Trace Nutrients Research 32 : 20－26 (2015)

Original Article

Lipid Components Prepared from an Oyster-extract By-product Decreases Triacylglycerol Contents by Suppressing Acetyl-CoA Carboxylase Activity and Lowering the Stearoyl-CoA Desaturase Index in Rat Livers

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Summary

In order to clarify the potential of an oyster-extract by-product (OEBP) as a health-maintaining and -promoting food material or supplement, this study investigated the effects of lipid prepared from an OEBP (L-OEBP) on serum and liver lipid contents in rats. L-OEBP was extracted by the methods of hexane/ethanol/water, and contained 3.7% eicosapentaenoic acid (EPA), 3.3% docosahexaenoic acid (DHA), and 17.7% phospholipid. Four-week-old male Wistar rats were divided into 2 dietary groups of 6 rats each, with one group receiving a control AIN-93G diet (7% soybean oil) and the other receiving L-OEBP diet (5% soybean oil + 2% L-OEBP). After the rats had been fed their respective diets for 27 days, their serum and liver lipid contents and hepatic fatty acid-metabolizing enzyme activities were measured. Hepatic triacylglycerol (TAG) and monounsaturated fatty acid contents as well as the stearoyl-CoA desaturase index (oleic acid/stearic acid) were lower in the L-OEBP group than in the control group. Hepatic acetyl-CoA carboxylase activity was also lower in the L-OEBP group than in the control group. Therefore, L-OEBP decreased the liver TAG content in part by suppressing hepatic fatty acid synthesis. Since the current formulation of L-OEBP did not sufficiently lower the serum content of TAG or the amounts of EPA and DHA were low, further improvements are needed in its formulation and concentration of *n*-3 polyunsaturated fatty acid prior to its application as a lipid-lowering functional food and supplement.

Introduction

Oysters (*Crassostrea gigas*) contain many nutrients and are widely consumed in Japan. The main components of oyster extract include zinc, glycogen, and taurine, and oyster-extract powder is currently being marketed as a supplement. The nutritional benefits of oyster extract for health maintenance and lifestyle-related diseases have been reported[1, 2)]. Our previous findings suggested that oyster-extract powder suppressed the initiator action of carcinogens in mice and promoted the recovery of proximal tubular epithelial cell function in *p*-aminophenol-induced nephrotoxicity in rats[3, 4)]. However, waste is produced at a high ratio in the manufacturing process of oyster-extract powder. Waste disposal and by-product management by the food processing industry for environmental protection and sustainability are challenging[5)]. Thereafter, the effective usage of an oyster-extract by-product (OEBP) is expected. By measuring its nutrient components, we found that a high content of protein and lipid remained in OEBP. We previously suggested that dietary OEBP decreased liver cholesterol contents by enhancing fecal cholesterol and bile acid excretion due to the proteins and phospholipid (PL) remaining in this by-product[6)]. However, the lipid-lowering effects of the current OEBP formulation are inadequate for its application as a lipid-lowering functional food or supplement[6)]; therefore, further improvements in the composition and concentra-

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tions of bioactive components in OEBP are needed. In the present study, we extracted the lipid of OEBP, which contains *n*-3 polyunsaturated fatty acid (PUFA) such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and PL. EPA and DHA have been shown to improve several metabolic issues associated with hypertriglyceridemia, blood pressure, platelet aggregation, and inflammation[7]. The daily intake of EPA and DHA is known to reduce the incidence and death from cardiovascular diseases. Therefore, national societies and public organizations have been recommending an intake of 1 g/day of long-chain *n*-3 PUFA for anti-atherosclerotic and anti-arrhythmic purposes or 2–4 g/day for lipid-lowering effects[7]. Furthermore, dietary PL have been shown to have more beneficial effects than dietary triacylglycerol (TAG)[8, 9]. In order to clarify the potential of OEBP as a health-maintaining and -promoting food material or supplement, the effects of lipid prepared from an OEBP (L-OEBP) on serum and liver lipid contents were investigated in rats.

