Trace Nutrients Research 29: 18－24 (2012)

Original Article

Effect of Dietary Oyster-Extract By-Product on Serum and Liver Lipid Contents in Rats

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Summary

To clarify the possibility of using an oyster-extract by-product as a health maintaining and promoting food material or supplement, this study investigated the effects of oyster-extract by-product powder (OEBP) on serum and liver lipid contents in rats. OEBP was prepared from washed and freeze-dried oyster-extract by-product, which contained 58.3% protein, 19.7% fat, and 19.7% carbohydrate. Male, 5-week-old Wistar rats were divided into 2 dietary groups of 7 rats each, one group receiving a control AIN-93G diet and the other receiving a 5% OEBP-containing diet. After the rats had been fed on their respective diets for 31 days, their serum and liver lipids contents and fecal cholesterol, bile acid, and nitrogen excretion were measured. The results demonstrated that OEBP decreased liver cholesterol content, in part owing to the enhancement of fecal cholesterol and bile acids excretion; however, serum cholesterol content in the OEBP diet did not decrease. In addition, the OEBP group exhibited lower protein digestibility during *in vitro* digestion analyses compared with the control diet. Therefore, in the OEBP group, the excretion of fecal cholesterol and bile acids was influenced by the low digestibility of OEBP protein. However, in order to develop OEBP as a lipid-lowering functional food and supplement, we concluded that OEBP required improvements in its formulation and further concentration of the bioactive components because OEBP has not the lowering effect of serum lipid contents.

Introduction

Seafood consumption has preventative effects against coronary heart disease according to epidemiological evidence gathered from Greenland Inuit[1] and Japanese fishing villages[2]. Many other studies have also described that seafood consumption assists in protecting against lifestyle-related diseases, including type 2 diabetes, hypertension, and hyperlipidemia[3]. The various beneficial effects of seafood have primarily been attributed to *n*−3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). There has been a great deal of research focused on the efficacy of EPA and DHA in seafood for human health. However, dietary regimes also include EPA and DHA, and in addition to these components whole seafood provides many additional nutrients. Dietary *n*−3 PUFAs decrease serum triacylglycerol (TG) content, although a meta-analysis indicated that they do not lower serum cholesterol content[4]. Therefore, there is a possibility that the health functions of seafood are not solely related to EPA and DHA. The beneficial effect of seafood protein may have been masked by EPA and DHA in seafood intake intervention studies. Seafood protein has beneficial effects such as hypocholesterolemic, anti-hypertensive, stimulation of fibrinolysis, and anti-obesity properties in animal stdies[5-8].

Oysters (*Crassostrea gigas*) contain many nutrients and are widely used in Japan. Oyster-extract contains main components including zinc, glycogen, and taurine, and oyster-extract powder is marketed as a supplement. Several studies have suggested that oyster-extract powder suppresses the initiator action of carcinogens in mice and promotes the recovery of proximal tubular epithelial cell function in *p*-aminophenol-induced nephrotoxicity in rats[9, 10]. Recently, the matter of food by-product disposal had become a concern. Thereafter, it is also expected that the effective usage of

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oyster-extract by-product. To measure the nutrient components of oyster-extract by-product, we found that a high content of protein remains in oyster-extract by-product. In this study, to clarify the possible use of oyster-extract by-product as a health maintaining and promoting food material or supplement, the effects of oyster-extract by-product powder (OEBP), obtained from washed and freeze-dried oyster-extract by-product, on serum and liver lipid contents were investigated in rats.

Materials and Methods

1. Preparation of oyster-extract by-product powder

Oyster (*Crassostrea gigas*) extract by-product, obtained from oyster meat after the procedure of oyster-extract preparation (extraction methods: hot water and 0.1 N HCl), was provided by Japan Clinic Co., Ltd. (Kyoto, Japan). Oyster-extract by-product was chopped into small pieces and then rinsed with water. The resulting meat was freeze-dried, ground using a mill (GM200, Retsch Co., Ltd. Haan, Düsseldorf, Germany) and then sieved to remove any lump of oyster meat. The resulting product was called OEBP (particle size: < 1 mm).

