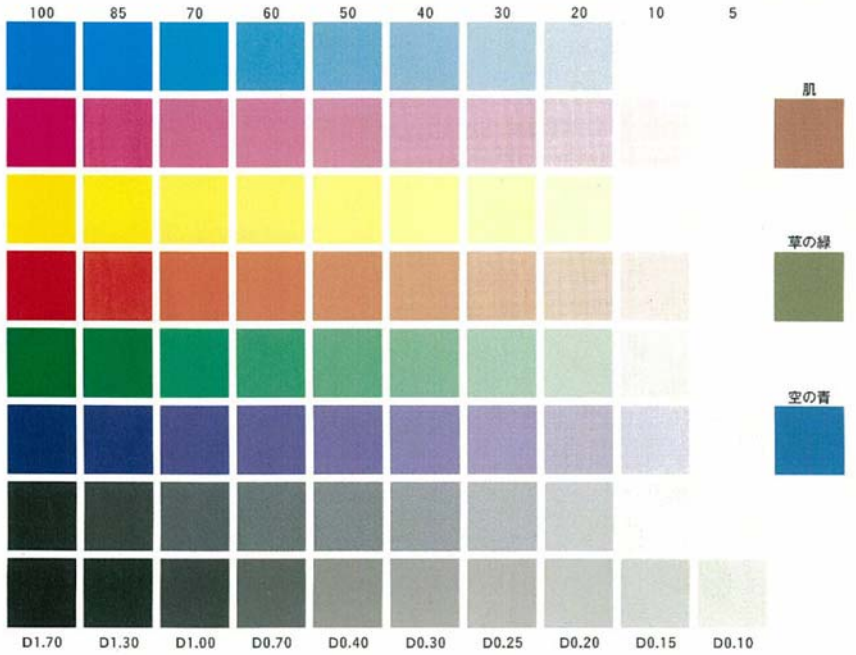

Studies on the Novel Functional Components Improving Lipid Metabolism Prepared from Marine Products

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Chapter 5

Abbreviations

ABCG5	ATP-binding cassette G5
ABCG8	ATP-binding cassette G8
ACAT-1	acyl-coenzyme:cholesterol acyltransferase-1
ACC	ATP-citrate lyase
ACL	acetyl-coenzyme A carboxylase
ACOX	acyl-CoA oxidase
ANOVA	analysis of variance
BAT	brown adipose tissue
BSA	bovine serum albumin
CHOL	cholesterol
CHD	coronary heart disease
CPT-2	carnitine palmitoyl transferase-2
CYP7A1	cholesterol 7α-hydroxylase
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
EPA	eicosapentaenoic acid
FAS	fatty acid synthas
FXRα	farnesoid X receptor-α
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gas chromatography
HDL-C	high density lipoprotein-cholesterol
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HNF-4α	hepatocyte nuclear factor-4α
IBAT	intestinal bile acid transporter
LDLR	low density lipoprotein receptor
LDL-C	low density lipoprotein-cholesterol
LRH-1	liver receptor homolog-1
LXRα	liver X receptor-α
ME	malic enzyme
MW	molecular weight
NCP1L1	niemann-pick C1 like 1 protein 1

PAGE	poly-acrylamide gel electrophoresis
PAP	phosphatidate phosphatase
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PLs	phospholipids
PPARα	peroxisome proliferation activated receptor-α
PUFAs	polyunsaturated fatty acids
SD	standard deviation
SE	standard error
SHP-1	small heterodimer parter-1
SPLs	soybean phospholipids
SR-B1	scavenger receptor class B type 1
SREBP	sterol regulatory element binding protein
TG	triacylglycerol
TL	total lipid
TLC	thin-layer chromatography
UCP-1	uncoupling protein-1
VLDL	very low density lipoprotein
WAT	white adipose tissue

Preface

In Japan, the changes in dietary habits that have occurred during the past 40 to 50 years seem to be a major factor in the concurrent alteration of disease patterns. Traditionally, fish, vegetables, and rice were the main foods in Japanese cuisine, but consumption of other animal products has increased over the past 40 to 50 years. Currently, 25% of calories in the average Japanese diet come from fat, 60% from carbohydrates, and 15% from protein, and the average rate of salt consumption is 12 g/day. The traditional Japanese diet, which was uncommonly high in salt and low in fat and protein, has been associated with a reduced incidence of stroke. Yet as dietary habits in Japan have rapidly become more similar to those in the United States and Europe, Japan has seen an increase in the incidence of coronary heart disease (CHD), which is responsible for about 30% of all deaths in Western countries.

Epidemiological evidence from Greenland Inuit and Japanese fishing villages suggests that eating fish and marine animals can prevent CHD. Dietary studies by numerous investigators have similarly shown that regular fish intake affects several humoral and cellular factors involved in CHD and may prevent atherosclerosis, thrombosis, and sudden cardiac death. Once- or twice-weekly consumption of fish (or daily intake of a small amount of fish, such as 30 to 60 g) is associated with reduced risk of CHD in Western countries. Fish intake is therefore important for health maintenance. Marine foods, unlike many livestock products, are a source of animal protein that can be continually harvested without causing environmental degradation, as long as marine resources are well managed. In the future, marine foods will play an important role in averting food shortages due to global population increase.

An important class of biomarkers for marine food intake is the n-3 polyunsaturated fatty acids (PUFAs), which can be measured in blood or tissue; high PUFA levels are also associated with reduced risk of CHD. Accordingly, most of the research on dietary fish consumption has focused on the effects of two PUFAs known as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which are found in fish oil. Several mechanisms have been proposed to explain how n-3 PUFA might beneficially influence cardiovascular disease; these include suggestions that n-3 PUFA can prevent arrhythmias or decrease serum triacylglycerol (TG) by stimulating

β-oxidation and inhibiting lipogenesis in the liver. Yet it is difficult to explain the health benefits associated with fish consumption only in terms of n-3 PUFAs such as EPA and DHA because their effect on plasma cholesterol (CHOL) levels is still controversial.

In most previous studies, the preventive effects of these fatty acids against arteriosclerotic disorders have been examined using TG or ester-type n-3 PUFAs. Yet large amounts of n-3 PUFAs are also found in phospholipids (PLs) from marine products, and it is expected that PLs containing n-3PUFAs may be more effective for the prevention and improvement of hyperlipidemia than TG containing n-3 PUFAs. Furthermore, the Japanese diet includes not only fish oil but fish tissue as well, which provides nutrients such as proteins as well as fat. Based on the evidence that lipids and protein in the diet strongly affect the incidence of coronary or cerebral artery disease, it is thought that improving lipid metabolism in the circulatory system can prevent the development of arteriosclerotic disease. In fact, dietary therapy is considered the first-choice treatment for arteriosclerotic disease, and is as important as medical treatment in such cases.

For these reasons, this study examined fish protein, one of the main nutritional components of fish, rather than fish oil; specifically, this study examined Alaska pollock fillet protein, which is widely used for human nutrition, and salmon protamine, a pharmaceutical product, which acts as an antidote to heparin. This study represents the first time that certain components, such as PLs and protein, have been examined in marine products.

I discuss the effects on lipid metabolism that are caused by various components of fish: n-3 PUFAs containing PLs in Chapter 1, fish protein in Chapter 2, and protamine in Chapter 3. Chapter 4 describes the mechanism by which dietary fish protein inhibits lipid absorption in the intestine. Finally, Chapter 5 describes the effects of fish peptides, prepared using papain from fish protein, on improving lipid metabolism.

Chapter 1

Effect of phospholipid containing n-3 polyunsaturated fatty acids on lipid metabolism in rats

Introduction

It has been suggested that the increase in total fat, saturated fatty acids, and the ratio of n-6 PUFAs to n-3 PUFAs in the diet strongly affect the incidence of arteriosclerosis and coronary or cerebral artery disease [1,2]. Therefore, it is thought that improvement of lipids metabolism in the circulatory system can prevent the development of arteriosclerotic disease. Dietary therapy is considered as the first-choice treatment for arteriosclerotic disease and is as important as medical treatment. In particular, the amount and type of dietary fat may contribute to hyperlipidemia, the initial symptom prior to the induction of arteriosclerosis [3-5].

Dietary PLs also have beneficial effects compared with dietary TG. It has been reported that phosphatidylcholine (PC) can decrease blood total lipids [6] and improve brain function [7], and phosphatidylethanolamine (PE) and phosphatidylserine can decrease blood CHOL [8] and improve brain function [9].

In general, n-3 PUFAs is commercially produced from extracted fish oil. EPA and DHA can be obtained by the purification from n-3 PUFAs as ethyl-type esters. In most previous studies, the preventive effects against arteriosclerotic disorders have been examined using TG or ester-type n-3PUFAs. PLs from marine resources have also a large amount of n-3 PUFAs. However, a few studies have focused on the beneficial function of dietary PLs containing n-3 PUFAs on lipid metabolism in experimental animals [3,10]. Therefore, it was expected that dietary PLs containing n-3PUFAs may be more effective for the prevention and improvement of hyperlipidemia compared with dietary fish oil and PLs. Then, the effects of PLs containing n-3 PUFAs on lipid contents in serum, liver, and feces, and liver mRNA expression levels of genes encoding proteins related to CHOL metabolism were investigated in rats. In this study, PLs containing n-3PUFAs were prepared from squid mantle muscle (*Todarodes pacificus*), which is a source of abundant PLs

containing n-3 PUFAs.

Materials and methods

Materials

Soybean PLs (SPLs) and PLs containing n-3 PUFAs extracted from squid mantle muscle (*Todarodes pacificus*) were provided by Bizen Chemical (Okayama, Japan). Fish oil extracted from bigeye tuna (*Thunnus obesus*) was provided by Yashima Shiyoji (Shizuoka, Japan). American Institute of Nutrition (AIN-93) vitamin mix, AIN-93G mineral mix, dextrinized cornstarch, cornstarch, cellulose, sucrose, and casein were purchased from Oriental Yeast (Tokyo, Japan). L-Cystine, choline bitartrate, and soybean oil were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were obtained from commercial sources and were of reagent grade.

Animal care and diets

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the ''Guide for the Care and Use of Experimental Animals'' of the Prime Minister's Office of Japan. Five-week-old male Wistar rats obtained from Shimizu Laboratory Supplies (Kyoto, Japan) were kept in an air-conditioned room (temperature, 21–22°C; humidity, 55–65%; lights on, 08:00–20:00) and had free access to drinking tap water and feed. Rats were fed a diet prepared according to the recommendations of the AIN-93G [11]. After acclimation for 5 days with AIN-93G diet, rats were divided into the following four dietary groups of seven rats each: soybean oil diet; fish oil plus soybean oil diet, SPLs plus soybean oil diet; PLs containing n-3 PUFAs (squid PLs) plus soybean oil (SQPL) diet. Table 1 shows the composition of the experimental diets prepared according to AIN-93G. SPLs contained 76.2% PC and 12.2% PE w/w, and squid PLs contained 75.9% PC and 9.3% PE w/w by thin-layer chromatography (TLC) methods [12]. The fatty acid composition in the experimental diets was analyzed using a fused silica capillary column, Omegawax 250 (Supelco, Bellefonte, USA) by gas chromatography (GC) (GC-14B, Shimadzu Co., Kyoto, Japan) after methylation using sodium methoxide.