Materials and Methods

1. Preparation of L-OEBP

An oyster (*Crassostrea gigas*) extract by-product, obtained from oyster meat after oyster-extract preparation (extraction methods: hot water and 0.1 N HCl), was obtained in June, 2014 from Japan Clinic Co., Ltd. (Kyoto, Japan). The crude protein content was determined by the Kjeldahl method, with an N-to-protein conversion factor of 6.25. The crude lipid content was measured by the Bligh and Dyer method[10]. The moisture content was estimated as the loss in weight after drying at 105℃ for 24 hours, and the amount of ash was analyzed by direct ignition at 550℃ for 24 hours. The remaining content after the elimination of protein, lipid, ash, and moisture was considered to be carbohydrates.

OEBP was cut into small pieces and then ground using a mill (GM200, Retsch Co., Ltd. Haan, Düsseldorf, Germany). L-OEBP was extracted using the method of hexane/ethanol/water or chloroform/methanol/water[10]. PL contents were measured using a phosphorus analysis[11]. The cholesterol content was analyzed using a gas-liquid chromatography system (GC-14B, Shimadzu Co.) with an SE-30 column (Shinwa Chemical Industries; Kyoto, Japan), in which 5 α-cholestane was used as an internal standard[12]. The TAG content was determined using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries; Osaka, Japan). The composition of fatty acids was determined in an analysis using a fused silica capillary column, Omegawax 250 (Supelco, Pennsylvania, USA) in a gas chromatography (GC) system (GC-2014, Shimadzu Co., Kyoto, Japan) after methylation with boron trifluoride-methanol[13]. The temperatures of injection and detection were 250 and 260℃ , respectively. The initial column temperature of 120℃ was increased to 240℃ at a rate of 2℃/min. The identification of each fatty acid species was conducted using a standard mixture of key fatty acid methyl esters (Supelco® 37 Component FAME Mix; Sigma-Aldrich Japan Co., Ltd.).

2. Animal care and experimental diets

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai University and followed the "Guide for the Care and Use of Experimental Animals" issued by the Prime Minister's Office of Japan. Four-week-old male Wistar rats obtained from Japan SLC, Inc. (Shizuoka, Japan) were kept in an air-conditioned room (temperature, 21–22℃ ; humidity, 55–65%; lights on, 08:00–20:00), with free access to tap water and feed. Twelve rats were divided into the following 2 dietary groups of 6 rats each: the control diet (AIN-93G) and L-OEBP diet groups. The experimental diet was prepared according to the AIN-93G formula[14] and contained 7% soybean oil (Control diet) or 5% soybean oil and 2% L-OEBP (extracted by the method of hexane/ethanol/water, L-OEBP diet), 20% casein, 3.5% AIN-93G mineral mixture, 1% AIN-93 vitamin mixture, 0.25% choline bitartrate, 0.3% L-cystine, 5% cellulose, 10% sucrose, 0.014% *tert*-butylhydroquinone, and 13.2% dextrinized corn starch, and was made up to 100% with corn starch. In this study, we planned that the amount of L-OEBP in experimental diet and the feeding period are often used for measurement of lipid contents in serum and liver[8].

Food consumption and body weights were recorded daily. After feeding for 27 days, rats were weighed and sacrificed under isoflurane (Intervet K.K.; Osaka, Japan) anesthesia between 09:00 and 11:00. Rats were not deprived of food prior to being sacrificed because food deprivation is known to significantly down-regulate the expression of genes involved in fatty acid synthesis and cholesterol metabolism[15]. Blood was collected from the abdominal aorta without the use of anti-coagulants, and serum was obtained by centrifugation at 1,500 × *g* for 15 min. The liver and abdominal white adipose tissue (WAT) from the epididymis, mesentery, perinephria, and retroperitoneum were removed rapidly, weighed, rinsed with saline, and then frozen in liquid nitrogen, followed by storage at −70℃ until later analysis.

