was speculated that high phosphatidylcholine- and phosphatidylethanolamine-bound DHA levels were involved in the improved storage stability of fish fillets.

Keywords: soybean phospholipids, phosphatidylcholine, phosphatidylethanolamine, oxidation, fish

Introduction

N-3 polyunsaturated fatty acids (PUFAs), such as α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have been attracting attention regarding their preventive effects against diseases such as atherosclerosis, coronary arterial heart diseases, and hypertension (Conner, 2000; Takeuchi *et al.*, 2007). N-3 PUFAs are easily oxidized because they contain many double bonds. We previously reported that soybean phospholipid administration inhibited lipid oxidation in fish fillets (Murano *et al.*, 2008). This finding is of interest as a new technique to improve the storage stability of muscle food containing n-3 PUFAs.

In addition to the main components, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), soybean phospholipids also contain other phospholipids, such as phosphatidylinositol and phosphatidylserine, with these other components accounting for about 55% (Schneider, 2006). The first objective of this study was to confirm the effects of the main components of soybean phospholipids, soybean PC and soybean PE, and identify the active components that increase the storage stability of fish fillets. Rainbow trout were fed

purified diets supplemented with soybean oil (Control), high-purity soybean phosphatidylcholine (S-PC), and high-purity soybean phosphatidylethanolamine (S-PE) for 4 weeks, and the storage stability of fish fillets was compared among the 3 groups.

The mechanism responsible for improving fish fillet storage stability using soybean phospholipids has not been clarified. Although the enhanced storage stability of muscle food with a high content of antioxidative substances, such as total tocopherol and phospholipids, has been reported (Kim *et al.*, 2006; Yamaguchi and Toyomizu, 1984), the feeding of soybean phospholipids did not alter the total tocopherol or phospholipid content of fish fillets (Murano *et al.*, 2008). The second objective was to clarify the reason for the soybean phospholipid-induced increase in the storage stability of fish fillets. It has been reported that the oxidative stability of muscle food varied depending on the fatty acid species bound to phospholipids, a biomembrane component (Stanley, 1991; Meynier *et al.*, 1999). We investigated the effects of high-purity dietary soybean PC and soybean PE on phospholipid-bound fatty acids in fish fillets.

Materials and Methods

Fish and feeding experiment We used a purified diet,

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Table 1. Experimental diet composition.

	Treatment group		
	Control	S-PC	S-PE
	(g/100g)		
Casein	50	50	50
Dextrin	20	20	20
Corn starch	9	9	9
Mineral mix	5	5	5
Vitamin mix	1	1	1
Lipids			
Fish oil	10	10	10
Soybean oil	5	4	4
Soybean phosphatidylcholine	0	1	0
Soybean phosphatidylethanolamine	0	0	1

the composition of which is shown in Table 1. Vitamins and minerals were combined according to the formulation reported by Kaushik *et al.* (1998) and Ogino *et al.* (1979). Three experimental diets were prepared: Control (no added phospholipids), S-PC (soybean PC added), and S-PE (soybean PE added). All diets were supplemented with 10 g/100 g fish oil, and 5, 4 and 4 g/100 g soybean oil was added to the Control, S-PC, and S-PE diets, respectively. S-PC was prepared by adding 1 g/100 g soybean PC, and S-PE by adding 1 g/100 g soybean PE (both from Sigma-Aldrich Co., St. Louis, MO, USA). We previously reported that 1% soybean phospholipids improved the storage stability of fish fillets. Therefore, 1% soybean phospholipids were substituted with 1% phosphatidylcholine or 1% phosphatidylethanolamine in the present study. The soybean phospholipids added to prepare the S-PC and S-PE diets were of high-level purity, 99% and 98%, respectively. The fatty acid compositions of the total lipids of the 3 diets are shown in Table 2.

The test fish were maintained as previously reported (Murano *et al.*, 2008). Juvenile rainbow trout (*Onchorhynchus mykiss*), purchased from a local seed supplier, were used for the feeding experiment. At the start of the feeding experiment, a total of 45 fish (each weighing 2.0 g on average) were divided into 3 groups (15 fish per group). The fish were biologically and biochemically analyzed after the feeding period.

Oxidation test The oxidation test was performed as previously reported (Murano *et al.*, 2008). The fillets were cut and adjusted to a weight of 1.0 ± 0.1 g. The samples were oxidized by incubation at $4 \pm 0.5^\circ\text{C}$ under fluorescent lighting for 3 days. The positions of individual samples in

Table 2. Fatty acid composition of the experimental diets.