As shown in Table 2, the fatty acid composition was almost the same between SO and SPL, or between FO and SQPL diets.

Table 1 Composition of the experimental diets (g/kg)

Components	SO	FO	SPL	SQPL
Dextrinized corn starch	132	132	132	132
Corn starch	397.5	397.5	397.5	397.5
Casein	200	200	200	200
Sucrose	100	100	100	100
Cellulose	50	50	50	50
AIN93G mineral mixture	35	35	35	35
AIN93 vitamin mixture	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	3	3	3	3
tert-Butylhydroquinone	0.01	0.01	0.01	0.01
Soybean oil	70	58	52	52
Fish oil		12		
Soybean phospholipid			18	
Squid phospholipid				18

Food consumption and body weight were recorded every 2 days. Feces were collected from each group every 24 h for 7 days prior to sacrifice. After feeding for 4 weeks, rats were weighed and sacrificed under pentobarbital (Nembutal®, Dainippon Sumitomo Pharma, Osaka, Japan) anesthesia. Rats were not fasted before being sacrificed because food deprivation prior to sacrifice leads to a significant downregulation of the genes involved in fatty acid synthesis and CHOL metabolism [13]. Blood was collected without anticoagulant, and serum was obtained by centrifugation at 1500 × g for 15 min and stored at −80°C until analysis. Liver and abdominal white adipose tissues (WAT) of the epididymis, mesentery, and perinephria were extirpated rapidly then weighed, rinsed with 0.9% NaCl, and frozen in liquid nitrogen followed by the storage at −80°C until analysis. Aliquots of the liver were taken for mRNA expression analysis and stored in RNA-Later Storage Solution (Sigma Chemical, St. Louis, USA).

Table 2 Fatty acid composition of experimental diets (wt%)

Fatty acid	SO	FO	SPL	SQPL
14 : 0		0.2		
16 : 0	10.9	11.2	11.9	14.8
16 : 1 n-7		0.3		
18 : 0	4.0	4.4	3.9	4.5
18 : 1 n-9	21.9	22.0	20.0	20.2
18 : 1 n-7	1.5	1.6	1.6	1.5
18 : 2 n-6	57.2	48.7	55.7	48.0
18 : 3 n-3		4.8	5.4	4.6
20 : 4 n-6		0.2		
20 : 5 n-3		1.2		1.0
22 : 6 n-3		3.4		3.4
others	4.5	2.0	1.5	2.0

Analysis of lipid parameters

Serum TG, CHOL, PL, high density lipoprotein-cholesterol (HDL-C), and low density lipoprotein-cholesterol (LDL-C) contents were determined by using an Olympus AU5431 automatic analyzer (Olympus Co., Tokyo, Japan).

Liver lipids were extracted by the method of Bligh and Dyer [14]. Liver TG content was determined as follows, i.e., liver lipids were dissolved with an equal volume of dimethyl sulfoxide (DMSO) and then used to determine the TG content using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries). Liver CHOL content was analyzed using a SE-30 column (Shinwa Chemical Industries, Kyoto, Japan) with a GC-14B using 5α-cholestane as an internal standard. Liver PL content was measured using phosphorus [15]. The liver protein content was determined according to the method of Bradford using bovine serum albumin (BSA) as a standard [16].

The fecal CHOL content was analyzed using SE-30 with a GC-14B instrument and 5α-cholestane as an internal standard, and fecal bile acid content was measured according to the method of Bruusgaard *et al.* [17].

Analysis of mRNA expression levels

Total RNA was extracted from 100 mg of liver using TRIZOL reagent (Invitrogen, Tokyo, Japan). Then, cDNA was synthesized from total RNA using a RevaTraAce® qPCR RT kit (TOYOBO, Osaka, Japan). Real-time quantitative polymerase chain reaction (PCR) analysis was performed with an automated sequence detection system (DNA Engine option 2, Bio-Rad Laboratories, CA, USA) using SYBR®GreenER™ qPCR SuperMix Universal (Invitrogen). The primer sequences used for the detection of ATP-binding cassette (ABC) G5, ABCG8, cholesterol 7α-hydroxylase (CYP7A1), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), acyl-coenzyme:cholesterol acyltransferase-1 (ACAT-1), low density lipoprotein receptor (LDLR), sterol regulatory element binding protein-1c (SREBP-1c), sterol regulatory element binding protein-2 (SREBP-2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows, for- ward: 5'-ATGGCCTGTACCAGAAGTGG-3' and reverse: 5'-GGATACAAGCCCAGAGTCCA-3'; for ABCG5, forward: 5'-TTCTGCTGCAACGCTCTCTA-3' and reverse: 5'-GGAGGAACGACATCTTGGAA-3'; for ABCG8, forward: 5'-CCCAGACCCTTTGACTTTCA-3' and reverse: 5'-GATCCGAAGGGCATGTAGAA-3'; for CYP7A1, forward: 5'-TACTCCTTGGTGATGGGAGC-3' and reverse: 5'-CCATTGGCACCTGGTACTCT-3'; for HMGR, forward: 5'-AAGTACGCCATCGGCTCTTA-3' and reverse: 5'-TCACCACCACGTCTGGTTTA-3'; for ACAT-1, forward: 5'-ACCGCCATGAGGTACGTAAG-3' and reverse: 5'-CGGCGCTGTAGATCTTTCTC-3' ; for LDLR, forward: 5'-GCACAGCAACCAGAAACTCA-3' and reverse: 5'-ACCACTTCAGGTTTCATGCC-3'; for SREBP-1c, for- ward: 5'-CACCTGTGGAGCAGTCTCAA-3' and reverse: 5'-TGCCAGAGTGTTGTCCTCAG-3'; for SREBP-2, forward: 5'-ATGACTCTACCCACGGCAAG-3' and reverse: 5'-TACTCAGCACCAGCATCACC-3'; for GAPDH, which were designed using GENETYX-MAC version 13.0.4 software (Genetyx, Tokyo, Japan). Results were quantified with a comparative method and were expressed as a relative level after

normalization to the GAPDH expression level.

Statistical analysis

Data are expressed as means ± standard error (SE). To determine the dietary fatty acids, lipid structure, and interactions between dietary fatty acids and lipid structure, data were analyzed to two-way analysis of variance (ANOVA). Statistical comparisons were made using the Turkey–Kramer test. Differences with $p < 0.05$ were considered significant. The analysis was performed using StatView-J version 5.0 software (Abacus Concept, Berkeley, CA, USA).

Results

Growth parameters and organ weights

Table 3 shows growth parameters and organ weights. No differences were observed in body weight, body weight gain, energy intake, or food efficiency among the groups. There were no significant differences in relative liver weights, epididymal WAT, mesentery WAT, perirenal WAT, or total WAT weights among the groups.

Lipid parameters

Figure 1 shows TG (A), CHOL (B), and LDL-C (C) contents in the serum. Serum TG contents in the FO and SQPL groups were significantly lower than that in the SO and SPL groups. The fatty acids, but not the lipid structure, affected the serum TG content ($p < 0.0001$). The serum CHOL content was significantly lower in the SQPL group than in the FO and SO groups. Moreover, the serum CHOL content of the SPL group tended to be lower compared with those in the FO and SO groups.

The lipid structure affected the serum CHOL content ($p = 0.0005$). The squid PLs diet resulted in a reduction in serum LDL-C content compared with the SO diet. There were no significant differences in serum PL or HDL-C contents among the groups (data not shown).

Table 4 shows liver TG and CHOL contents, and daily fecal CHOL and bile acid excretions for seven days prior to sacrifice. Liver TG content was decreased in the SQPL group, and it was significantly lower than that in the SO group. The fatty acids

affected liver TG content ($p = 0.003$). Liver CHOL content in the SQPL group was significantly lower than that in the SO group. The lipid structure affected ($p = 0.002$) liver CHOL content. There was no significant difference in liver PL content among the groups (data not shown). Fecal CHOL excretion was significantly higher in the SPL and SQPL groups than in the SO and FO groups. The lipid structure affected fecal CHOL content ($p < 0.0001$). There were no significant differences in fecal bile acid excretion among the groups.

mRNA expression levels

Table 5 shows the relative liver mRNA expression levels of genes encoding proteins involved in lipid metabolism. The ABCG5 expression level was significantly higher in the SQPL group than that in the SO, FO, and SPL groups. In addition, the ABCG8 expression levels in the SPL and SQPL groups were significantly higher than those in the SO and FO groups. The lipid structure affected ABCG5 and ABCG8 expression levels ($p < 0.0001$). The CYP7A1 expression level was decreased by dietary n-3PUFAs compared with soybean oil fatty acids ($p = 0.04$). Furthermore, the HMGR expression level tended to decrease due to n-3 PUFAs compared with soybean oil fatty acids ($p = 0.06$). LDLR and SREBP-1c expression levels in the SQPL group showed a tendency to decrease as compared with the SO group. The SREBP-2 expression level tended to decrease with dietary n-3 PUFAs compared with dietary soybean oil fatty acids ($p = 0.08$). There were no significant differences in ACAT-1 expression levels among the groups.

Table 3. Growth parameters and organ weights of rats fed the experimental diets for 4 weeks.

	SO	FO	SPL	SQPL
Growth parameters				
Initial body weight (g)	122 ± 2.0	125 ± 2.8	122 ± 1.5	122 ± 2.0
Final body weight (g)	312 ± 5.2	315 ± 7.3	316 ± 6.5	318 ± 5.6
BW gain (g/day)	7.1 ± 0.2	7.0 ± 0.2	7.2 ± 0.2	7.2 ± 0.2
Energy intake (kcal/day)	73.8 ± 3.8	75.3 ± 5.4	77.7 ± 4.2	78.4 ± 4.5
Food efficiency (g/kcal)	0.096 ± 0.003	0.093 ± 0.003	0.094 ± 0.003	0.093 ± 0.002
Relative organs weights (g/100g BW)				
Liver weight	3.9 ± 0.1	3.8 ± 0.1	3.9 ± 0.1	3.9 ± 0.1
Epididymal WAT weight	1.4 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
Mesentery WAT weight	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.2
Perirenal WAT weight	1.2 ± 0.1	1.1 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
Total WAT weight	3.9 ± 0.2	3.7 ± 0.2	4.0 ± 0.2	4.0 ± 0.3

Data represent means ± SE (n=7).

BW, body weight; WAT, white adipose tissue.

SO; soybean oil diet, FO; fish oil diet, SPL; soybean phospholipid diet, SQPL; squid phospholipid diet.

Table 4. Triacylglycerol and cholesterol contents in the liver, and cholesterol and bile acid contents in daily excreted feces for seven days prior to sacrifice in rats fed experimental diets for 4 weeks.