Table 1 shows the nutrient composition of oyster-extract by-product and OEBP. The crude protein content was determined by the Kjeldahl method, with an N-to-protein conversion factor of 6.25. The crude fat content was measured by the Soxhlet method. 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, fat, ash, and moisture was considered as carbohydrate.

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

	Oyster-extract by-product †	OEBP
Crude protein	21.5	58.3
Crude fat	7.2	19.7
Ash	0.8	1.2
Moisture	69.7	1.1
Carbohydrate §	0.8	19.7

† Consists of oyster meat after the procedure of oyster-extract preparation.
§ Carbohydrate = 100 − (crude protein + crude fat + ash + moisture)
OEBP, oyster-extract by-product powder.

Table 2 shows the fatty acid compositions of OEBP. The composition of fatty acids was determined by analysis using a fused silica capillary column, Omegawax 250 (Supelco, Pennsylvania, USA) in a gas-liquid chromatography system (GC-14B, Shimadzu Co., Kyoto, Japan) after methylation with sodium methoxide[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]. Phospholipid content was measured by phosphorus analysis[13].

Table 2 Fatty acid composition of OEBP total lipid (%)

Fatty acid	
14 : 0	8.7
15 : 0	1.0
16 : 0	33.4
16 : 1	6.4
17 : 0	2.1
18 : 0	6.2
18 : 1 *n*−9	5.0
18 : 2 *n*−6	11.9
20 : 0	3.0
20 : 1 *n*−9	4.3
20 : 4 *n*−6	2.0
22 : 2	5.1
22 : 6 *n*−3	1.2
Others	9.8

OEBP, oyster-extract by-product powder.

Casein and OEBP were hydrolyzed using 6 mol/L HCl for 24 hours. The amino acid composition in casein and OEBP was determined by reversed phase high-performance liquid chromatography (HPLC) with UV detection using derivatization with phenylisothiocyanate (Table 3)[14]. The identification and quantitation of each amino acid were carried out using commercially available authentic standard mixtures.

Table 3 Amino acid composition of casein and OEBP (g/100g protein)

Amino acid	Casein	OEBP
Alanine	2.9	3.6
Arginine	3.6	9.2
Aspartic acid †	6.8	11.1
Glutamic acid §	20.5	14.3
Glycine	1.7	4.4
Histidine	2.9	1.9
Isoleucine	5.3	2.8
Leucine	9.1	8.4
Lysine	7.7	6.6
Methionine	2.8	3.2
Phenylalanine	4.8	6.4
Proline	10.8	5.0
Serine	5.0	5.9
Threonine	4.0	7.5
Tyrosine	5.4	4.6
Valine	6.5	5.2

† Aspartic acid + asparagine.
§ Glutamic acid + glutamine.
OEBP, oyster-extract by-product powder.

2. Animals care and experimental diets

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical Uni-

versity and followed the "Guide for the Care and Use of Experimental Animals" issued by the Prime Minister's Office of Japan. Male 5-week-old 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. Fourteen rats were divided into the following 2 dietary groups of 7 rats each: control diet (AIN-93G) and OEBP diet. Table 4 lists the composition of the experimental diets, prepared based on the AIN-93G formulation[15].

Table 4 Composition of the experimental diets (g/kg diet)

	Control	OEBP
Casein	200	170.9
OEBP	–	50
Dextrinized corn starch	132	132
Corn starch	397.486	386.486
Sucrose	100	100
Cellulose	50	50
AIN-93G mineral mixture	35	35
AIN-93 vitamin mixture	10	10
L-Cystine	3	3
Choline bitartrate	2.5	2.5
Soybean oil	70	60.1
tert-Butylhydroquinone	0.014	0.014

Diets were prepared based on the AIN-93G formula.
OEBP, oyster-extract by-product powder.

