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Separation of Sardine Oil without Heating from Surimi Waste and Its Effect on Lipid Metabolism in Rats

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Sardine oil was obtained by centrifugation of surimi wastewater without heating or chemical refining. This oil (CE) showed light yellow color and the peroxide value was less than 1.0 meq/kg. The main lipid class of CE was triacylglycerol (TG) (>99%). These features indicate that CE can be directly used as food materials without further purification. Commercial sardine oil (CO) is usually prepared via some kind of refining process with high temperature (250 °C) and chemical treatment. The comparative study on the physiological effects of these sardine oils (CE and CO) revealed that the dietary sardine oils were more effective in reducing abdominal fat pads, plasma total cholesterol, and TG levels of rats than was a soybean oil diet (control). Furthermore, these effects were greater in CE than CO, although there was little difference in the fatty acid composition of both oils. Although the main lipid class of CE was TG (>99%), CE was prepared by centrifugation from surimi waste and directly used as dietary fat without further purification. Therefore, CE may contain some kinds of minor components, which could be attributed to the higher physiological activity of CE. To reveal the involvement of the minor compounds in CE, we prepared TG from CE by column chromatography and measured its effect on lipid metabolism of rats. TG from CE also showed the reducing effects on abdominal fad pads and plasma lipid levels. The effect of TG from CE was almost the same as that of original CE, suggesting that the higher nutritional activity of CE than CO may not be due to the minor compounds in CE.

KEYWORDS: Fish oil; nonheating separation; EPA; DHA; anti-obesity effect; hypolipidemic effect

INTRODUCTION

Eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are the predominant long-chain n-3 polyunsaturated fatty acids (PUFA) found in fish oils. These n-3 PUFA are effective in lowering blood pressure, reducing hyperlipidemia and arrhythmias, preventing arterial thrombosis and cardiovascular disease, and are antiinflammatory, anticancer, and anti-obesity (*1*). However, the intakes of EPA and DHA in Western countries such as USA and UK are below the lowest of the recommended intakes. Therefore, there is a need for fish oils in functional foods or nutraceuticals.

For the separation of fish oil from oily fish bodies, cooking is first carried out. The main objective of cooking is protein denaturation to achieve release of oil and water. The species of fish, size, condition, and oil content are representative of factors that determine the cooking conditions. Typical cooking conditions are a cooking timer of 15 min, during which temperature is raised to 90 °C. After cooking, the oils are drained by press through a strainer. In Japan, cooking with boiling water is carried out. The raw material is placed into boiling water in an iron pan. The oil separated from fish floats to the surface and is removed from the pan. After boiling, the fish are pressed to separate the remaining oil. The crude fish oil production is completed by oil and stick−water separation in desludging or self-cleaning centrifuges.

The color of the crude oil is black and still contains a large amount of impurities. Therefore, further refining is needed for edible and industrial uses. The refining process involves degumming, neutralization (alkaline refining), drying, bleaching, deodorization and distillation. These processes reduce the impurities such as phosopholipids, sugars, proteinaceous compounds, free fatty acids, pigments, water, etc. However, the high temperature (250 °C) and chemical treatment during the refining step may cause isomerization of highy unsaturated PUFA such

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as EPA and DHA to produce trans-isomers and result in the decrease in the nutritional value of these PUFA.

In this study, we report the new method for the fish oil separation from wastewater of surimi production without heating and chemical refining. Surimi is a basic food material in Japan for many kinds of seafood products. Surimi is usually prepared as follows; after separating the fish flesh from bones, fin, skin, and viscera, the fillets are minced. Following mincing, the meat particles are thoroughly leached in freshwater. The purpose of leaching is the removal from the minced meat of water soluble matter, lipids, and blood to improve the color and flavor as well as to increase the gel strength of the surimi. The greatests quantities of surimi are manufactured from Alaska Pollock, but surimi can be also produced from fatty fish species such as sardine and mackerel (*2*). In this case, lipid is present at high levels in the mince water and leach water. This wastewater may be good source for fish oil production. However, there has been no attempt to use the fish oil separated from surimi wastewater. The present study describes the chemical and nutritional properties of the sardine oil from the surimi waste.

MATERIALS AND METHODS

Samples and Chemicals. Japanese anchovy (*Engraulis japonicus*) was caught on December, 2002, off the coast of Choshi, Japan. The raw material was frozen as soon as possible after catching and stored until analysis. Commercial sardine oil prepared from anchovy was obtained from a Japanese fish company (Nippon Chemical Feed Ltd., Hakodate, Japan). Soybean oil was obtained from Wako Pure Chemical Ind., Osaka, Japan. All organic solvents (analytical grade) and acetic acid were obtained from Wako Pure Chemical Ind.. BF_3 methanol solution, activated carbon, and Celite 545 were obtained from Nacalai Tesque Inc., Kyoto Japan.