3. Lipid analysis

Serum TAG, PL, and cholesterol contents were determined using a commercial service (Japan Medical Laboratory, Osaka, Japan). Briefly, total liver lipids were extracted using the chloroform/methanol/water method as described previously[10]. Each total lipid sample was dissolved in an equal volume of 2-propanol, and the TAG content was determined using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries; Osaka, Japan). Cholesterol and PL contents were determined by GC and phosphorus analyses, respectively, as described previously[11, 12]. The fatty acid composition of total lipids was analyzed with a fused silica capillary column (Omegawax 250, Supelco) after methylation using boron trifluoride-methanol on a GC (Shimadzu Co.), as described previously[13]. The amounts of individual fatty acids in the liver were quantified using tridecanoic acid (C13:0) as an internal standard.

4. Analysis of enzyme activities

The liver was homogenized with 10 volumes of 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA-2Na. The homogenate was centrifuged at 500 × *g* at 4℃ for 10 min, and the supernatant was obtained. The supernatant was recentrifuged at 9,000 × *g* at 4℃ for 10 min to sediment the mitochondria (mitochondrial fraction). The remaining supernatant was then ultracentrifuged at 105,000 × *g* at 4℃ for 60 min, and the supernatant was obtained (cytosolic fraction).

Carnitine palmitoyl transferase (CPT)-2 activity in the mitochondrial fraction was measured by the method of Markwell *et al.*[16]. Acetyl-CoA carboxylase (ACC)[17], fatty acid synthase (FAS)[18], malic enzyme (ME)[19], and glucose-6-phosphate dehydrogenase (G6PDH)[20] activities in the cytosolic fraction were assayed spectrophotometrically. The protein content of each fraction was determined according to the method of Lowry *et al.*[21] using bovine serum albumin as a standard.

5. Statistical analysis

Data represent the means and standard errors of the mean (SEM). The significance of differences was evaluated using the Student's *t*-test and means and were considered significantly different at $p < 0.05$. Analyses were performed using GraphPad Prism6 software (GraphPad Software, California, USA).

Results and Discussion

We previously suggested that OEBP decreased liver cholesterol contents in part by enhancing fecal cholesterol and bile acid excretion[6]. The composition of OEBP is presented in Table 1. OEBP contained high levels of crude proteins (22.6 g/100 g) and crude lipid (9.6 g/100 g). We previously reported that a high content of *n*-3 PUFA and PL remained in OEBP. The present study examined the effects of dietary L-OEBP on lipid metabolism in rats.

The lipid compositions of OEBP extracted by hexane/ethanol/water or chloroform/methanol/water are presented in Table 2. The method of hexane/ethanol/water extracted a lower rate of total lipid and PL than chloroform/methanol/water, but did not affect the cholesterol or TAG content. Hexane is a good solvent of low polarity lipids, and its main use is to extract neutral lipids from mixtures of water with ethanol. Chloroform is also a popular solvent, particularly for intermediate polarity lipids, and, when mixed with methanol, becomes a general extraction solvent. However, chloroform and methanol cannot be used as extract solvents in food sanitation, whereas hexane and ethanol can. Therefore, we used L-OEBP extracted by hexane/ethanol/water in animal experiments. The fatty acid compositions of L-OEBP extracted by hexane/ethanol/water are presented in Table 3. The main fatty acids of L-OEBP were as follows (% w/w): DHA, 3.3%; EPA, 3.7%; palmitic acid, 26.8%; and oleic acid, 1.8%.

Table 1 Chemical compositions of the oyster-extract by-product (g/100 g)

Composition	Oyster-extract by-product[†]
Crude protein[‡]	22.6
Crude lipid	9.6
Moisture	63.7
Ash	0.8
Carbohydrate[§]	3.3

[†] Consisted of oyster meat after oyster-extract preparation. Oyster-extract by-product was obtained in June, 2014 from Japan Clinic Co., Ltd.
[‡] The N-to-protein conversion factor used was 6.25.
[§] Carbohydrate = 100 − (crude protein + crude lipid + ash + moisture).