	SO	FO	SPL	SQPL	ANOVA (*P* values)		
					Fatty acid (F)	Structure (S)	F × S
Liver (mg/g Protein)							
TG	296 ± 14.1 a	232 ± 21.6 ab	281 ± 29.2 a	187 ± 17.3 b	0.003	0.21	0.54
Cholesterol	29.5 ± 2.4 a	25.3 ± 1.4 ab	21.7 ± 1.3 ab	19.1 ± 1.4 b	0.10	0.002	0.68
Feces							
Cholesterol (mg/day)	47.4 ± 2.5 a	48.8 ± 2.1 a	69.4 ± 2.9 b	64.9 ± 1.9 b	0.53	<0.0001	0.25
Bile acid (μmol/day)	14.4 ± 1.5	15.6 ± 0.6	14.1 ± 0.6	15.6 ± 1.5	0.14	0.66	0.83

Data represent means ± SE (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test.

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TG, triacylglycerol.

SO; soybean oil diet, FO; fish oil diet, SPL; soybean phospholipid diet, SQPL; squid phospholipid diet.

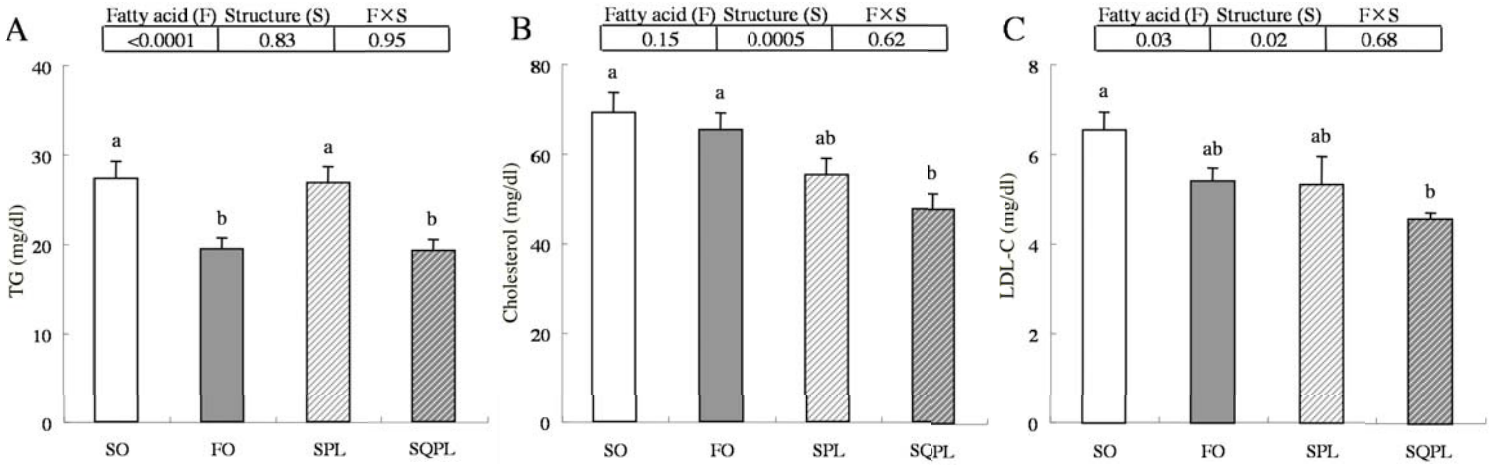


Figure 1 Serum TG (A), cholesterol (B), and LDL-C (C) contents in rats fed experimental diets for 4 weeks.
Data are means ± SE (n=7). Values not sharing a common letter are significantly different at $p < 0.05$.
TG, triacylglycerol; LDL-C, low density lipoprotein-cholesterol.
SO; soybean oil diet, FO; fish oil diet, SPL; soybean phospholipid diet, SQPL; squid phospholipid diet.

Table 5. Relative mRNA expression levels of genes encoding proteins related to lipid metabolism in rats fed experimental diets for 4 weeks.

	SO	FO	SPL	SQPL	ANOVA (*P* values)		
					Fatty acid (F)	Structure (S)	F × S
	(Relative expression levels)						
ABCG5	1.00 ± 0.05 a	1.22 ± 0.08 a	1.40 ± 0.17 a	2.08 ± 0.15 b	0.002	<0.0001	0.08
ABCG8	1.00 ± 0.11 a	1.20 ± 0.15 ab	1.79 ± 0.20 bc	2.01 ± 0.11 c	0.18	<0.0001	0.95
CYP7A1	1.00 ± 0.26	0.91 ± 0.09	0.52 ± 0.11	0.59 ± 0.13	0.96	0.04	0.68
HMGR	1.00 ± 0.09	0.80 ± 0.07	1.02 ± 0.14	0.80 ± 0.08	0.06	0.95	0.93
ACAT-1	1.00 ± 0.11	0.95 ± 0.08	0.91 ± 0.11	1.13 ± 0.07	0.39	0.65	0.19
LDLR	1.00 ± 0.08	0.98 ± 0.09	0.96 ± 0.07	0.82 ± 0.10	0.36	0.27	0.50
SREBP-1c	1.00 ± 0.14	0.82 ± 0.12	0.84 ± 0.15	0.59 ± 0.10	0.11	0.14	0.78
SREBP-2	1.00 ± 0.06	0.79 ± 0.06	1.08 ± 0.07	0.96 ± 0.09	0.08	0.26	0.76

Data represent means ± SE (n=7). The expression levels of mRNAs were determined by quantitative RT-PCR. Relative expression levels are indicated as the ratio of mRNA to GAPDH mRNA. Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test
ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; CYP7A1, cholesterol 7α-hydroxylase; HMGR, 3-hydroxy-3methylglutaryl-coenzyme A reductase; LDL-R, low density lipoprotein receptor; SREBP-1c, sterol regulatory element binding protein-1c; SREBP-2, sterol regulatory element binding protein-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
SO; soybean oil diet, FO; fish oil diet, SPL; soybean phospholipid diet, SQPL; squid phospholipid diet.

Discussion

Many researchers have shown that dietary n-3 PUFAs, such as EPA and DHA, have beneficial health properties, i.e., decrease in serum TG [18], anti-inflammation [19], anti- platelet aggregation [20], and anti-cancer [21]. Thus, most research attention is directed to the effects of EPA and DHA in oils. In addition, experimental reports have also shown that dietary PLs have various physiological effects compared with TG [6-9, 22, 23]. However, a few studies have focused on the effect of dietary PLs containing n-3PUFAs in marine resources on blood and liver lipid contents in experimental animals. This study examined the effects of dietary PLs containing n-3PUFAs extracted from squid mantle muscle on lipid metabolism in rats compared with dietary fish oil and SPLs.

Dietary squid PLs decreased serum and liver TG contents, as shown in Fig. 1A and Table 4. It is thought that the decrease in TG contents in serum and liver might be mainly due to the suppression of fatty acid synthesis and enhancement of fatty acid β-oxidation in the liver. Shirouchi *et al.* [3] suggested that dietary omega-3 PUFA in PC can decrease fatty acid synthesis through the transcriptional suppression of SREBP-1c compared with egg-PC in Otuka Long-Evans Tokushima fatty rats. It is known that SREBP-1c plays a key role in hepatic transcriptional regulation of lipogenic enzymes such as acetyl-CoA carboxylase, fatty acid synthase, and stearoyl-CoA desaturase [24]. This study, the SQPL diet decreased SREBP-1c expression levels by 41.5% as compared with the SO diet. Although liver fatty acid synthesis and fatty acid β-oxidation enzymes, such as carnitine palmitoyl transferase-2 (CPT-2) and acyl-CoA oxidase (ACOX) activities, were not analyzed, it is at least in part predicted that the decrease in TG contents by dietary squid PLs was attributable to the suppression of fatty acid synthesis through the suppression of SREBP-1c at the mRNA expression level. Furthermore, the interactions between dietary fatty acids and lipid structure on SREBP-1c expression level was not significant (p = 0.78). Dietary squid PLs generated decreased SREBP-1c expression levels with an additive effect of PLs and n-3PUFAs.

As indicated in Fig. 1B and Table 4, dietary squid PLs decreased serum and liver CHOL contents, which were lower than in the SO and FO groups. To clarify the mechanism for the decrease in serum and liver CHOL contents, the effects of squid PLs

on fecal CHOL and bile acid excretion was analyzed. Nagata *et al.* [25] found that the decrease in serum CHOL c ontent depends on the extent of fecal steroids excretion. The SPL and SQPL diets increased fecal CHOL excretion whereas the SO and FO diets did not (Table 4). It was suggested that the increased fecal CHOL excretion was due to dietary PLs but not to dietary n-3 PUFAs. Imaizumi *et al.* [26] suggested that dietary SPLs increased the fecal excretion of neutral sterols, and dietary PE and PC increased the excretion of fecal neutral steroids to a similar extent [8]. In this study, SPLs and squid PLs were rich in PC and PE, respectively (76.2% PC and 12.2% PE in SPLs, 75.9% PC and 9.3% PE in squid PLs w/w). On the other hand, dietary PC and PE did not affect extent the excretion of fecal acidic steroids [8] while dietary phosphatidylinositol (PI) increased fecal bile acid excretion compared with dietary soybean oil in Zucker (fa/fa) rats [27]. This study, SPLs and squid PLs contained very low levels of PI (data not shown), resulting in no effect on fecal bile acid excretion. Therefore, it was suggested that the decrease in serum and liver CHOL contents by dietary PLs containing n-3PUFAs was attributable to increased fecal CHOL excretion, which was due to the effects of PC and PE in PLs but did not affect fecal bile acid excretion because of dietary n-3 PUFAs and PC and PE in PLs.

CHOL homeostasis is maintained by a balance of export, biosynthesis, storage, catabolism, and uptake. Therefore, the liver mRNA expression levels of genes encoding proteins involved in CHOL homeostasis were analyzed. ABCG5 and ABCG8 play a major role in the export of CHOL into bile when they form heterodimers. The expression levels of ABCG5 and ABCG8 were increased by the PLs diet compared with the TG diet. A previous study showed that dietary soybean lecithin stimulates bile formation and biliary lipid secretion, and particularly CHOL secretion into the bile [22f]. Dietary PLs increased the export of CHOL into bile through the increases in ABCG5 and ABCG8 expression levels. Furthermore, dietary n-3 PUFAs increased ABCG5 and ABCG8 expression levels compared with dietary soybean oil fatty acids (respectively, p = 0.002 and 0.14). It was suggested that the decrease in serum and liver CHOL contents by dietary PLs containing n-3PUFAs was due to the increased export of CHOL into bile through the enhancement liver ABCG5 and ABCG8 mRNA expression levels. The increased ABCG5 and ABCG8 expression levels also influence fecal CHOL excretion. It is necessary to perform further experiments in order to clarify the mechanism that determines the level of biliary excretion of CHOL.