	Treatment group		
	Control	S-PC	S-PE
	(g/100g)		
12:0	0.1	0.1	0.1
14:0	4.0	4.0	4.0
15:0	0.3	0.3	0.3
16:0	12.7	12.9	13.1
16:1	4.9	4.9	4.9
17:0	0.2	0.2	0.2
18:0	3.1	3.1	3.0
18:1	21.4	20.6	20.6
18:2 (n-6)	18.3	19.0	18.9
18:3 (n-3)	2.7	2.7	2.7
20:0	0.3	0.2	0.2
20:1	9.0	9.0	9.0
20:2 (n-6)	0.2	0.2	0.2
22:0	0.6	0.6	0.6
22:1	8.0	8.0	8.0
20:5 (n-3)	7.1	7.1	7.1
24:0	0.4	0.4	0.4
24:1	0.5	0.5	0.5
22:5 (n-3)	1.0	1.0	1.0
22:6 (n-3)	5.2	5.2	5.2

the incubator were strictly standardized during the oxidation period. The degree of lipid oxidation in the fish fillets was measured before and after the oxidation test.

Biochemical analysis Lipids were extracted from the samples by the method reported by Folch *et al.* (1957). The phospholipid content, fatty acid composition, thiobarbituric acid reactive substance (TBARS), and tocopherol content were analyzed as previously reported (Murano *et al.*, 2008). The extracted lipids were fractionated into triacylglycerol (TG) and phospholipids by thin layer chromatography with chloroform : methanol (9 : 1) (Silica gel 70 Plate; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The fractionated TG was extracted from resin and measured by weight (Murano *et al.*, 2007). Total phospholipids, PC, and PE were analyzed using the method established by the Japan Oil Chemists' Society No. 2. 4. 11 (2003). Fatty acids at positions *sn*-1 and *sn*-2 in PC and PE were analyzed by the phospholipase A2 method (Wells and Hanahan, 1969). The fatty acid analysis was performed by gas chromatography (Murano *et al.*, 2008) after the conversion of fatty acyl groups in the TG to their methyl esters by heating in a sealed tube at 100°C for 1h with methanolic sulfuric acid under nitrogen. Gas chromatography was performed using an Agilent Technologies 6869 series (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame-ionization detector with a capillary column (OMEGAWAX320; Supelco, Bellefonte, PA, USA). Tricosanoic acid was used as an internal standard in quantitative gas chromatographic analyses (Shantha and Ackman, 1990). Tocopherol concentration was measured by a modified procedure of Huang and Shaw (1994). Analysis was carried out by HPLC (YMC-Pack SIL A-012 column, YMC Co., Ltd., Kyoto, Japan; F1100 Fluorescence Spectrophotometer, Hitachi High-Technologies Corporation, Tokyo, Japan). The eluting solvent was hexane : isopropyl alcohol (100 : 0.5) at flow rate of 2 mL / min.

Statistical analysis Data are expressed as mean $\pm$ SD. Significant differences between groups were determined by one-way ANOVA followed by Tukey's multiple comparison

test. Differences with a *P*-value of < 0.05 were considered significant. A statistical software package (Toukei version 2, Esumi, Tokyo, Japan) was used for all statistical analyses.

Results

The growth performance of the rainbow trout fed the 3 diets for 4 weeks is shown in Fig. 1 and Table 3. There were no significant differences in body weight, feed conversion efficiency, or liver weight among the 3 diet groups. Dietary soybean PC or soybean PE did not affect growth performance.

There were no significant differences in the total lipid, TG, or phospholipid content of fish fillets at the end of the feeding period among the 3 groups, nor were there significant differences in the PC, PE, tocopherol (Table 4), or the total fatty acid composition (Table 5).

To investigate oxidative stability, fish fillets were subjected to the oxidation test after completion of the feeding experiment. As a parameter of lipid oxidation in fish fillets, the TBARS level was measured before and after the oxidation test. No significant difference in the TBARS level was noted among the 3 groups before the test. After the oxidation

Fig. 1. Growth of rainbow trout during feeding period. Data are represented as mean $\pm$ SD (n=15).

Table 3. Growth and feed performance of rainbow trout after 4-week feeding period.

	Treatment group		
	Control	S-PC	S-PE
Initial body weight (g)	2.0 $\pm$ 0.1	2.1 $\pm$ 0.1	2.0 $\pm$ 0.1
Final body weight (g)	6.5 $\pm$ 1.1	6.4 $\pm$ 0.9	6.1 $\pm$ 1.3
Feed conversion efficiency (%)	136	132	124
Liver weight (g)	0.14 $\pm$ 0.04	0.14 $\pm$ 0.05	0.15 $\pm$ 0.04

Data are represented as mean $\pm$ SD, n=15.