Table 2 Lipid compositions of the oyster-extract by-product (OEBP) prepared by different extracted methods (g/100 g of OEBP)

	Hexane /ethanol/water	Chloroform /methanol/water
Total lipid	6.2	9.6
PL	1.1	4.5
Cholesterol	0.16	0.17
TAG	4.9	5.0

OEBP, oyster-extract by-product; PL, phospholipid; TAG, triacylglycerol.

Table 4 shows the growth parameters, organ weights, and lipid components of the serum and liver in rats. No significant differences were observed in growth parameters, including the initial body weight, final body weight, body weight gain, food intake, and food efficiency, between the experimental groups. Furthermore, no significant differences were noted in relative liver, epididymal, mesenteric, perirenal, retroperitoneal, or total WAT weights between the groups. Liver TAG contents were significantly lower, while serum TAG contents were slightly lower in the L-OEBP group than in the control group. Liver PL contents were significantly higher in the L-OEBP group than in the control group. The increase in liver PL might be a reflection of the PL content of L-OEBP. No significant differences were observed in serum or liver cholesterol contents between the control and L-OEBP groups. A previous study demonstrated that dietary OEBP reduced cholesterol contents in the liver[6]; however, L-OEBP did not affect serum or liver cholesterol contents in the present study. Serum and liver cholesterol contents were found to be lower with a diet containing PL than with a

Table 3 Fatty acid composition of lipid prepared from an oyster-extract by-product (wt%)

Composition	L-OEBP†
C14:0	5.4
C16:0	26.8
C16:1 *n*-7	0.3
C18:0	6.7
C18:1 *n*-9	1.8
C18:1 *n*-7	4.0
C18:2 *n*-6	12.7
C18:3 *n*-3	0.4
C20:0	3.2
C20:1 *n*-9	5.2
C20:4 *n*-6	2.5
C20:5 *n*-3 (EPA)	3.7
C22:1 *n*-9	7.2
C22:6 *n*-3 (DHA)	3.3
Others	16.6

† Total lipid was extracted by the hexane/ethanol/water method.

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; L-OEBP, lipid prepared from an oyster-extract by-product.

Table 4 Growth parameters, organ weights, and lipid parameters of serum and liver in rats fed experimental diets for 4 weeks

	Control	L-OEBP
Growth parameters		
Initial BW (g)	82.3 ± 1.6	81.3 ± 2.0
Final BW (g)	298.7 ± 7.7	298.8 ± 4.4
BW gain (g/day)	7.7 ± 0.3	7.8 ± 0.2
Food intake (g/day)	17.7 ± 1.6	18.6 ± 1.7
Food efficiency (g/g)†	0.45 ± 0.017	0.432 ± 0.006
Organ weight (g/100g BW)		
Liver	4.36 ± 0.12	4.46 ± 0.13
Epididymal WAT	1.26 ± 0.06	1.37 ± 0.08
Mesentery WAT	1.32 ± 0.11	1.51 ± 0.17
Perirenal and retroperitoneal WAT	1.41 ± 0.06	1.47 ± 0.08
Total WAT‡	3.99 ± 0.16	4.36 ± 0.26
Serum lipids (mg/dL)		
TAG	97.3 ± 14.6	71.7 ± 9.3
PL	162 ± 7	150 ± 6
Cholesterol	92.0 ± 6.5	86.2 ± 3.8
Liver lipids (mg/g liver)		
TAG	34.5 ± 0.4	21.1 ± 0.7**
PL	20.7 ± 0.3	23.0 ± 0.2**
Cholesterol	2.1 ± 0.2	2.1 ± 0.3

Data represent means ± SEM (n = 7). Values were significantly different from the control diet at* $p < 0.05$ and** $p < 0.01$ using the Student's *t*-test.

† Food efficiency (g/g) = BW gain (g/day) / food intake (g/day);

‡ Total WAT reshows the sum of WAT weights from the epididymis, mesentery, perinephria, and retroperitoneum.