Food consumption and body weight were recorded daily. Feces were collected from each group every 24 hours for 7 days before being sacrificed. After feeding for 31 days, rats were weighed and sacrificed under pentobarbital (Nembutal®, Dainippon Sumitomo Pharma Co., Ltd.; Osaka, Japan) anesthesia for 08:00–10:00. Rats were not deprived of food prior to being sacrificed because food deprivation leads to a significant down regulation of the genes involved in fatty acid synthesis and cholesterol metabolism[16]. Blood was collected from the abdominal aorta without the use of anti-coagulant, and serum was obtained by centrifugation at 1,500×*g* for 15 min and then stored at −70 ℃ until analysis. The liver and abdominal white adipose tissue (WAT) from the epididymis, mesentery, perinephria, and retroperitoneum were removed rapidly, then weighed, rinsed with saline, and frozen in liquid nitrogen, followed by storage at −70 ℃ until analysis.

3. Serum biochemical test

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea nitrogen (SUN), total lipid, phospholipids, cholesterol, and TG were measured using an automatic analyzer (AU5431, Olympus Co. Tokyo, Japan).

4. Liver lipids analysis

Liver lipids were extracted using the method of Bligh and Dyer[17]. Each total lipid sample was dissolved in an equal volume of dimethylsulfoxide, and the TG content was determined using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries; Osaka, Japan). Cholesterol and phospholipid contents were determined by gas-liquid chromatography and phosphorus analysis, respectively, as described previously.

5. Fecal lipid and nitrogen analysis

Fecal cholesterol content was determined by gas-liquid chromatography as described above. Fecal bile acids content was determined as 3α-hydroxysteroid based on the molar extinction coefficient of NADH at 340 nm[18]. Fecal nitrogen content was determined by the Kjeldahl method.

6. *In vitro* digestion

Control and OEBP diets were digested by the methods of Hosomi *et al.* with some modifications[5]. Pepsin hydrolysis parameters were as follows: diet concentration, 10 % (w/v); enzyme/substrate ratio, 1:100 (w/w); adjusted to pH 2.0 with HCl at 37 ℃ (Sigma Chemical Co., Missouri, USA). After incubation for 180 min, pepsin was inactivated by neutralization with NaOH.

Porcine pancreatin (Sigma Chemical Co.) was added; pancreatin hydrolysis parameters were as follows: enzyme/substrate ratio, 1:30 (w/w); adjusted to pH 7.4 with NaOH at 37 ℃. After hydrolysis for 360 min, digestion was stopped by heating to 95 ℃ for 15 min. The digested sample was centrifuged at 4,500×*g* for 20 min. The sediment was washed with distilled water two times, centrifuged at 4,500×*g* for 20 min, dried at 105 ℃ for 24 hours, and then weighed.

7. Protein digestibility

For the monitoring of protein digestibility at intervals during digestion, aliquots of the digestion reaction were removed at 0, 2, 5, 10, 15, 20, 30, 45, 60, 120, and 180 min during the pepsin and pancreatin *in vitro* digestions. The aliquots were submerged in a boiling water bath for 15 min to inactivate pepsin and pancreatin, and then cooled and stored at −35 ℃ until analysis.

Protein digestibility was calculated by the determination of free amino groups by reaction with 2,4,6-trinitrobenzene sulfonic acid[19]. L-Leucine was used as a standard in the protein digestibility assays.

8. Statistical analysis

Data represent means and standard deviations (SD). The statistical significance of differences was evaluated using Student's *t*-test and means were considered signifi-

cantly different at $p<0.05$. The correlation was determined by simple analysis. Analyses were performed using StatView-J version 5.0 software (Abacus Concepts, California, USA)

Results and Discussion

OEBP, prepared by rinsing and freeze-drying oyster-extract by-product, contained 58.3% protein, 19.7% fat, and 19.7% carbohydrate. In this study, OEBP was added to the control diet (AIN-93G) at the expense of cornstarch, casein, and soybean oil in the preparation of the OEBP diet, hence the contents of carbohydrate, protein, and fat between the control and OEBP diets were nearly the same. The phospholipids and cholesterol contents in OEBP were 85.2% and 1.2%, respectively. The amino acid composition in OEBP, compared with casein, showed higher levels of arginine, aspartic acid (aspartic acid + asparagine), glycine, and threonine but lower levels of glutamic acid (glutamic acid + glutamine), isoleucine, and proline.