Table 4. Fillet total lipid, triacylglycerol, phospholipid, and total tocopherol contents at the end of feeding.

	Treatment group		
	Control	S-PC	S-PE
Total lipids (g/100g)	5.6 ± 1.2	5.6 ± 0.4	5.5 ± 1.0
Triacylglycerol (g/100g)	3.6 ± 0.5	3.5 ± 0.5	4.0 ± 0.5
Phospholipids (g/100g)	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
Phosphatidylcholine (g/100g)	0.74 ± 0.14	0.75 ± 0.03	0.72 ± 0.19
Phosphatidylethanolamine (g/100g)	0.25 ± 0.12	0.27 ± 0.10	0.31 ± 0.03
Total tocopherol (µg/g)	25 ± 5	24 ± 3	24 ± 3

Data are represented as mean ± SD, n=5.

Table 5. Fatty acid composition of the fillet at the end of feeding.

Fatty acids	Treatment group		
	Control	S-PC	S-PE
	(g/100g)		
14:0	4.0 ± 0.3	4.1 ± 0.2	3.7 ± 0.2
16:0	21.2 ± 1.4	21.7 ± 1.6	21.7 ± 0.7
16:1	7.2 ± 1.0	7.4 ± 1.1	7.5 ± 1.1
18:0	5.0 ± 0.2	4.3 ± 2.1	4.9 ± 0.4
18:1	26.9 ± 1.1	26.6 ± 2.6	25.4 ± 0.8
18:2 (n-6)	11.4 ± 0.5	9.2 ± 0.8	10.9 ± 0.9
18:3 (n-6)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
18:3 (n-3)	1.3 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
20:1	6.1 ± 0.7	6.3 ± 0.5	5.8 ± 0.4
20:2 (n-6)	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
20:3 (n-6)	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
20:4 (n-6)	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.1
22:1	3.6 ± 0.2	3.9 ± 0.4	3.4 ± 0.2
20:5 (n-3)	2.4 ± 0.2	2.8 ± 0.3	2.6 ± 0.3
24:0	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.0
24:1	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.1
22:5 (n-3)	0.6 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
22:6 (n-3)	7.4 ± 1.0	8.5 ± 1.6	8.9 ± 1.6

Data are represented as mean ± SD, n=5.

test, the TBARS level was increased to more than double in all groups, and the level was significantly lower in the S-PC (120.9 nmol / g) and S-PE (111.2 nmol / g) groups than in the Control group (157.4 nmol / g) (Fig. 2).

The fatty acid composition of the fish fillet phospholipids was analyzed after completion of the feeding experiment

Fig. 2. Effects of dietary phospholipids on thiobarbituric acid reactive substances (TBARS) in rainbow trout fillets before and after the oxidation test. Data are represented as mean ± SD (n=8). Letters indicate statistically significant differences ($P < 0.05$).

(Table 6). The analysis revealed that the ratios of 14:0, 16:0, 22:1 and 24:1 in the S-PE group were significantly lower than those of the control group, and 18:1 in the S-PC and S-PE groups were significantly lower than that of the control group. The ratios of 20:5 and 22:5 in the S-PC and S-PE groups were significantly higher than in the control group, and 22:6 in the S-PE group was higher than in the control group. The ratios of saturated and monounsaturated fatty acids were high in the Control group, while the ratio of PUFAs was high in the S-PC and S-PE groups. The ratios of n-3 PUFAs were especially high in the S-PC and S-PE groups. However, the ratios of n-6 PUFAs were not different. No significant differences were present in any fatty acid between the S-PC and S-PE groups.

Fatty acids bound to PC and PE were analyzed. Signifi-

Table 6. Fatty acid composition of the fillet phospholipids at the end of the feeding.