Excess CHOL is normally eliminated from the liver mainly via bile acids via CYP7A1 [28]. It was suggested that the decrease in CYP7A1 expression level in the dietary PLs groups was due to decreases in liver CHOL accumulation. The expression level of ACAT-1, a rate-limiting enzyme of CHOL storage, was not different among the groups. Previous studies have shown that dietary soybean lecithin decreases liver ACAT-1 activities [22] and dietary PI decreases ACAT-1 mRNA expression levels [27]. However, dietary SPLs and squid PLs did not affect ACAT-1 expression levels in this study although the reason was not clear. The expression level of HMGR, a rate-limiting enzyme of CHOL synthesis, tended to decreased due to n-3 PUFAs compared with soybean oil fatty acids ($p = 0.06$). A previous study showed that fish oil suppresses the activity of HMGR [29]. Hence, it was suggest that a decrease in HMGR expression level in the dietary squid PLs groups was due to dietary n-3 PUFAs, but dietary PLs did not show any effect. The gene expression and proteolytic activation of SREBP-2 in liver is controlled by the content of sterols in the liver [30]. SREBP-2 regulates target genes, such as HMGR and LDLR. SREBP-2 expression levels in dietary n-3 PUFAs tended to decrease compared with those in the dietary soybean oil fatty acid group ($p = 0.08$), but dietary squid PLs did not show any effect. Therefore, it was predicted that the decreased HMGR expression levels in the dietary n-3PUFAs group were due to the suppression of SREBP-2.

Serum LDL-C content was lower in SQPL diet fed rats than in soybean oil diet fed rats (Fig. 1C). A major regulator of circulating blood LDL-C is LDLR [31], a key enzyme required for CHOL uptake into cells. The expression level LDLR in rats fed an SQPL diet tended to decrease compared with rats fed an SO diet (82%). Although the decrease in serum LDL-C content was observed in rats fed squid PLs, a previous study showed that the suppression of SREBP-1c leads to a reduction in the formation and secretion of very low density lipoprotein (VLDL), which is the precursor of LDL [32]. In this study, dietary PLs containing n-3 PUFAs decreased SREBP-1c expression levels. The results suggested that the decrease in serum LDL-C content was due to the decreased secretion of VLDL by the suppression of SREBP-1c.

Summary

It has been demonstrated that the amount and type of dietary fat are factors involved in the risk of arteriosclerosis and coronary or cerebral artery disease through lipid metabolism. In this study, we investigated the effects of phospholipids (PLs) containing n-3 polyunsaturated fatty acids (PUFAs) on lipid metabolism in rats. PLs containing n-3 PUFAs were prepared from squid (*Todarodes pacificus*) mantle muscle. Groups of male Wistar rats were fed AIN93G diet containing soybean oil (SO, 7%), fish oil (1.2%) + SO (5.8%), soybean PLs (1.8%) + SO (5.2%), or PLs containing n-3PUFAs (1.8%) + SO (5.2%). The following indicators were assayed as indexes of lipid metabolism: triacylglycerol (TG) and cholesterol (CHOL) in serum and liver, fecal CHOL, bile-acid excretion, and liver mRNA expression levels of genes encoding proteins involved in CHOL homeostasis. Serum and liver TG contents decreased significantly in the group fed PLs containing n-3 PUFAs as compared to other groups, accompanied by a significant decline in the expression level of sterol regulatory element binding protein-1c. The decrease in CHOL content in the group fed PLs containing n-3PUFAs was due to the increase in fecal CHOL excretion and the increase of mRNA expression levels of ATP-binding cassette (ABC) G5 and ABCG8 in liver.

Chapter 2

Effects of dietary fish protein on serum and liver lipid contents in rats and the expression of hepatic genes involved in lipid Metabolism

Introduction

Epidemiological evidence from Greenland Inuit and Japanese fishing villages suggests that eating fish and marine animals can prevent CHD [33,34]. Dietary studies from many investigators have similarly shown that regular fish intake affects several humoral and cellular factors involved in CHD and may prevent atherosclerosis, thrombosis, and sudden cardiac death. Some reports have indicated that the beneficial effects of fish are attributable to n-3 PUFAs such as EPA and DHA [18,35]. However, Japanese dietary habits include not only fish oil but also the fish itself, which provides nutrients such as proteins as well as fat. Therefore, this study focused on fish protein, which is important as one of the main nutritional components of fish.

The effects of various nutrients on plasma CHOL have been reported: initially with dietary CHOL and later with amounts of dietary fat. Recently, dietary proteins such as plant and animal proteins have also been found to influence lipid metabolism in human subjects and animals [36,37]. For example, rats fed soy protein had lower levels of plasma CHOL accompanied by an increased fecal excretion of steroids when compared with rats fed casein [38]. Similarly, the effect of dietary fish protein influenced blood lipid contents in rats or rabbits as experimental animals [39-42]. However, few studies have focused on the influence of dietary fish protein on blood and liver lipid contents in experimental animals. In addition, few studies have evaluated the mRNA expression levels, enzyme activities of hepatic lipid metabolism-related enzymes, and fecal lipid contents with reference to dietary animal protein. Therefore, to obtain detailed information, this study examined the effects of fish proteins on gene expression levels and the activity of enzymes involved in CHOL and fatty acid metabolism and the role of nuclear receptors in the metabolism of CHOL and bile acids. This study examined for the first time the effect of fish protein on the mRNA expression levels of nuclear receptors in the metabolism of CHOL and bile acids.

The antiarteriosclerotic effect, which is represented by a decrease in plasma triacylglycerol of fish lipids, that is, EPA and DHA, has been demonstrated in animal models and an epidemiological survey in humans. However, it is not possible to explain the health function of fish-based foods only in terms of EPA and DHA. In fact, it is not necessary to eat only fish oil in the daily diet. Currently, no information is available concerning the antiarteriosclerotic effects of fish protein. Therefore, this study investigated the antiarteriosclerotic disease inhibition mechanism derived from eating fish protein. In addition, there is a report that fish oil does not present an anticholesterol effect. This study, attention was paid to fish protein, which is also an important nutrient derived from fish. This study demonstrated that protein isolated from fish protein affects the serum and liver lipid contents and the activities of hepatic lipid metabolism.

Materials and methods

Sample preparation

Alaska pollock (*Theragra chalcogramma*), widely supplied as raw material of surimi, were used as fish protein. Alaska pollock fillets were obtained from Suzuhiro Co., Ltd. (Odawara, Japan). The fillets were chopped into small pieces, mixed with an equal volume of distilled water, and homogenized in a Waring blender (Waring Products Division, New Hartford, CT) for 2 min after water deprivation. The resulting meat was treated with cold acetone, ethyl acetate, and *n*-hexane to remove protein-associated lipids. Then, the meat was dried under N_2 gas and stored at -30°C.

The composition of fish protein was determined, and the crude protein content, which was assayed according to the Kjeldahl method, was 90.6%. The crude fat content, assayed by the Soxhlet method, was <0.5%. The moisture content was determined as the loss in weight after drying at 105 °C for 24 h and was approximately 7%. Fish protein contained 1.5% ash as measured by the direct ignition method by heating at 550 °C for 24 h.

The molecular weight (MW) distribution of the dietary proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE, 12.5%) electrophoresis with SDS-PAGE standard (Broad) standards (Bio-Rad Laboratories).

Animal care

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the "Guide for the Care and Use of Experimental Animals" of the Prime Minister's Office of Japan. Five-week-old male Wistar rats obtained from Shimizu Laboratory Supplies Co., Ltd., were kept in an air-conditioned room (temperature, 20-22 °C; humidity, 55-65%; lights on, 8:00 a.m.-8:00 p.m.) and had free access to drinking tap water. The rats were fed a diet prepared according to the recommendations of AIN-93G [11]. After acclimation for 3 days with an AIN-93G diet, the rats were divided into the following eight dietary groups of seven rats each: control (Cont) diet; fish protein (FP) diet; high-fat (HF) diet; high-fat fish protein (HF-FP) diet; control plus CHOL (Cont+C) diet; fish protein plus CHOL (FP+C) diet; high-fat plus CHOL (HF+C) diet; and high-fat fish protein plus CHOL (HF-FP+C) diet. The experimental diets were prepared according to the AIN-93G scheme (Table 6). All of the diet ingredients were products of Oriental Yeast Co., Ltd.. After treatment with the experimental diets for 4 weeks, the rats were weighed and sacrificed under diethyl ether anesthesia. Blood was collected, and serum was harvested by centrifugation at 1500× g for 15 min before being stored at -80°C until analysis. Liver, abdominal WAT, and interscapular brown adipose tissue (BAT) were rapidly removed in their entirety and weighed, rinsed, frozen in liquid nitrogen, and kept at -80°C. The liver was taken for mRNA expression analysis and stored in RNA- Later Storage Solution (Sigma Chemical Co.,).

Table 6. Composition of the experimental diets (g/kg)

Components	Normal fat content diet				High fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
Dextrinized corn starch	132	132	132	132	60.2	60.2	60.2	60.2
Corn starch	397.5	397.5	391.5	391.5	181.3	181.3	175.3	175.3
Casein	200	100	200	100	258	129	258	129
Fish protein	-	100	-	100	-	129	-	129
Sucrose	100	100	100	100	100	100	100	100
Cellulose powder	50	50	50	50	50	50	50	50
AIN93G mineral mixture	35	35	35	35	35	35	35	35
AIN93 vitamin mixture	10	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Soybean oil	70	70	70	70	70	70	70	70
Lard	-	-	-	-	230	230	230	230
Cholesterol	-	-	5	5	-	-	5	5
Cholic acid	-	-	1	1	-	-	1	1

Diets were prepared based on the AIN-93G composition.

FP: fish protein group.

Analysis of the serum parameters

Total lipid (TL), PL, TG, CHOL, HDL-C, and LDL-C analyses were performed by a commercial service (Japan Medical Laboratory, Osaka, Japan).

Analysis of the liver lipid composition and enzyme activity measurements.

Liver lipids were extracted with a mixture of chloroform/methanol/water (1:1:0.9, v/v). PL were separated by silica gel column chromatography using chloroform and methanol as elution solvents. The CHOL content was analyzed by GC (GC-14B, SHIMAZU Co.,) using 5α-cholestane as an internal standard. The TG content was calculated by subtracting the PL and CHOL contents from the TL content.

The liver was homogenized in 6 volumes of a 125 mM NaCl containing 1 mM EDTA-2Na and 10 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 500 × g for 10 min at 4 °C. The supernatant was recentrifuged at 9000 × g for 10 min at 4 °C to sediment the mitochondria, and the remaining supernatant was collected. The activity of CPT-2 in the mitochondrial fraction was measured as described by Markwell *et al.* [43]. The activities of fatty acid synthase (FAS) [44], acetyl-coenzyme A carboxylase (ACC) [45], glucose-6-phosphate dehydrogenase (G6PDH) [46], malic enzyme (ME) [47], ATP-citrate lyase (ACL) [48], and microsomal phosphatidate phosphatase (PAP) [49] in the supernatant fraction were measured spectrophotometrically. Protein contents were determined according to the method of Bradford [16] using BSA as a standard.

Analysis of fecal bile acid, cholesterol, and lipid content

Feces were collected for 7 days after acclimation of the rats to the experimental conditions and diets. Total bile acids in the feces were determined as micromoles of 3α-hydroxysteroid based on the molar extinction coefficient of NADH at 340 nm [50]. Fecal CHOL was determined using 5α-cholestane as an internal standard, and the lipid content was extracted with a mixture of chloroform/methanol/ water (1:1:0.9, v/v).