BW, body weight; L-OEBP, lipid prepared from an oyster-extract by-product; PL, phospholipid; TAG, triacylglycerol; WAT, white adipose tissue.

diet containing soybean oil alone (AIN-93G), and this was attributed to the enhanced fecal and biliary excretion of cholesterol[8, 22]. Since the L-OEBP diet in the present study contained 2.2 g/kg PL, these PL may have affected cholesterol metabolism, but not cholesterol contents in the serum and liver. One of the reasons why no significant changes were observed in serum and liver cholesterol contents between the two groups may have been because the L-OEBP diet contained approximately 320 mg/kg cholesterol.

The activities of liver fatty acid synthesis and β-oxidation-related enzymes are presented in Table 5. The activity of ACC, which is a key enzyme for malonyl-CoA synthesis, was significantly lower in the L-OEBP group than in the control group. The activity of FAS, which is a rate-limiting enzyme for fatty acid synthesis, was slightly lower in the L-OEBP group ($p = 0.10$). No significant differences were observed in the activity of G6PDH or ME, which are key enzymes in the production of cellular NADPH, between the groups. A previous study reported that dietary *n*-3 PUFA decreased serum TAG contents through lipogenic gene (ACC and FAS) transcription via the suppression of sterol regulatory element binding protein (SREBP)-1c in the liver[23]. Therefore, the TAG-lowering effects induced by *n*-3 PUFA in L-OEBP may have affected the activities of enzymes related to fatty acid synthesis. The intake of *n*-3 PUFA has been shown to enhance the expression of the peroxisome proliferator-activated receptor (PPAR)-α, which is regulated by acyl-CoA oxidase and CPT-1 genes[24]. The activity of CPT-2, a key enzyme in fatty acid β-oxidation in mitochondria, was significantly lower in the L-OEBP group than in the control group. Previous studies reported that the activities of CPT-1 and CPT-2 in the liver did not show parallel changes[24, 25]. Furthermore, malonyl-CoA, which is synthesized by ACC and a natural inhibitor of CPT-1, inhibited the transfer of long-chain fatty acids into the mitochondria via CPT-1[26]. The L-OEBP diet may have activated CPT-1 in the liver due to the suppression of ACC activity. Further research is needed in order to clarify the effects of OEBP on hepatic CPT-1 activity with or without malonyl-CoA.

Table 5 Enzyme activities related to fatty acid metabolism in rats fed experimental diets for 4 weeks (nmol/min/mg protein)

	Control	L-OEBP
FAS	6.6 ± 1.3	4.1 ± 0.5
ACC	390 ± 21	290 ± 19**
G6PDH	145 ± 26	114 ± 28
ME	10.1 ± 0.9	10.2 ± 1.8
CPT-2	2.13 ± 0.15	1.64 ± 0.13*

Data represent means ± SEM (n = 7). Values were significantly different from the control diet at *$p < 0.05$ and **$p < 0.01$ using the Student's *t*-test.

ACC, acetyl-CoA carboxylase; CPT-2, carnitine palmitoyl transferase-2; FAS, fatty acid synthase; G6PDH, glucose-6-phosphate dehydrogenase; L-OEBP, lipid prepared from an oyster-extract by-product; ME, malic enzyme.

The amounts of hepatic fatty acid (A) and fatty acid desaturase indices (B, C16:1 *n*-7/C16:0; C, C18:1 *n*-9/C18:0) are presented in Figure 1. A high monounsaturated fatty acid (MUFA) content has been associated with various disease states, including obesity, diabetes, and cardiovascular disease[27]. The amounts of C16:0, C16:1 *n*-7, C18:0, C18:1 *n*-9, C18:1 *n*-7, C18:2 *n*-6, C18:3 *n*-3, and C20:4 *n*-6 were significantly lower in the L-OEBP group than in the control group. No significant differences were observed in the amount of DHA between the L-OEBP and control groups