	Treatment group		
	Control	S-PC	S-PE
	(g/100g)		
14:0	3.3 ± 0.2	3.3 ± 0.5 ^a	2.8 ± 0.4 ^b
15:0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
16:0	31.0 ± 3.0 ^a	27.9 ± 3.7 ^{ab}	25.6 ± 1.9 ^b
16:1	3.7 ± 1.4	3.9 ± 0.8	4.0 ± 0.5
17:0	0.4 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
18:0	8.0 ± 0.9	7.5 ± 0.6	6.0 ± 0.3
18:1	20.1 ± 1.1 ^a	18.5 ± 1.0 ^b	17.4 ± 1.6 ^b
18:2 (n-6)	6.5 ± 0.6	7.0 ± 0.5	6.3 ± 0.2
18:3 (n-6)	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
18:3 (n-3)	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
20:1	2.6 ± 0.1	2.6 ± 0.1	2.3 ± 0.3
20:2 (n-6)	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0
20:3 (n-6)	0.6 ± 0.3	0.8 ± 0.1	0.7 ± 0.1
20:4 (n-6)	1.0 ± 0.1	1.0 ± 0.3	1.2 ± 0.2
20:3 (n-3)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:1	1.3 ± 0.1 ^a	1.4 ± 0.1 ^a	1.2 ± 0.2 ^b
20:5 (n-3)	2.8 ± 0.8 ^a	3.9 ± 0.8 ^b	4.4 ± 0.7 ^b
24:1	0.8 ± 0.1 ^a	0.5 ± 0.3 ^{ab}	0.6 ± 0.0 ^b
22:5 (n-3)	0.9 ± 0.2 ^a	1.1 ± 0.2 ^b	1.2 ± 0.1 ^b
22:6 (n-3)	13.2 ± 3.7 ^a	16.1 ± 4.9 ^{ab}	22.1 ± 3.2 ^b

Data are represented as mean ± SD, n=5. Letters indicate statistically significant differences ($P < 0.05$).

cant differences were noted in the fatty acid composition of phospholipids, and we focused on the highly abundant fatty acids (16:0, 18:1, 20:5, and 22:6). To identify the binding positions of these fatty acids, *sn*-1 and *sn*-2 positions were individually analyzed. More than half of the *sn*-1 positions in fish fillet PC were bound by 16:0 fatty acid, with no significant difference among the diet groups. Significant differences were noted in the 20:5 and 22:6 fatty acid compositions, but their binding rates were extremely low (Fig. 3A). The *sn*-2 positions in PC were bound by 18:1, 20:5 and 22:6 at a high rate (Fig. 3B). The 20:5 and 22:6 rates were higher in the S-PC and S-PE groups than in the Control group. The 18:1 rate was lower in the S-PE than in the Control group. Regarding *sn*-1 positions in fish fillet PE, a significant difference was noted only in the rate of 20:5 bound to *sn*-1 position in PE between the Control and S-PC groups (Fig. 4A).

At the *sn*-2 positions in PE, the 18:1-bound rate was lower in the S-PC and S-PE groups than in the Control group, while the 22:6-bound rate was higher than in the Control group (Fig. 4B).

Discussion

This study showed that the main components of soybean phospholipids, soybean PC and soybean PE, increased the storage stability of fish fillets. When fish were fed soybean phospholipids, the rates of DHA bound to PC and PE increased in fish fillets.

Soybean phospholipids are known to exhibit an ex-vivo antioxidative effect on oil and fat. Zambiasi and Pryzybylski (1998) reported that the addition of 3% or more phospholipids to vegetable oil was effective. Judde *et al.* (2003) showed that the addition of 1% soybean phospholipids inhibited oxidation in various oils, such as rapeseed, sunflower, soybean, walnut, palm, and fish oil, by measuring rancimat induction time (110°C) and peroxide value (40°C). The antioxidative mechanisms of phospholipids have been proposed to be due to the amino function of PC, PE and PS, which have been shown to have metal-chelating properties (Judde *et al.*, 2003). Kashima *et al.* (1991) and Hamilton *et al.* (1998) reported that soybean PC and soybean PE increased the oxidative stability of soybean oil. Takeuchi *et al.* (1997) reported that the addition of soybean PE synergistically inhibited oxidation of n-3 PUFA with tocopherol. Recently, we reported that the oral administration of soybean phospholipids increased the oxidative stability of the meat in administered animals (Murano *et al.*, 2008).

The oxidative stability of muscle food varies depending on the fatty acid composition and content of antioxidative substances. Morrissey *et al.* (1998) reported that oxidative stability was high when the PUFA content of meat was low. However, there was no significant difference in the fatty acid composition of fish fillets among the groups after the feeding period. The increased storage stability of meat with a high tocopherol or phospholipid content has been reported (Kim *et al.*, 2006; Yamaguchi and Toyomizu, 1984), but no significant difference was noted in the tocopherol and phospholipid content. Based on these findings, enhanced fish fillet storage stability by dietary soybean phospholipids cannot be explained by the reduction in the PUFA content of fish fillets or increases in the tocopherol and phospholipid contents.