Lipid Separation from Sardine Surimi Waste. A block of frozen fish (15 kg) was smashed, and the particles were leached in freshwater below 8 °C. The material is decanted and dewatered through screens. The leached mince was strained to produce 5.9−6.5 kg sardine surimi. For each block of frozen fish, 80−90 L of wastewater was obtained. The total wastewater was centrifuged continuously at 7370*g* and 9940*g* by two types of three-phase nozzle centrifuge (Alpha-Laval Co., Tokyo, Japan) at 8−15 °C. Sludge, oil phase, and water phase was separated with this machine and discharged through each nozzle. The oil phase was dewatered at 15500G at 10−17 °C by high-speed continuous centrifuge (Alpha-Laval Co., Tokyo, Japan). In addition, the sardine oil was extracted from the wastewater or from the fish body with chloroform/methanol (2:1, v/v) (*3*). When the oil was extracted with organic solvent from the fish body, the body was cut and homogenized in the chloroform/methanol solution.

Lipid Analysis. Lipid profile of commercial sardine oil (CO), sardine oil obtained by centrifugation method (CE), and sardine oil obtained by solvent extraction was checked by thin-layer chromatography (TLC) (*4*). TLC was carried out on 0.25-mm silica gel plates (Merck, Darmstadt, Germany) developed with chloroform/methanol/water (25:10:1, v/v/v) or with *n*-hexane/diethyl ether (70:30, v/v). The lipid composition was determined quantitatively by TLC-flame ionization detector (FID) on Chromarod S−III using an Iatroscan TH-10 (Iatron, Tokyo, Japan) equipped with a Shimadzu C−R6A integrator (Shimadzu Seisakusho Co., Kyoto, Japan). Chromarods (Iatron, Tokyo, Japan) were activated by passing them through the FID scanner, and 1 μL of the sample solution was applied on each rod. The rods were developed 10 cm from the origin with chloroform/methanol/water (65:25:4, v/v/v) or *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v). After developing the rods were dried in a desiccator for a few minutes and then scanned by the Iatroscan. The fatty acid composition of sample oil was determined by gas chromatography (GC) after conversion of fatty acyl groups in the oil to their methyl esters by heating in a sealed tube at 90−100 °C for 1 h with 7% BF_3 methanol under nitrogen. The methyl ester obtained by the transesterification was purified on a silicic acid column (Silica gel 60; Merck, Darmstadt, Germany) by eluting with *n*-hexane and a mixture of *n*-hexane/diethyl ether (95:5, v/v/v). The methyl ester eluted with *n*-hexane/diethyl ether (95:5, v/v) was subjected to GC. GC analysis was performed on a Shimadzu GC-14B (Shimadzu Seisakusho, Kyoto, Japan) equipped with an FID and a capillary column (Omegawax 320 (30-m × 0.32-mm i.d.); Supelco, Bellefonte, PA). The peroxide value of each oil sample was determined by the AOCS Official Method (*5*).

Preparation of Triacylglycerol (TG) of Sardine Oil from Surimi Waste. Sardine oil from surimi waste (CE) was passed through a column packed with a 1:1 *n*-hexane slurry mixture (w/w) of activated carbon and Celite 545 to remove tocopherols and pigments. The recovered oil was refined on a silicic acid column (Silicagel 60, Merck) by eluting with *n*-hexane and a mixture of *n*-hexane/diethyl ether solution (95:5, 90:10, and 80:20, v/v). TG fraction eluted with *n*-hexane/diethyl ether (90:10, v/v) was used for the present study as TG from CE. The purified oil sample gave only a single spot corresponding to TG on the TLC with normal-phase silica plates (Merck) developed with *n*-hexane/diethyl ether/acetic acid (60:40:1, v/v/v).