Western blot analysis

BAT tissue was homogenized in 5 volumes of 10 mM Tris-HCl and 1 mM EDTA-2Na (pH 7.4) for 30 sec using a Polytron (Pellet Pestles, Kontes, Vineland, NJ). After centrifugation at 9000 × g for 5 min, the fat cake was discarded, and the infranant (fat-free extract) was used for the Western blotting analysis of uncoupling protein-1 (UCP-1). The total protein content in BAT was measured according to the method of Bradford [16]. The supernatants (BAT 30 μg of protein/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membrane. The membrane was incubated with an antibody against UCP-1 (Sigma Chemical Co.,) for 1 hour and then incubated with a secondary rabbit IgG-antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz) for 1 hour at room temperature. The membranes were treated with ECL Western Blotting Detection reagent (GE Healthcare UK Ltd., U.K.) according to the manufacturer's instructions. β-Actin was detected as a control with a β-actin antibody (Santa Cruz Biotechnology).

mRNA analysis

Total RNA was extracted from liver using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Then, cDNA was synthesized from total RNA using a High- Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Real-time quantitative RT-PCR analysis was performed with an automated sequence detection system (ABI Prism 7000; Applied Biosystems Japan Ltd.). PCR cycling conditions were 50 °C for 2min and 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1 min. HMGR, CYP7A1, ACAT1, SREBP-2, peroxisome proliferation activated receptor alpha (PPARα), and GAPDH mRNA expression levels were measured using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.). PCR Primers (HMGR: Rn00565598_m1; CYP7A1: Rn00564065_m1; ACAT1: Rn00567139_m1; SREBP-2: Rn01502638_m1; PPARα: Rn00566193_m1; GAPDH: Rn99999916_s1) were purchased from Applied Biosystems Japan Ltd. The expression signal of the housekeeping gene GAPDH served as an internal control for normalization.

Moreover, LDLR, farnesoid X receptor-α (FXRα), small heterodimer parter-1

(SHP-1), liver receptor homolog-1 (LRH-1), and GAPDH mRNA expression levels were measured using SYBR Green PCR Master Mix (Applied Biosystems). The PCR solution (25 μL) was composed of 12.5 μL of SYBR Green PCR Master Mix solution, 5 μL of template cDNA, 1 μL of forward primer, 1 μL of reverse primer, and 5.5 μL of RNase free water. The primer sequences used for the detection of LDLR, FXRα, SHP-1, LRH-1, and GAPDH were as follows: forward, 5'CACCCCCTCGTTGAAAACCT3', and reverse, 5'CCTTAGCCAGCTCTTCCAGATC3', for LDLR; forward, 5'GGGCCTTGGACGTCTCTGA3', and reverse, 5'CTGGGA-TGGTGGTCTTCAAATAA3', for FXRα; forward, 5' CGCCTGGCCCGAATC3', and reverse, 5' GAAGGGTACAGGAGATGTTCTTGAG3', for SHP-1, forward, 5'TCCGGGCAATCAGCAAA3', and reverse, 5'CCCCATTCACGTGCTTGTAGT3', for LRH-1; and forward, 5'GAAGACACCAGTAGACTCCACGACATA3', and reverse, 5'GAAGGTCGGTGTGAACGGATT3', for GAPDH. The PCR cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60°C for 1min.

Statistical analysis

Data are expressed as means ± standard deviation (SD) of seven rats. Statistical analyses between multiple groups were determined by one-way ANOVA. Statistical comparisons were made using the Tukey-Kramer test. Differences with $p < 0.05$ were considered to be significant. The analyses were performed using Stat View-J version 5.0 software (Abacus Concept, Berkeley).

Results

Molecular weight of fish protein

Figure 2 shows coomassie blue stained SDS-PAGE of casein and fish protein The fish protein was detected mostly as two bands (ca. 200 and 45 kDa), which may be derived from myofibrillar protein.

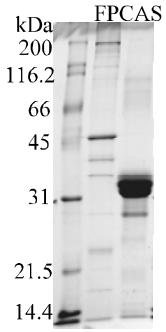


Figure 2 Coomassie blue stained SDS-PAGE of casein (CAS) and fish protein (FP)

Growth parameters

Body weight gain, food intake, food efficiency ratio, liver weight, and WAT weight are presented in Table 7. The diet intake tended to be higher in the HF-FP+C group than in the other groups, but the energy efficiency ratio was not significantly different among the groups. No significant differences in these parameters were observed between the normal fat content and high fat content diets. There were no significant differences in body weight, body weight gain, liver weight, or WAT weight among the groups.

Serum lipid content

Serum lipid contents are presented in Table 8. TL and TG contents tended to be lower in rats fed the fish protein-containing diets compared with rats fed casein. When the rats were fed the normal fat content diet, HDL-C content tended to be higher in rats fed the fish protein-containing diet compared with the casein diet, but no differences were noted compared with rats fed the high fat content diet. When the rats

were fed CHOL-enriched diets, serum CHOL was lower in FP+C and HF-FP+C rats and was significantly lower than in the casein group, whereas rats fed the CHOL-free diets of FP or HF-FP tended to show lower CHOL levels. Dietary fish protein resulted in a reduction in LDL-C compared to casein diets. PL content was not significantly different among the groups.

Liver lipid content and enzyme activities

The liver lipid contents are presented in Figure 3. In the normal fat content diet, hepatic PL was significantly higher in rats fed the CHOL-free diet than in rats fed the CHOL-enriched diet, although in the high fat content diet hepatic PL was significantly lower in rats fed the CHOL-free diet than in rats fed the CHOL-enriched diet. When the rats were fed the CHOL- enriched diet, TL content was significantly lower in rats fed the diet containing fish protein than in rats fed casein. There tended to be a lower content of TG in rats fed the fish protein diets than in rats fed the casein diets. When the rats were fed a CHOL-enriched diet, the CHOL content was markedly lower in rats fed the fish protein diet than in rats fed the casein diet.

The hepatic enzyme activities of the fatty acid metabolic pathway are presented in Table 9. When the rats were fed the normal fat content diet, the hepatic activities of FAS, ACC, G6PDH, and ME were higher in rats fed the diet containing fish protein than in the rats that were fed casein. When the rats were fed the high fat content diet, the hepatic activities of FAS and ACC were higher in rats fed the fish protein diet than in rats fed the casein diet. There were no differences in the hepatic activities of G6PDH, ME, and CPT-2 among the four groups. The activity of PAP, which is the rate-limiting enzyme for TG synthesis in the liver, showed no difference among all groups.

Fecal lipid content

The fecal lipid contents are presented in Figure 4. There was a tendency to higher contents of TL in rats fed the fish protein diet than in rats fed the casein diet. In the CHOL-free diets, the fecal excretion of bile acid and CHOL tended to be higher in rats fed the fish protein diet than in rats fed the casein diet, and for the CHOL-enriched

diets the rats fed the fish protein diet showed significantly greater fecal excretion of bile acid and CHOL than those fed the casein diet.

UCP-1 protein expression in BAT

Figure 5 shows the UCP-1 expression of BAT. There was no difference in the UCP-1 protein expression level in the BAT or BAT weight among all groups.

mRNA expression of hepatic lipid metabolism related enzymes

To examine the effect of dietary fish protein on the level of mRNA lipid metabolism related enzymes, the expression levels of mRNAs related to CHOL metabolism in the liver tissue were determined using quantitative real-time PCR (Table 10). The level of CYP7A1 was significantly increased by feeding fish protein. SHP-1, the nuclear receptor regulating CYP7A1 expression, tended to be less abundant in rats fed the fish protein diet than in rats fed the casein diet (Figure 3). The gene expression levels of HMGR, ACAT1, LDLR, SREBP-2, FXRα, LRH-1, and PPARα were not significantly different among the groups.

Table 7. Initial body weight, final body weight, body weight gain, energy intake, food efficiency ratio, and relative weights of liver and WAT of rats fed the experimental diets for 4 weeks

	Normal fat content diet				High fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
Growth parameters								
Initial BW (g)	101 ± 9.9	101 ± 3.5	109 ± 8.3	103 ± 4.5	109 ± 3.5	109 ± 6.4	110 ± 5.3	97 ± 4.5
Final BW (g)	307 ± 26.6	311 ± 16.4	314 ± 16.8	303 ± 14.8	291 ± 23.6	290 ± 23.3	306 ± 23.2	316 ± 23.2
BW gain (g/day)	7.1 ± 0.6	7.2 ± 0.6	6.9 ± 0.6	6.7 ± 0.4	6.6 ± 0.7	6.4 ± 0.7	6.9 ± 0.8	7.8 ± 0.9
Energy intake (kcal/day)	68.3 ± 7.2 ab	73.1 ± 7.7 b	67.0 ± 3.8 ab	65.2 ± 4.5 a	64.5 ± 9.1 a	64.8 ± 11.5 a	73.6 ± 9.0 ab	81.9 ± 16.5 b
Food efficiency (g/kcal)	0.106 ± 0.010	0.101 ± 0.008	0.105 ± 0.009	0.105 ± 0.007	0.098 ± 0.011	0.099 ± 0.011	0.092 ± 0.011	0.095 ± 0.011
Organs weights (g/100g BW)								
Liver weight	3.9 ± 0.2	3.6 ± 0.2	5.4 ± 0.5	5.0 ± 0.2	3.8 ± 0.2	3.6 ± 0.4	5.3 ± 0.6	5.0 ± 0.3
WAT weight	4.8 ± 1.0	5.0 ± 0.7	4.4 ± 0.6	4.1 ± 0.4	5.5 ± 0.7	4.9 ± 0.7	5.3 ± 1.1	5.3 ± 0.8

Food consumption and body weight were recorded every two days. Liver and WAT figures were obtained after sacrifice and weight measured.

Data are means ± SD (n=7).

Food efficiency = The data were calculated from the BW gain per energy intake.

Table 8. Lipid concentrations in serum of rats fed the experimental diets for 4 weeks (mg/dl)

	Normal fat content diet				High fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
TL	244 ± 16	232 ± 26	252 ± 33	239 ± 23	258 ± 19	242 ± 22	271 ± 28	260 ± 25
TG	33.0 ± 3.2	30.6 ± 3.9	32.9 ± 6.0	32.9 ± 6.0	36.3 ± 5.7	32.3 ± 4.2	38.3 ± 6.8	35.1 ± 3.9
PL	131 ± 19	126 ± 20	128 ± 23	141 ± 23	129 ± 18	144 ± 24	145 ± 19	136 ± 20
CHOL	77.6 ± 8.7 ac	70.7 ± 5.5 bc	84.9 ± 10.6 a	75.9 ± 6.7 c	83.4 ± 6.3 ac	75.9 ± 5.8 a	91.0 ± 8.9 ab	80.7 ± 6.9 c
HDL-C	67.9 ± 7.8	74.3 ± 5.8	66.3 ± 7.8	70.0 ± 7.8	61.7 ± 7.4	62.6 ± 9.8	65.7 ± 8.1	63.9 ± 6.5
LDL-C	5.3 ± 0.8 ab	4.8 ± 0.7 ab	5.7 ± 0.4 a	4.6 ± 1.1 b	5.4 ± 1.0 ab	4.7 ± 0.5 ab	5.7 ± 0.9 a	5.1 ± 0.8 b

Data are means ± SD. Values in the same row not sharing a common letter are significantly different at $p<0.05$ by Turkey-Kramer test.