We analyzed the fatty acid composition of fish fillet phospholipids and identified differences in the composition of fatty acids, such as 16:0, 18:1, 20:5, and 22:6. Thus, we analyzed these fatty acid compositions in the main phospholipid species, PC and PE, and found that the 20:5 and 22:6 contents were high in the S-PC and S-PE groups. We also

Fig. 3. Fatty acid composition of phosphatidylcholine at the *sn*-1(A) and *sn*-2 (B) positions in the fillets at the end of feeding. Data are represented as mean $\pm$ SD ($n=5$). Letters indicate statistically significant differences ($P < 0.05$).

analyzed the amount of fatty acids bound to *sn*-1 and *sn*-2 positions in PC and PE. In both PC and PE, the *sn*-2-bound 22:6 content was significantly higher in the S-PC and S-PE groups. PC and PE are the main biomembrane components, and the increased oxidative stability of biomembranes by DHA-bound phospholipids has been reported. Lyberg *et al.* (2005) and Vikbjerg *et al.* (2007) showed, using a biomembrane model, that the storage stability of DHA bound to *sn*-2 positions of PC was increased. It has been clarified that PC containing DHA at *sn*-2 positions shows a variety of morphologies in the lipid bilayer, and is more dynamic than other fatty acid residues. Gawrisch *et al.* (2003) reported that DHA chains bend, tilt and back-fold. Therefore, DHA chains in membranes are, on average, much shorter

Fig. 4. Fatty acid composition of phosphatidylethanolamine at the *sn*-1 (A) and *sn*-2 (B) positions in the fillets at the end of feeding. Data are represented as mean $\pm$ SD ($n=5$). Letters indicate statistically significant differences ($P < 0.05$).

than DHA chains in extended conformations such as angle-ion or helical. Feller *et al.* (2001) reported that the presence of polyunsaturation in a hydrocarbon chain intrinsically increases the amount of conformational space accessible about the central methylene group as compared to saturated hydrocarbon chains. They showed a preferred model of extreme flexibility for the DHA chain, as opposed to one rigid structure or a few selected structures. The polyunsaturated chains rapidly convert between a large number of conformers when located in the lipid matrix. Accordingly, a gap is produced in the membrane, and a water molecule can easily enter near the double bond of DHA (Huster *et al.*, 1997; Saiz and Klein, 2001). When a water molecule is present near this double bond, chain oxidation reactions may not easily occur

because of steric interference by the water molecule (Miyashita, 2006). Regarding PE, the increased oxidative stability of a bilayer by DHA-bound PE has been reported. Lyberg *et al.* (2005) showed, using an emulsion system, that oxidative stability was higher when PE was bound by DHA than by other fatty acids. In the S-PC and S-PE groups, the oxidative stability of the biomembrane may be increased, which may have maintained the biomembrane function and antioxidative defense system, increasing the storage stability of fish fillets. Biomembrane deterioration is considered to be a general mechanism that markedly reduces the quality of muscle food (Stanley, 1991). N-3 PUFA contained in biomembranes are easily affected by radicals, and which triggers lipid peroxide production. The lipid peroxide produced induces further oxidation of membrane lipids, facilitating the deterioration of meat quality, as characterized by odor production, discoloration, and impaired texture (Stanley, 1991).

Takenaka *et al.* (2007) reported on the effect of PE, having saturated and unsaturated fatty acids, on lipid oxidation. They demonstrated that under the presence of α -tocopherol, unsaturated PE has a synergistic effect. In the present study, the TG oxidation in fish fillets might be inhibited by the synergistic effect of unsaturated PE in combination with α -tocopherol. Therefore, it would be of interest to investigate extracted lipids from fish fillets administered soybean phospholipids.

The PC- and PE-bound EPA contents were also increased by dietary high-purity phospholipids. Since only limited studies on the oxidative stability of EPA-bound phospholipids have been performed, the degree of contribution of an increase in the phospholipid-bound EPA content on the storage stability of fish fillets is unclear. The fatty acid composition in fish fillet total lipids was not affected by dietary high-purity phospholipids, and this was not contradictory to the findings of the previous report (Murano *et al.*, 2008). Feeding phospholipids increased the PC- and PE-bound DHA and EPA contents of fish fillets, and these were not due to the dietary fatty acid compositions, because the DHA and EPA contents of the diets were equivalent.

To identify the active component of soybean phospholipids involved in improving the storage stability of fish fillets, rainbow trout were fed purified diets containing 1% soybean PC and soybean PE with purities greater than 99% and 98%, respectively, for 4 weeks. The storage stability of fillets from fish fed the diets containing high-purity phospholipids was greater than that of the Control group; showing that both soybean PC and soybean PE were components that increased storage stability. In fillets of fish fed the high-purity soybean PC and PE, DHA-binding to PC and PE, at the *sn*-2 position was significantly increased. These results indicate that the

high phospholipid-bound DHA content has been one factor leading to the increased storage stability of fish fillets.

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