Animals and Diets. Male Wistar rats (4 weeks of age, mean body weight 62 g) were purchased from Japan CREA Co., Tokyo, Japan. They were housed at a constant humidity (55%) and temperature (20 ± 1 °C) with a 12 h light/dark cycle throughout the experiment. After acclimation for 1 week by feeding control diets, rats were randomly divided into three groups of 7 or 5 rats and given free access to the water and the experimental diet. The diet was prepared according to the recommendation of the American Institute of Nutrition (AIN-93G) (*6*). Thus, the control diet contained the following ingredients, in % by weight diet, cornstarch, 39.8; casein, 20.0; dextrinized cornstarch, 13.2; sucrose, 10.0; soybean oil, 7.0; AIN-93 mineral mixture, 3.5; AIN-93 vitamin mixture, 1.0; L-cystine, 0.3; choline bitartrate, 0.25; cellulose, 5.0; *tert*-butylhydroquinone, 0.002. When the diet was supplemented with sardine oil, this oil was added at the expense of soybean oil in the AIN-93G diet to give a final dietary fat mixture 3.0% sardine oil and 4.0% soybean oil. The study protocol was approved by the committee of the Kansai Medical University.

Sample Collections. After 4 weeks of feeding, the animals were starved for one night and anesthetized with diethyl ether. Rats were killed by exsanguination, and their blood was withdrawn at the abdominal artery. The liver was then excised and weighed. The liver was perfused with 0.9% NaCl, and liver lipids were extracted with chloroform/methanol (2:1, v/v) as described previously by Folch et al. (*7*). The abdominal fat pads composed of epididymal adipose tissues were also excised and weighed.

Analysis of Plasma Lipids. Total cholesterol (*8*), high-density lipoprotein (HDL) cholesterol (*9*), free cholesterol (*8*), TG (*10*), and phospholipids (*11*) of plasma were analyzed according to the standard methods.

Statistical Analysis. The results were expressed as means ± SE. ANOVA was used to test for significant difference between control and sardine oil administered rats.

RESULTS AND DISCUSSION

The oil extracted with organic solvent from the fish body was 7.0 g/100 g wet weight. Therefore, 1050 g of oil should be contained in a block of frozen fish (15kg). On the other hand, more than 80 L of surimi wastewater was obtained from the one block of frozen fish, and the oil (>4.5 g) was obtained by centrifugation method from the wastewater (1 L). This means that more than 360 g of oil (>34% of fish oil) have be recovered from the one block of frozen fish by the centrifugation method from surimi wastewater. Furthermore, the oil obtained by solvent (chloroform/methanol) extraction from the water (1L) was 5.7 g, showing that more than 70% of oil could be recovered from surimi wastewater by the continuous centrifugation method. CE showed light yellow color. The peroxide value of CE depended on the storage time of the frozen fish. If the storage time was less than 1 month, the peroxide value was less than 1.0 meq/kg. This indicates that the sardine oil can be directly applied as

Table 1. Fatty Acid Profile of Soybean Oil and Sardine Oil

		sardine oil			
				solvent extraction	
	soybean oil (control)	CO (heat)	CE (non-heat)	from fish body	from waste water
14:0	ND	8.2	6.9	8.0	7.5
16:0	10.5	16.7	15.9	18.7	15.7
18:0	3.8	3.2	2.8	3.4	2.9
16:1n-7	ND	8.4	7.8	8.8	7.7
18:1n-7	1.4	3.1	2.6	3.1	2.3
18:1n-9	24.4	9.6	8.9	9.9	8.7
18:2n-6	52.6	0.9	1.0	0.7	1.1
18:3n-3	5.6	0.5	1.0	0.5	1.1
18:4n-3	ND	2.2	2.4	2.0	2.1
20:5n-3	ND	16.3	15.9	14.4	16.0
20:5n-3	ND	2.1	3.2	2.0	3.0
22:6n-3	ND	10.7	11.6	12.5	10.9

Table 2. Final Body Weight, Liver Weight, and Fat Pad Weight of Rats Fed Two Kinds of Sardine Oils (CO and CE)[a]

	group		
	control	CO	CE
body weight (g)	232.2 ± 7.6	240.6 ± 7.6	238.7 ± 4.0
liver weight (g)	9.58 ± 0.35	9.37 ± 0.51	9.91 ± 0.58
liver/body wt (g/100 g of BW)	4.17 ± 0.26	3.89 ± 0.14	4.14 ± 0.20
abdominal fat pad weight (g)[b]	2.61 ± 0.27	2.34 ± 0.23	1.99 ± 0.11
fat pad/body wt (g/100 g of BW)	1.14 ± 0.13	0.97 ± 0.08	0.83 ± 0.04[c]

[a] The values are mean ± SE for seven rats. [b] Abdominal fat pads are made up of epididymal and perirenal adipose tissues. [c] Significantly different from control ($P < 0.05$).

food materials without further purification, although commercial fish oils are usually prepared via a number of purification steps from crude oils obtained by the cooking of fish.