TL, total lipid; TG, triacylglycerol; PL, phospholipid; T-Chol, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

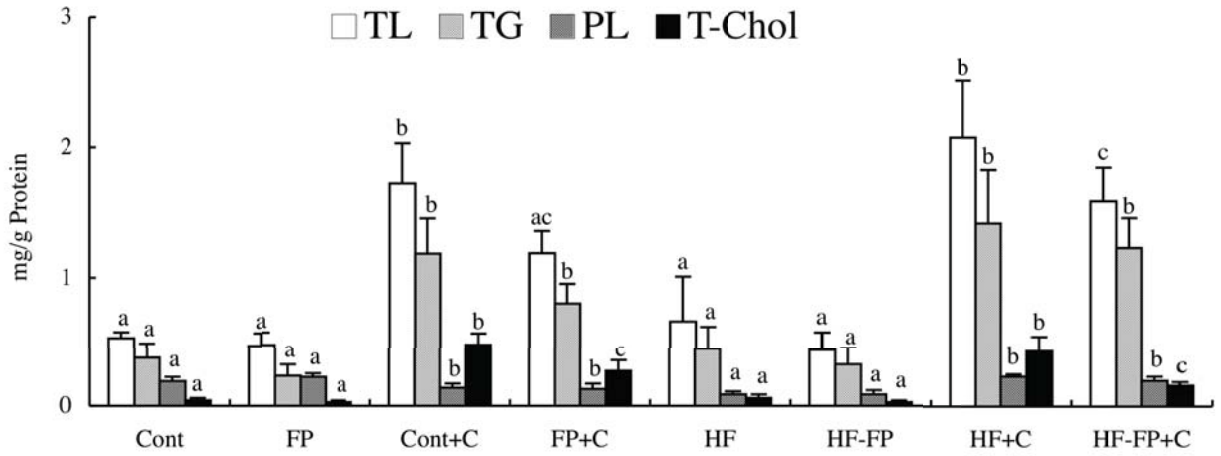


Figure 3. Lipid concentrations in the liver of rats fed the experimental diets for 4 weeks.
Data are means ± SD. Values not sharing a common letter are significantly different at $p < 0.05$.
TL, total lipid; TG, triacylglycerol; PL, phospholipid; T-Chol, total cholesterol.

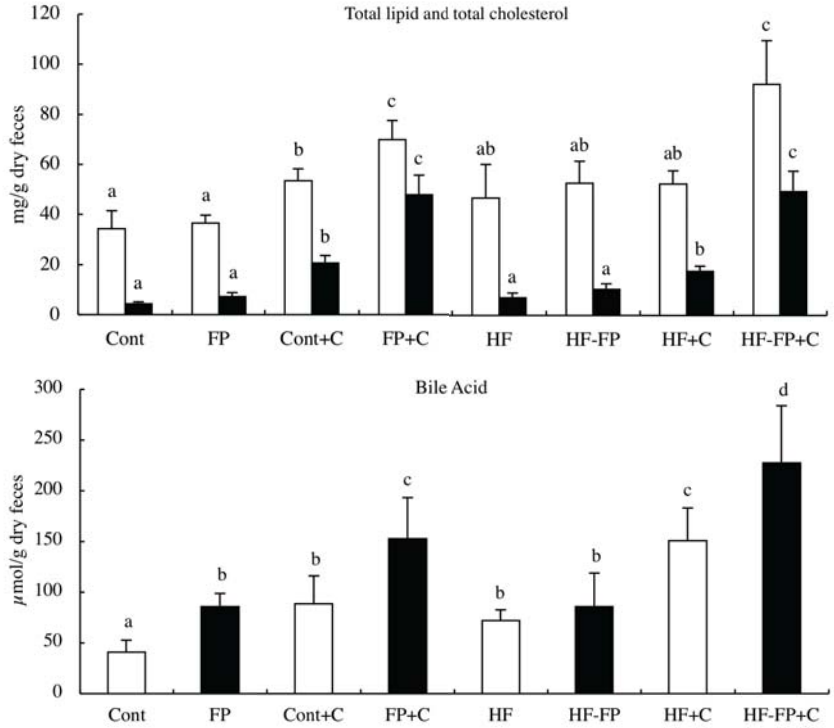


Figure 4. Total lipid and total cholesterol (a) and bile acid (b) in the feces of Wistar rats fed the diets containing casein (white bars) or fish protein (black bars).
Data are means ± SD.Values not sharing a common letterare significantly different at $p < 0.05$.TL, total lipid; T-Chol, total cholesterol.

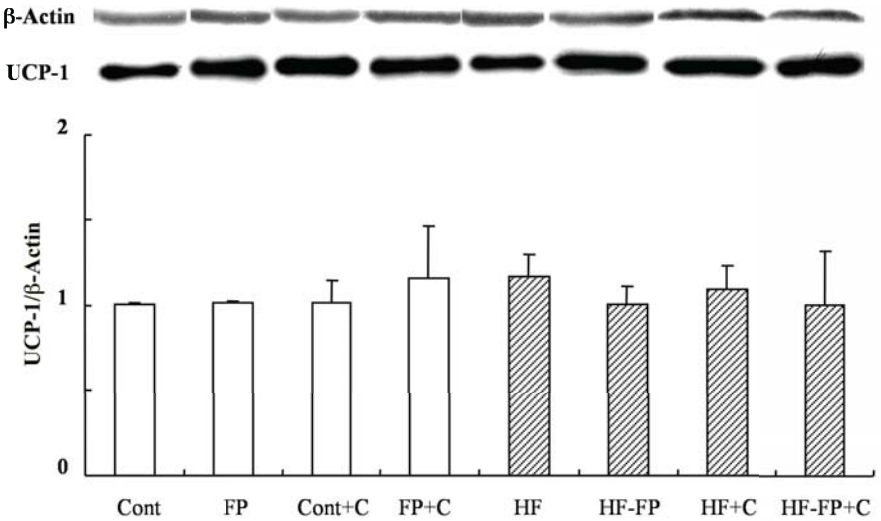
Table 9. Activities of enzymes related to the fatty acid metabolic pathways in the livers of wistar rats (nmol / min mg protein)

	Normal fat content diet				High fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF+C	HF-FP+C
FAS	1.91± 0.44 a	2.36± 0.51 ab	3.09± 0.39 b	2.72± 0.45 b	1.60± 0.35 a	2.04± 0.47 ab	1.76± 0.37 ab	2.51± 0.42 b
ACC	65.0± 7.5 ab	92.3± 8.7 a	72.4± 9.2 ab	75.9± 8.8 b	90.4± 9.3 a	89.8± 8.5 a	88.7± 12.9 a	118.2± 14.6 b
G6PDH	8.65± 2.34 ab	15.60± 5.94 a	5.59± 1.50 ab	5.18± 1.97 b	4.11± 1.27	4.74± 8.52 a	4.00± 0.89	4.19± 0.73
ME	2.84± 1.37 ab	6.50± 1.46 a	1.90± 0.49 ab	1.68± 0.38 b	1.42± 0.39	1.50± 0.44	1.15± 0.89	1.06± 0.37
ACL	26.7± 4.3 a	27.4± 4.4 a	13.4± 4.4 b	13.5± 4.6 b	9.0± 0.5	7.5± 1.8	5.7± 1.3	5.7± 0.7
PAP	6.58± 0.35	6.47± 1.16	7.09± 0.59	6.43± 0.42	5.62± 0.54	5.38± 0.49	5.41± 0.61	5.49± 0.58
CPT-2	2.40± 0.76	2.26± 0.64	1.95± 0.47	2.21± 0.62	1.57± 0.33	2.35± 0.43	2.02± 0.44	1.93± 0.65

Data are means ± SD. Values in the same row not sharing a common letter are significantly different at $p<0.05$ by Turkey-Kramer test.

FAS, fatty acid synthase; ACC, acetyl-coenzyme carboxylase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme; ACL, ATP-citrate lyase; PAP, phosphatidate phosphatase; CPT-2, carnitine palmitoyl transferase-2.

Figure 5. The UCP-1 expression of BAT of rats fed the experimental diets for 4 weeks.



Protein expression levels of UCP-1 are shown relative to the BAT of rats fed the Cont diet (= 1.00).
UCP-1; uncoupling protein-1.

Table 10. Expression Levels of mRNA in Rat Liver Estimated by Quantitative Real-Time RT-PCR (mRNA per GAPDH)

	Normal fat content diet				High fat content diet			
	Cont	FP	Cont+C	FP+C	HF	HF-FP	HF-C	HF-FP+C
	(Relative expression levels)							
HMGR	1.00 ± 0.35	1.05 ± 0.18	0.84 ± 0.27	0.76 ± 0.09	1.49 ± 0.44	1.13 ± 0.45	1.12 ± 0.45	1.06 ± 0.24
ACAT-1	1.00 ± 0.14 a	1.24 ± 0.25 a	1.85 ± 0.32 b	1.48 ± 0.12 ab	1.70 ± 0.30	1.80 ± 0.34	2.10 ± 0.66	2.20 ± 0.42
CYP7A1	1.00 ± 0.39 ab	1.77 ± 0.45 ab	3.28 ± 2.52 b	7.93 ± 2.71 c	2.31 ± 0.89 a	5.55 ± 3.69 ab	6.26 ± 1.84 b	18.34 ± 4.47 c
LDL-R	1.00 ± 0.33	1.12 ± 0.24	1.17 ± 0.13	0.96 ± 0.18	1.45 ± 0.37	1.37 ± 0.26	1.96 ± 0.54	2.01 ± 0.61
SREBP-2	1.00 ± 0.25	1.17 ± 0.20	1.13 ± 0.13	0.93 ± 0.26	1.52 ± 0.39	1.66 ± 0.42	1.20 ± 0.35	1.25 ± 0.14
FXRα	1.00 ± 0.37	1.36 ± 0.46	1.11 ± 0.31	0.79 ± 0.08	1.56 ± 0.65	1.51 ± 0.17	1.96 ± 0.32	1.96 ± 0.43
LRH-1	1.01 ± 0.60	0.96 ± 0.35	0.95 ± 0.34	0.73 ± 0.28	1.66 ± 0.43	1.35 ± 0.41	2.83 ± 0.91	2.18 ± 1.13
SHP-1	1.01 ± 0.60 ab	0.78 ± 0.34 ab	0.95 ± 0.34 a	0.66 ± 0.22 b	1.66 ± 0.43	1.23 ± 0.50 a	2.51 ± 0.53 b	1.53 ± 0.65 ac
PPARα	1.00 ± 0.32	1.15 ± 0.28	1.35 ± 0.42	1.16 ± 0.22	1.79 ± 0.45	1.79 ± 0.32	2.05 ± 0.88	1.87 ± 0.30

Data are means ± SD. Values in the same row not sharing a common letter are significantly different at $p<0.05$ by Turkey-Kramer test. Relative values are presented as the ratio of mRNA to GAPDH mRNA.