Table 5 shows the growth parameters and organ weight. There were no differences in body weight gain, food intake, liver weight, and total abdominal WAT weight between the control and OEBP groups.

Table 6 shows the biochemical parameters of the serum, liver, and feces. Differences in serum AST, ALT, and SUN were not observed as a result of OEBP administration. The serum total lipid, phospholipid, cholesterol, and TG contents did not differ between the control and OEBP diets, but the OEBP group showed significantly lower liver cholesterol content than the control group. The liver total lipid, phospholipid, and TG contents did not differ between the control and OEBP group.

One of the reasons for the decrease in liver cholesterol content was thought to be related to the absorption of cholesterol and bile acids from the small intestine[20]. Previ-

Table 5 Growth parameters and organ weights for rats fed experimental diets for 4 weeks

	Control	OEBP
Growth parameters		
Body weight gain (g/day)	7.16 ± 0.20	6.76 ± 0.29
Food intake (kcal/day)	18.0 ± 2.0	17.5 ± 1.8
Organ weight (g/100 g BW)		
Liver weight	3.61 ± 0.26	4.01 ± 0.53
Epididymal WAT weight	1.44 ± 0.10	1.54 ± 0.21
Mesentery WAT weight	2.13 ± 0.20	2.28 ± 0.34
Perirenal and retroperitoneal WAT weight	1.34 ± 0.42	1.39 ± 0.43
Total WAT weight †	4.98 ± 0.90	5.18 ± 0.73

Data represent means ± SD (n = 7).
† Total WAT reshows the sum of WAT weights from the epididymis, mesentery, perinephria, and retroperitoneum
BW, body weight; OEBP, oyster-extract by-product powder; WAT, white adipose tissue.

Table 6 The biochemical parameters of serum, liver and feces in rats fed experimental diets for 4 weeks

	Control	OEBP
Serum		
AST (IU/L)	79.9 ± 6.8	81.3 ± 5.5
ALT (IU/L)	43.1 ± 3.6	47.4 ± 6.1
SUN (mg/dL)	23.1 ± 3.0	17.9 ± 5.5
Total lipid (mg/dL)	205 ± 21	220 ± 19
Phospholipids (mg/dL)	119 ± 12	129 ± 11
Cholesterol (mg/dL)	66.1 ± 7.8	71.3 ± 5.8
TG (mg/dL)	22.0 ± 5.3	20.4 ± 5.3
Liver (mg/g Liver)		
Total lipid	7.03 ± 0.90	6.89 ± 0.53
Phospholipids	2.62 ± 0.06	2.66 ± 0.18
Cholesterol	0.45 ± 0.15	0.24 ± 0.05 *
TG	3.96 ± 0.80	3.77 ± 0.97
Feces		
Dry weight (g/day)	3.92 ± 0.35	4.33 ± 0.38
Cholesterol (mg/day)	5.58 ± 1.03	12.04 ± 1.04 *
Bile acids (μmol/day)	8.18 ± 0.64	13.62 ± 2.48 *
Nitrogen (mg/day)	32.9 ± 5.3	51.5 ± 6.7 *

Data represent means ± SD (n = 7). Value statistically different at *$p<0.05$ using a Student's t-test compared to control group.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; OEBP, oyster-extract by-product powder; SUN, urea nitrogen; TG, triacylglycerol.

ous studies suggested that the extent of the decrease in serum and liver cholesterol depends on the extent of fecal cholesterol and bile acids excretion[5, 21]. Therefore, we analyzed the effect of dietary OEBP on cholesterol and bile acids absorption by determining the total amount of cholesterol and bile acids in feces. Table 6 shows the fecal dry weight, cholesterol, bile acids, and nitrogen excretion in rats fed on control and OEBP diets. The fecal dry weight in the OEBP group tended to be higher than that in the control group. The fecal cholesterol and bile acids contents were significant higher in the OEBP group than in the control group. The enhancement of fecal cholesterol excretion by OEBP administration was partly influenced by the cholesterol content of the OEBP diet. Based on the fecal steroids excretion, the decreased liver cholesterol content in rats fed the OEBP diet was in part as a result of the enhancement of fecal cholesterol and bile acids excretion.