TLC−FID analysis showed that oil obtained by centrifugation and by solvent extraction of wastewater mainly consisted of TG (>99%) with small amounts of free fatty acids, sterols, and phospholipids. The oil from the whole body consisted of TG (85%), phospholipids (13.5%), and free fatty acid (1%). This result indicates that only TG passed into the leached water in the process of surimi production. Fatty acid composition of CE is shown in **Table 1**. The main fatty acids of CE were EPA (20:5n-3, 15.9%), palmitic acid (16:0, 15.9%), and DHA (22:6n-3, 11.6%). The fatty acid composition was almost the same as that of the sardine oil obtained by solvent extraction from the wastewater and whole fish body (**Table 1**).

Table 2 shows the final body weight, liver weight, and fat pad weight of the rats fed the control and two kinds of sardine oils (CO and CE). The peroxide value of both oils was less than 1.0 meq/kg. Fatty acid composition of the oils was shown in **Table 1**. All animals remained healthy through the experimental period. The body weight and liver weight of the rats did not differ between the animals. On the other hand, fat pad weight and fat pad/body weight (BW) of the CE diet-fed rats was significantly lower than that of the control-fed rats.

Parrish et al. (*12*) reported that lard-fed rats had 77% more fat in perirenal fat pads and 51% more fat in epididymal fat pads compared with fish oil-fed rats. The same result was obtained by Kawada et al. (*13*). They also found that the expression of uncoupling protein (UCP) in interscapular brown adipose tissue (BAT) was significantly higher in the fish oil diet-fed rats compared to that in the lard-fed group. In BAT mitochondria, substrate oxidation is poorly coupled to ATP synthesis because of the presence of UCP, thereby leading to energy dissipation, that is, heat production. Kawada et al. (*13*) suggested that the intake of PUFA found in fish oil such as EPA and DHA causes UCP induction and enhancement of thermogenesis, resulting in suppression of the excessive growth of abdominal fat pads. PUFA from vegetable oils also suppressed the excessive accumulation of adipose tissue, as compared to animal fats (*14*, *15*). However, the activity of PUFA from vegetable oils was less than that of EPA and DHA from fish oil (*13*). Our present study supports these previous results in terms of the higher anti-obesity effect of EPA and DHA in fish oils (**Table 2**).

Table 3. Effects of Experimental Diets on the concentration of Plasma Lipids[a]

	group		
	control	CO	CE
total cholesterol (mg/dL)	68.9 ± 5.2	54.3 ± 2.1[b]	48.1 ± 2.7[c,e]
HDL cholesterol (mg/dl)	29.9 ± 1.9	30.1 ± 0.9	26.1 ± 1.3
free cholesterol (mg/dL)	16.6 ± 1.3	14.0 ± 0.7	13.1 ± 0.8[b]
TG (mg/dL)	99.4 ± 19.1	59.3 ± 9.8	41.9 ± 7.4[b]
phospholipids (mg/dL)	135.9 ± 10.1	112.3 ± 3.2[b]	95.0 ± 4.6[c,e]
total lipids (mg/dL)	338.7 ± 30.7	253.3 ± 13.7[b]	209.4 ± 14.4[c,d]

[a] The values are mean ± SE for seven rats. [b,c] Significantly different from control (b, $P < 0.05$; c, $P < 0.01$). [d,e] Significantly different from CO (d, $P < 0.05$; e, $P < 0.01$).

As shown in **Table 2**, abdominal fat pad/BW of rats fed CO diet was lower than that of rats fed control, but not significantly. On the other hand, CE diet significantly reduced the fat pad accumulation of the rats as compared with the control, although there was little difference in the fatty acid composition of CO and CE (**Table 1**). Higher activity of CE than CO was also observed in the reducing plasma lipid levels (**Table 3**). Total cholesterol, phospholipid, and total lipid levels significantly decreased by feeding two kinds of sardine oils, as compared with control diets. This effect was recognized more significantly in CE diet. TG level in the CE-diet-fed rats was significantly lower than that of control. The level in the CO diet-fed rats was also lower than that of control, but not significantly. The effects of fish oils on plasma lipid levels are known to be due to the n-3 PUFA in the fish oils (*1*). The lowering effect of CO and CE on the plasma cholesterol and TG levels would be also due to EPA and DHA in these oils. However, the difference in the effect of CO and CE could not be explained by the contents of EPA and DHA (**Table 1**).

Although the main lipid class of CE was TG (>99%), CE was prepared by centrifugation from surimi waste and directly used as dietary fat without further purification, while CO was obtained after some refining steps. Therefore, CE may contain some kinds of minor components not in CO, which could be attributed to the higher physiological activity of CE. To reveal the involvement of the minor components in CE, we prepared TG from CE by two kinds of choromatographic techniques and analyzed its effects on body weight, liver weight, fat pad weight (**Table 4**), and plasma lipid levels (**Table 5**).