HMGR, 3-hydroxy-3methylglutaryl-coenzyme A reductase; ACAT-1, acyl-coenzyme:cholesterol acyltransferase-1; LDL-R, low-density lipoprotein receptor; SREBP-2, sterol regulatory element-binding protein-2; FXRα, farnesoid X receptor-α; LRH-1, liver receptor homologue-1; PPARα, peroxisome proliferation activated receptor alpha.

Discussion

The results of this study demonstrate that fish protein compared to casein led to a decrease in the liver CHOL and serum CHOL and LDL-C contents. Brandsch *et al.* suggested that protein extracts from beef, pork, and turkey meat did not alter the CHOL content in the plasma or liver compared with casein [36]. These results indicate a possibility that CHOL metabolism changes in the liver and serum were involved in the anti-atherosclerotic disease potential of fish protein compared with animal proteins such as casein, beef, pork, and turkey.

The liver TG content tended to be lower in fish protein-fed rats than in the casein-fed rats (Figure 3). The reason for the decrease in TG content in the liver was thought to be the absorption of lipid from the small intestine and/or the metabolism of lipids and fatty acids in the liver. The enzyme activities of fatty acid β-oxidation in the mitochondria for fatty acid synthesis in the liver were examined when the rats were fed the normal fat content diet, and the hepatic activities of fatty acid synthesis in rats fed fish protein were higher than in rats fed casein, whereas the enzymes of β-oxidation in liver mitochondria showed no differences. When the rats were fed high fat content diets, there were no differences in the hepatic activities of fatty acid synthesis and the hepatic activities of fatty acid oxidation of the rats fed fish protein were greater than in those fed casein. However, the activity of PAP, which is the rate-limiting enzyme for TG synthesis in the liver, showed no differences among all of the diets. Moreover, the expression level of PPARα, which is a transcription factor that has important effects on lipid homeostasis via regulation of the expression of genes involved in hepatic fatty acid uptake and oxidation in peroxisomes, showed no differences among all of the groups [51]. The UCP-1 expression level in BAT, which is known as a significant component of whole body energy expenditure and its dysfunction contributes to the development of obesity, showed no differences among all of the diets [52]. Therefore, it was considered that the decrease in liver TG was not caused by the enzyme activities of fatty acid β-oxidation and synthesis in the liver but rather by a decrease in the absorption of lipids from the small intestine in rats fed on fish protein. When the rats were fed CHOL-enriched diets, the hepatic activities of fatty acid synthesis and oxidation were not sufficient, and the livers of CHOL-fed rats exhibited hypertrophy due to accumulated lipids. The fecal TL content of rats fed fish protein was greater than that

of rats fed the casein diet (Figure 4). Consequently, it was hypothesized that the decrease in the liver TG content was due to the inhibition of lipid absorption from the small intestine by digested fish protein. The digested fish protein may inhibit lipase activity and micelle formation in the small intestine. Therefore, it is probable that the fish protein decreased the TG content in the liver.

Remarkable differences were observed in the CHOL content in the serum and liver in rats fed fish protein (Table 8; Figure 3). In the CHOL-enriched diet, dietary fish protein, as compared with dietary casein, significantly decreased the serum CHOL and LDL-C and the liver CHOL content. In the CHOL-free diets, a similar decrease was observed. The reason for the decrease in CHOL content in the liver was thought to be the down-regulation of genes involved in CHOL synthesis and CHOL uptake via decreased levels of mRNA coding for SREBP-2 and the up-regulation CYP7A1, which is the initial and rate-limiting enzyme in the conversion of CHOL to 7α-hydroxylated bile acid, via a FXRα-mediated pathway. In this study, fish protein did not affect the hepatic gene expression levels of HMGR, LDLR, or SREBP-2, and the CYP7A1 expression level was higher in rats fed the fish protein diet than in rats fed the casein diet. Hence, in the livers of rats of fed fish protein, enhanced rates of the hepatic catabolism of CHOL via bile acid were observed, but the synthesis and uptake of CHOL were not changed.

In this study, the fecal excretion of CHOL and bile acid was greater in rats fed fish protein than in the rats fed casein. Hence, the dietary fish protein effectively inhibited the absorption CHOL and bile acid in the small intestine. These results suggest the suppression of CHOL absorption by the micellar solubility of CHOL and the binding capacity of cholate of digested fish protein in the jejunal epithelia. Furthermore, the digestion of fish protein may also inhibit the reabsorption of bile acids in the ileum.

Recently, attention has been focused on the roles of nuclear receptors in the metabolism of CHOL and bile acids [53]. Bile acids negatively regulate the transcription of CYP7A1 via the activation FXRα, that is, a SHP-dependent pathway. In this study, the mRNA expression level of hepatic SHP-1 was significantly lower in rats fed fish protein compared with rats fed a CHOL-enriched diet. It is known that the reduction of SHP-1 activates LRH-1, which binds to the promoter of CYP7A1 and increases its mRNA expression level. This study suggests that the CYP7A1

expression level was higher in rats fed the fish protein diet than in rats fed the casein diet, which resulted from the digested fish protein inhibiting the reabsorption of bile acids in the ileum. The FXRα/SHP-dependent pathway was negatively regulated by the decreased reabsorption of bile acid, and increased binding to the promoter of CYP7A1 by LRH-1 activated the expression of CYP7A1.

The serum LDL-C content was lower in the fish protein fed than in the casein-fed rats. In the case of dietary soy protein, the LDL-C content was decreased as a result of an increase in LDLR activity [54]. In this study, the mRNA expression level of hepatic LDLR was not significantly different among the diets. The decreased LDL-C content observed in the rats fed fish protein could possibly be related to a decrease in VLDL secretion, which is the precursor of low density lipoprotein (LDL), or an increase in the removal of VLDL by VLDL receptors in hepatocytes [55]. In a previous study, Shukla *et al*. showed that a decrease in the serum CHOL content was due to reduced serum HDL-C content on feeding fish protein from Alaska pollock to rats [39]. However, in this study, dietary fish protein did not affect the serum HDL-C content. The reason for the difference in HDL-C metabolism due to the intake of fish protein is unclear at present.

Summary

Dietary proteins influence the lipid metabolism of human subjects and animals. This study evaluated the effects of fish protein on lipid metabolism in rats. Alaska pollock fillets, widely supplied as raw materials of surimi, were used as fish protein. As parameters of lipid metabolism, cholesterol and triacylglycerol concentrations in the serum and liver, the fecal excretion of bile acids, and the hepatic expression of genes encoding proteins involved in lipid homeostasis were examined. Rats fed fish protein showed decreased cholesterol concentrations in the serum and liver, and fecal bile acid and cholesterol concentrations were increased. This was caused by the increased expression of cholesterol 7α-hydroxylase as the digested fish protein inhibited the absorption of bile acid and cholesterol in the small intestine. In addition, it was found that dietary fish protein affects the farnesoid X receptor/small heterodimer partner-dependent pathway, which is negatively regulated by the decreased reabsorption of bile acid. Furthermore, it increased the binding to the promoter of cholesterol 7α-hydroxylase through activated liver receptor homologue-1.

Chapter 3

Effect of dietary protamine on lipid metabolism in rats

Introduction

In the past, the potential health functions of fish tissues, except for muscle, have attracted little attention. For example, for the testes and ovaries, which are considered as edible parts, there is only information concerning the high levels of CHOL and nucleic acids. Therefore, we focused on the effect of protamine from salmon milt on lipid metabolism. Protamine from salmon milt, also called salmine, has a low molecular weight of around 4000 - 5000, which plays a role in protecting DNA from being damaged. As a pharmaceutical product, protamine acts as an antidote to heparin, maintains antihyperglycemic effects together with insulin, and also functions to restrict fat absorption in the intestine [56]. In addition, protamine has also been widely used as a natural food preservative, because protamine has a strong antibacterial effect [57]. Previous studies showed that protamine strongly inhibited the hydrolysis of trioleoylglycerol emulsified with PC [58] and protamine reduce weight gain and body fat accumulation through the inhibition of dietary fat absorption [59]. However, few studies have evaluated the beneficial effect of protamine besides the inhibition of fat absorption. To examine the effect of dietary protamine on lipid metabolism, we evaluated serum and liver lipid contents, lipid metabolizing enzyme activities in the liver, the excretion of fecal lipid, and the expression mRNA levels of CHOL-related enzymes in rats.

Materials and Methods

Materials

Salmon protamine was provided by Asama Chemical Co., Ltd. (Tokyo, Japan). AIN-93 vitamin mix, AIN-93G mineral mix, dextrinized cornstarch, cornstarch, cellulose, sucrose, and casein were purchased from Oriental Yeast Co., Ltd. L-Cystine, choline bitartrate, and soybean oil were purchased from Wako Pure

Chemical Industries, Ltd.. All other chemicals used were of reagent grade.

Animal care and experimental diets

Five-week-old male Wistar rats obtained from Shimizu Laboratory Supplies Co., Ltd. were housed in plastic cages in an air-conditioned room (temperature, 20-22°C; humidity, 55-65%; lights on, 08:00-20:00 h). After acclimation for 3 days with a diet prepared according to that recommended by AIN-93G [11], rats were divided randomly into three groups and given free access to water and the experimental diet. Table 11 shows the composition of the experimental diets prepared according to AIN-93G. Experimental diet consumption and body weight were recorded every two days. Feces were collected from each group every 24 hours for seven days prior to sacrifice. After treatment with the experimental diets for 4 weeks, rats were weighed and sacrificed under pentobarbital (Nembutal®, Dainippon Sumitomo Pharma) anesthesia. Rats were not fasted before being sacrificed because food deprivation prior to sacrifice leads to a significant down-regulation of the genes involved in fatty acid synthesis and CHOL metabolism [13]. Blood was collected from the descending aorta, and serum was prepared by centrifugation at 1,500 × g for 15 min and then stored at –80°C until analysis. Liver and abdominal WAT were rapidly removed in their entirety and were weighed, rinsed, frozen in liquid nitrogen, and kept at –80°C. Aliquots of the liver were taken for mRNA expression analysis and stored in RNA-Later Storage Solution (Sigma Chemical Co.). The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the "Guide for the Care and Use of Experimental Animals" of the Prime Minister's Office of Japan.

Table 11. Composition of experimental diets

Components	Diet group		
	Control	ProtL	ProtH
Dextrinized corn starch	132	132	132
Corn starch	397.5	397.5	397.5
Casein	200	180	150
Salmon protamine*	-	20	50
Sucrose	100	100	100
Cellulose	50	50	50
AIN93G mineral mixture	35	35	35
AIN93 vitamin mixture	10	10	10
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Soybean oil	70	70	70

Diets were prepared based on the AIN-93G composition.

*Protamine purity of salmon protamine was 83.5%.