In this study, the OEBP diet increased fecal nitrogen excretion compared with the control diet. Previous studies showed that dietary soybean, buckwheat, and fish protein simultaneously increased fecal steroids and nitrogen contents; moreover, positive correlation has been observed between fecal steroids and nitrogen contents[5, 22, 23]. In this study, the fecal cholesterol content correlated positively with the fecal nitrogen content, and a significant correlation between fecal bile acids and nitrogen was also observed (Fig. 1-A and 1-B). Therefore, the effect of OEBP on the liver cholesterol content in partly due to reflect the enhancement of fecal cholesterol and bile acids excretion owing to the increased fecal nitrogen content.

To clarify the enhancement of fecal nitrogen content by the OEBP diet, we determined the production rate of insoluble sediment weight and the time course of protein digestibility of control and OEBP diets using an *in vitro* digestion model. The OEBP diet had a significantly higher contained insoluble sediment compared with the control diet (Control and OEBP: 16.0 ± 0.33 and 19.4 ± 0.45, respectively). Fig. 2 shows the time course of protein digestibility of the control and OEBP diets measured using *in vitro* digestion. The protein digestibility of the control and OEBP diets with pepsin was very high in the first 10 min, after that hydrolysis proceeded more slowly, and at 180 min, the protein digestibility of the OEBP diet was significantly lower than that of the control diet. During pancreatin digestion, the protein digestibility of the control and OEBP diets were dramatically increased, and the protein digestibility of the OEBP diet was significantly lower compared with that of the control diet from 210 min to 360 min. The high production rate of insoluble sediment weight and the low protein digestibility of OEBP using this *in vitro* diges-

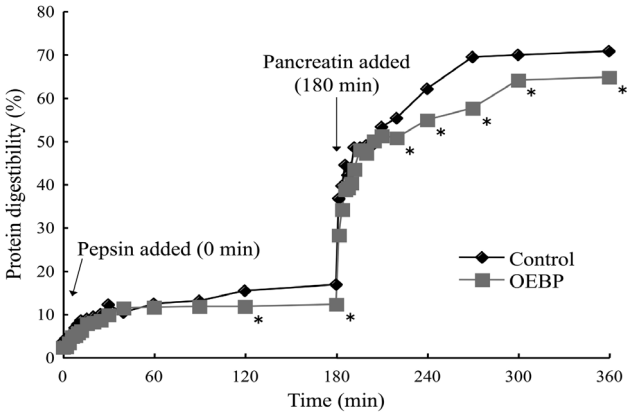

Fig. 2 Course of protein digestibility of control (◆) and OEBP (■) diets using pepsin (added at time 0 min) and pancreatin (added at time 180 min).
Each point represents means ± SD (n = 3). Values are statistically different compared with the control diet at $^{*}p < 0.05$ using Student's *t*-test.