As shown in **Table 4**, fat pad/BW of CE and TG from CE-fed rats was lower than that of control-fed rats, although the differences were not significant. On the other hand, the difference in the fat pad/BW of CO-diet-fed rats and control-fed rats was small. Total cholesterol of plasma of three kinds of sardine oil (CO, CE, and TG from CE)-fed rats was significantly lower than that of control-fed rats (**Table 5**). This difference was observed more significantly in CE diet and TG from CE diet than it was in CO diet. TG level of plasma of

Table 4. Final Body Weight, Liver Weight, Liver Lipid Weight, Fat Pad Weight of Rats Fed Two Kinds of Sardine Oils (CO and CE) and TG from CE[a]

	group			
	control	CO	CE	TG from CE
body weight (g)	255.0 ± 4.9	265.0 ± 2.7	257.2 ± 2.4	261.7 ± 4.5
liver weight (g)	9.76 ± 0.50	10.43 ± 0.26	8.95 ± 0.52	8.25 ± 0.91
liver/body weight (g/100g of BW)	3.84 ± 0.24	3.67 ± 0.31	3.48 ± 0.23	3.13 ± 0.35
liver lipid/liver weight (mg/10g of LW	8.49 ± 0.66	8.63 ± 0.96	7.64 ± 0.58	7.39 ± 0.46
abdominal fat pad weight (g)[b]	4.66 ± 0.36	4.50 ± 0.21	3.25 ± 0.24[c]	3.44 ± 0.46
fat pad/body weight (g/100g of BW)	2.10 ± 0.31	1.95 ± 0.26	1.26 ± 0.08	1.30 ± 0.16

[a] The values are mean ± SE for five rats. [b] Abdominal fat pads are made up of epididymal and perirenal adipose tissues. [c] Significantly different from control ($P < 0.05$).

Table 5. Effects of Experimental Diets on the Concentration of Plasma Lipids[a]

	group			
	control	CO	CE	TG from CE
total cholesterol (mg/dL)	82.6 ± 7.2	66.7 ± 2.8[b]	59.4 ± 4.7[c]	61.0 ± 4.2[c]
HDL cholesterol (mg/dL)	34.0 ± 2.6	36.9 ± 2.0	34.1 ± 2.6	33.9 ± 2.1
free cholesterol (mg/dL)	13.4 ± 1.3	10.4 ± 0.8	9.6 ± 1.2b	10.0 ± 1.0[b]
TG (mg/dL)	28.0 ± 7.5	18.0 ± 12.5	14.3 ± 4.0	10.9 ± 2.5
phospholipids (mg/dL)	127.3 ± 17.9	117.9 ± 6.3	92.4 ± 16.3	106.9 ± 6.3
total lipids (mg/dL)	279.3 ± 30.6	252.0 ± 22.8	196.0 ± 25.0[b]	209.4 ± 11.7[c]

[a] The values are mean ± SE for five rats. [b,c] Significantly different from control (*b*, $P < 0.05$; *c*, $P < 0.01$).

these sardine oils-fed rats was lower than that of control-fed rtas, but not significantly, while total lipid level of CE and TG from CE fed-rats was significantly lower than that of control-fed rats. However, there was little difference in these effects between CE and TG from CE diets, showing little relationship of minor components in CE with the higher activity of CE than CO.

CO was prepared from crude sardine oil by refining with high temperature (250 °C) and chemical treatment. These steps may result in the isomerization of highly unsaturated PUFA such as EPA and DHA to produce geometrical and positional isomers. On the other hand, CE was obtained directly from surimi waste without any treatments of heating and chemical procedures, in which the isomerization of EPA and DHA would have scarcely occurred. Although there have been no data on the adverse effects of these isomers derived from EPA and DHA, it may be suggested that these isomers may be formed from EPA and DHA in CO during refining process and may decrease the relative physiological activities of CO, as compared with that of CE.

ABBREVIATIONS USED

ANOVA, analysis of variance; ATP, adenosine 5′-triphosphate; BAT, brown adipose tissue; CE, sardine oil obtained by centrifugation method; CO, commercial sardine oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FID, flame ionization detector; GC, gas chromatography; HDL, high-density lipoprotein; PUFA, polyunsaturated fatty acids; SE, standard error; TG, triacylglycerol; TLC, thin-layer chromatography; UCP, uncoupling protein.

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