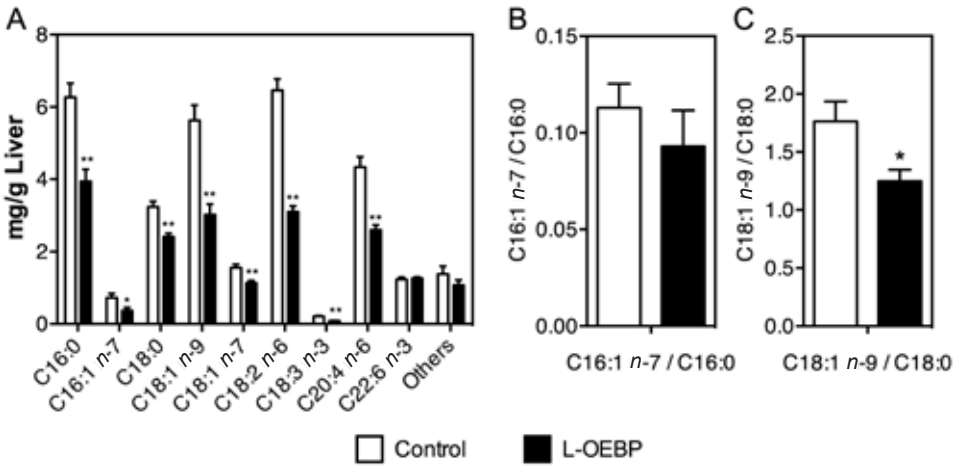

Fig. 1 Compositions of fatty acids and fatty acid desaturase indices (C16:1 *n*-7/C16:0 and C18:1 *n*-9/C18:0) in the liver.
(A) Compositions of fatty acids, (B) the fatty acid desaturase index (C16:1 *n*-7/C16:0), and (C) the fatty acid desaturase index (C18:1 *n*-9/C18:0) in the liver.
C16:0, palmitic acid; C16:1 *n*-7, palmitoleic acid; C18:0, stearic acid; C18:1 *n*-9, oleic acid; C18:1 *n*-7, cis-11-octadecenoic acid; C18:2 *n*-6, linoleic acid; C18:3 *n*-3, α-linolenic acid; C20:4 *n*-6, arachidonic acid; C22:6 *n*-3, docosahexaenoic acid (DHA).

because L-OEBP contained 3.3% (w/w) DHA (Table 3). In addition, the hepatic stearoyl-CoA (SCD) desaturase index (C18:1 *n*-9/C18:0) was significantly lower in the L-OEBP group than in the control group (Fig. 1C). A previous study showed that the liver SCD index positively correlated with liver SCD-1 mRNA expression levels and also with the TAG content[28]. SCD-1 is a key enzyme in the biosynthesis of MUFA and catalyzes the delta-9-*cis* desaturation of fatty acid substrates[29]. SCD-1 was previously reported to prevent the development of metabolic syndrome, including obesity and diabetes[30]. The regulation of SCD-1 activity has been proposed as a potential target in the prevention of obesity and diabetes. Therefore, L-OEBP may have the potential to prevent the development of obesity-related diseases by suppressing the accumulation of MUFA and lowering the SCD index.

Conclusions

We herein demonstrated that L-OEBP decreased the liver TAG content in part by suppressing hepatic fatty acid synthesis. Furthermore, L-OEBP decreased the MUFA content and SCD index, but did not change the amount of DHA in the liver. We predicted that these effects of L-OEBP were in partly due to the EPA, DHA, and PL in L-OEBP. However, the current L-OEBP formulation does not contain sufficient amounts of EPA and DHA (3.7 and 3.3% w/w, respectively) for its application as a lipid-lowering functional food and supplement. The food industry already supplies many kinds of food products fortified with *n*-3 PUFA. Fish oil is used as a supplement and contains approximately 20% (w/w) EPA and DHA. Therefore, we concluded that L-OEBP requires further improvements in its composition and concentration of *n*-3 PUFA before its application as a functional food or supplement.

Acknowledgements

We would like to thank Kazumasa Miyauchi and Ren Otsuka of Kansai University for their help with animal care and lipid analyses.

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