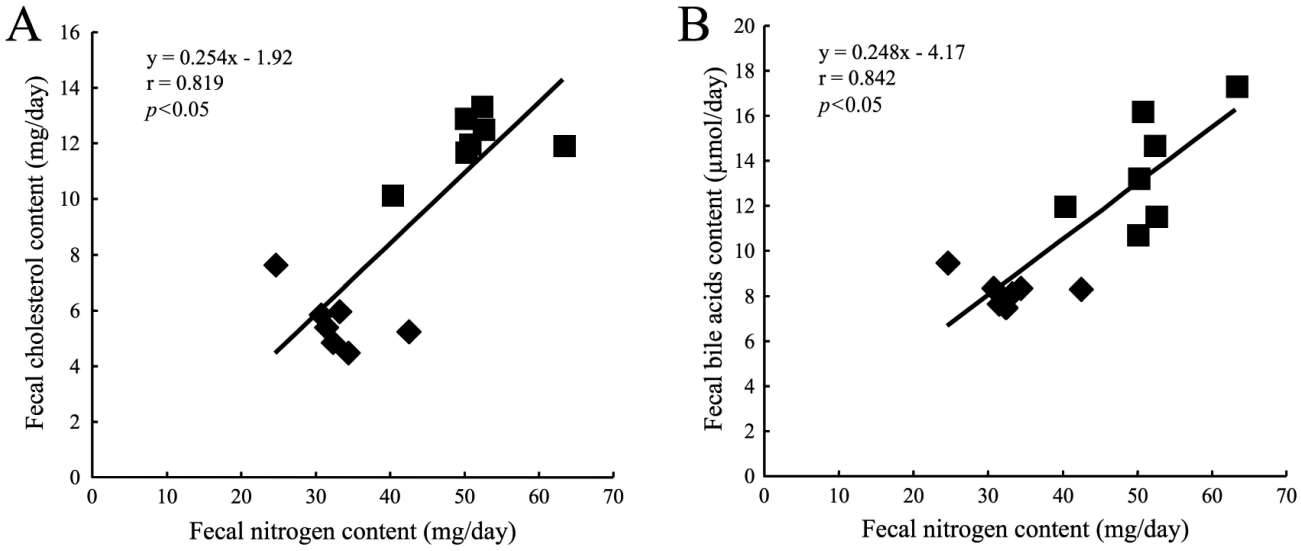

Fig. 1 Correlation between fecal cholesterol and nitrogen contents (A), fecal bile acids and nitrogen contents (B) in the control (◆) and OEBP (■) group. The values were significantly different ($p < 0.05$), as determined by simple correlations.

tion model and the increased fecal nitrogen excretion in OEBP group were probably caused by the low digestibility of OEBP. From these results, OEBP contains resistant proteins that are undigested remnants of dietary protein, which have the same function of fiber in the intestine[24]. A previous study suggested that a high-molecular-weight fraction of dietary soybean, which has 25–30% indigestible remnants, decreased serum and liver cholesterol contents owing to its enhancement of fecal cholesterol and bile acids excretion[24]. Because digestion-resistant proteins in OEBP inhibit cholesterol and bile acids absorption in the intestine, this results in a decrease in the liver cholesterol content. However, in this study dietary OEBP did not affect the serum cholesterol content despite the increase in fecal cholesterol and bile acids excretion. We estimated that the increase in the serum cholesterol content associated with the OEBP diet contained approximately 118 mg/kg cholesterol and liver cholesterol homeostasis. Further research is required to clarify this issue.

Our previous studies suggested that dietary phospholipids (18 g/kg) decreased the serum cholesterol and liver contents through the enhancement of fecal cholesterol excretion compared with a diet containing soybean oil alone (AIN-93G)[25]. The OEBP diet contained 8.4 g/kg phospholipids, thus there is a possibility that phospholipids in the OEBP diet affected cholesterol metabolism. Further research is necessary to clarify the effects of phospholipids in OEBP on cholesterol metabolism.

In conclusion, this study showed that OEBP decreased the liver cholesterol content in part as a result of the enhancement of fecal cholesterol and bile acids excretion; however, the serum cholesterol content in the OEBP group did not decrease. In addition, the observed effects of OEBP on fecal cholesterol and bile acids excretion were influenced by the low digestibility of OEBP protein. However, in terms of the aim of developing lipid-lowering functional foods or supplements, the current formulation of OEBP has not the lowering effect of serum lipid contents. We concluded that OEBP requires further improvement in its composition and concentration of its bioactive components before it can be considered for development as a functional food or supplement.

Acknowledgments

We would like to thank Hiroe Shimizu, Hiromitsu Imaike, Kana Uemura, and Ryosuke Inayoshi of Kansai University for help with animal care and lipid analyses.

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