Analysis of experimental diets and Salmon protamine amino acid composition

The experimental diets and salmon protamine amino acid composition was analyzed by HPLC as follows. The samples were hydrolyzed using 6 M HCl for 24 hours. The hydrolyzed samples were subjected to HPLC, in which the samples were automatically derivatized using *o*-phthalaldehyde and separated on a reversed-phase column (GL Sciences Inertsil ODS-3, 5 μm, 250 × 4.6 mm) at 40°C with 25 mM sodium phosphate buffer (pH 7.0) and acetonitrile using a gradient program at a flow rate of 1.0 mL/min. The eluate was monitored by fluorescence detection with an excitation wavelength of 340 nm and emission wavelength of 450 nm. The identification and quantitation of each amino acid were carried out using commercially available authentic standard mixtures and a CR-6A Chromatopac integrator (SHIMAZU Co.). Table 12 summarizes the amino acid composition of each diet and protamine.

Table 12. Amino acid contents of the experimental diets and salmon protamine (g/kg protein)

Amino acid	Diet group			Salmon protamine
	Control	ProtL	ProtH	
Alanine	33	108	220	6
Arginine	71	64	53	780
Aspartamic	27	24	20	ND
Cysteine	45	41	34	ND
Glutamic acid	50	45	38	ND
Glycine	84	76	63	32
Histidine	49	45	39	ND
Isoleucine	26	23	20	9
Leucine	60	58	54	ND
Lysine	27	25	22	ND
Methionine	16	18	20	ND
Phenylalanine	100	97	92	ND
Proline	190	171	143	67
Serine	46	48	52	69
Threonine	37	33	28	ND
Tryptophan	63	57	47	ND
Tyrosine	11	10	8	ND
Valine	4	4	3	38

ND: Not detected

Analysis of serum and liver lipids

Serum TL, PL, TG, CHOL, HDL-C, and LDL-C contents were analyzed using an Olympus AU5431 automatic analyzer. The VLDL fraction was isolated from the fresh serum by ultracentrifugation (Himac CS-100, Hitachi Koki Co., Ltd., Tokyo, Japan) at a density of <1.006 g/ml after adjustment with NaCl, and then the chylomicron fraction was removed because the rats were not fasted before sacrifice [60]. Serum VLDL TG and CHOL contents were analyzed using enzymatic kits obtained from Wako Pure Chemical Industries, Ltd..

Liver lipids were extracted using a mixture of chloroform:methanol:water (1:1:0.9, v/v). The liver TG content was determined as follows; liver lipids were dissolved in an equal volume of dimethyl sulfoxide and then used to determine the TG content using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical

Industries, Ltd.). The liver CHOL content was analyzed using a SE-30 column with a GC-14B gas chromatograph and 5α-cholestane as an internal standard. The liver PL content was measured by phosphorus analysis of liver lipids, and the liver protein content was determined by the method of Lowry *et al.* using BSA as a standard [61].

Preparation of the liver and enzyme activity assay

Liver tissue was homogenized in 10 volumes of a 0.25 M sucrose solution containing 1 mM EDTA-2Na in 3 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 500 × g for 10 min at 4°C. The supernatant was recentrifuged at 9,000 × g for 10 min at 4°C to sediment the mitochondria, and the remaining supernatant was collected. Furthermore, the supernatant was ultracentrifuged at 105,000 × g for 60 min at 4°C to give a remaining supernatant and a microsome fraction.

ACOX (EC 1.3.3.6) activity was assayed in the 500 × g supernatant fraction of the liver homogenate [62]. CPT-2 (EC 2.3.1.21) activity in the mitochondrial fraction was assayed as described by Markwell *et al.* [43]. The activities of FAS [44], ACC (EC 6.4.1.2) [45], and G6PDH (EC 1.1.1.49) [46] in the 105,000 × g supernatant fraction and PAP (EC 3.1.3.4) [49] in the microsome fraction were assayed spectrophotometrically. The protein content of various fractions was determined according to the method of Lowry *et al.* [61].

Analysis of the fecal lipids and nitrogen

Total bile acid content in the feces was determined as μmoles 3α-hydroxysteroid based on the molar extinction coefficient of NADH at 340 nm, and fecal CHOL content was determined by gas-liquid chromatography using a SE-30 column with a GC-14B gas chromatograph (SHIMAZU Co.) and 5α-cholestane as an internal standard. Fecal fatty acid content was determined by the method of Van de Kamer *et al.* [63]. Fecal protein content was determined by the Kjeldahl method. The apparent digestibility of protein was calculated as Apparent digestibility (%) = (Protein intake – Fecal protein)/Protein intake × 100.

Analysis of liver mRNA expression

Total RNA was extracted from the liver using an RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. Then, cDNA was synthesized from total RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd.). Real-time quantitative PCR analysis was performed using an automated sequence detection system (ABI Prism 7000; Applied Biosystems Japan Ltd.). HMGR, CYP7A1, and GAPDH mRNA expression levels were determined using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.). PCR Primers (HMGR: Rn00565598_m1; CYP7A1: Rn00564065_m1; GAPDH: Rn99999916_s1) were purchased from Applied Biosystems. In addition, LDLR, scavenger receptor class B type 1 (SR-B1), ABCA1, ABCG5, ABCG8, and GAPDH mRNA expression levels were determined using SYBR Green PCR Master Mix (Applied Biosystems). PCR primers (Table 13) for LDL-R, SR-B1, ABCA1, ABCG5, ABCG8, and GAPDH were designed using Primer Express 3.0 software (Applied Biosystems). The PCR cycling conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The expression level of the housekeeping gene GAPDH served as an internal control and was used for normalization.

Statistical analysis

Data are presented as means ± SD of seven rats. Statistical differences between multiple groups were determined by one-way ANOVA. Statistical comparisons were made using the Tukey-Kramer test. Difference were considered significant at $p<0.05$.

Table 13. Sequences of the specific primers used for reverse transcriptase polymerase chain reaction analysis

Gene name	5' → 3' primer sequence	Accession number
GAPDH	F:GAAGACACCAGTAGACTCCACGACAT	NM017008
	R:GAAGGTCGGTGTGAACGGATT	
LDLR	F:CACCCCCTCGTTGAAAACCT	NM175762
	R:CCTTAGCCAGCTCTTCCAGATC	
SR-B1	F:GCATTCGGAACAGTGCAACA	NM031541
	R:TCATGAATGGTGCCCACATC	
ABCA1	F:CCCGGCGGAGTAGAAAGG	NM178095
	R:AGGGCGATGCAAACAAAGAC	
ABCG5	F:CCTCAAGGGCTCCGAGAACT	NM053754
	R:ACCACACTGCCCCATAAGCT	
ABCG8	F:GCCATGGACCTGAACTCACA	NM130414
	R:GCTGATGCCAATGACGATGA	

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL-R, low density lipoprotein receptor; SR-B1, scavenger receptor class B type 1; ABCA1, ATP-binding cassette A1; ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; F, forward; R, reverse.

Results

Growth parameters and organ weights

Table 14 shows body weight, body weight gain, food intake, food efficiency, relative liver, and various WAT weights. There were no significant differences in body weight, body weight gain, food intake, or food efficiency among the groups. Dietary protamine did not affect the growth of rats. In addition, relative liver and various WAT weights did not show any differences among the groups.

Serum and liver lipid parameters

Table 15 shows the serum lipid and liver lipid contents. The serum TL, VLDL-TG, and VLDL-C contents in the low protamine (2%; ProtL) and high protamine (5%; ProtH) groups were significantly lower than in the control group, and there was no difference between the ProtL and ProtH groups. The serum TG, CHOL, and LDL-C contents were significantly lower the ProtH group compared with the control group. The serum HDL-C content in rats fed protamine tended to be lower than in rats fed casein, but not to a significant degree. No differences were found in serum PL among the groups.

The liver TL, TG, and CHOL contents in rats fed protamine were markedly lower than in rats fed casein. Furthermore, there was no difference between the ProtL and ProtH groups in the liver TL, TG, and CHOL contents. No differences were found in liver PL among the groups.

Liver enzyme activity

Table 16 shows the activities of fatty acid β-oxidation and fatty acid synthesis related enzymes to investigate the regulatory effect of dietary protamine on fatty acid metabolism in the liver. The activity of CPT-2, a key enzyme of fatty acid β-oxidation in mitochondria, was significantly higher in the ProtH group than in the control group. ACOX activity, a key enzyme of fatty acid oxidation in peroxisomes, was significantly higher in protamine fed rats than in the control group, and FAS activity, a key enzyme in fatty acid synthesis, was significantly higher in the ProtH group than in the control group. In contrast, there was no difference among the

groups in the activity of ACC, another key enzyme in fatty acid synthesis. The activity of G6PDH, a key enzyme in the production of cellular NADPH, which is required for the biosynthesis of fatty acids and CHOL, was significantly lower in the ProtH group compared with the control group. The activity of PAP, a rate limiting enzyme for TG synthesis in the liver, showed no differences among the groups.

Fecal lipids and protein digestibility

Table 17 shows the fecal lipid and protein contents to examine the effect of dietary protamine on fecal lipid excretion and apparent protein digestibility. The fecal fatty acid content, which is an index of fat absorption in the small intestine, was significantly higher in the ProtH group than in the control or ProtL groups. The fecal CHOL content was significantly higher in the ProtH group than in the control group, and those in the ProtL group tended to be higher than in the control group. The fecal bile acid content was significantly higher in rats fed protamine than in rats fed casein. Fecal dry weight, nitrogen content, and apparent protein digestibility were not different among the groups.

mRNA expression levels

Figure 6 shows the mRNA expression levels of enzymes and receptors related to CHOL metabolism in the liver tissue to evaluate the effect of dietary protamine using real-time quantitative PCR. The expression levels of CYP7A1, HMGR, LDLR, SR-B1, and ABCA1 were not different among the groups. In contrast, the expression levels of ABCG5 and ABCG8, which play major roles in the secretion of CHOL into bile, were higher in the ProtH group than in the control group, while those in the ProtL group tended to be higher than in the control group.

Table 14. Initial body weight, final body weight, body weight gain, energy intake, energy efficiency ratio, relative liver weight, and various WAT weights of rats fed the experimental diets for 4weeks

	Diet group		
	Control	ProtL	ProtH
Growth parameters			
Initial BW (g)	128 ± 7	129 ± 5	130 ± 8
Final BW(g)	312 ± 13	322 ± 14	324 ± 22
BW gain (g/day)	6.6 ± 0.3	6.9 ± 0.4	6.9 ± 0.6
Energy intake (kcal/day)	68.3 ± 5.2	69.7 ± 7.7	71.4 ± 8.0
Food efficiency (g/kcal)	0.096 ± 0.004	0.096 ± 0.006	0.097 ± 0.009
Organs weights (g/kg BW)			
Liver weight	0.38 ± 0.02	0.39 ± 0.07	0.36 ± 0.04
Epididymal WAT weight	0.15 ± 0.01	0.15 ± 0.03	0.15 ± 0.02
Mesentery WAT weight	0.13 ± 0.03	0.13 ± 0.04	0.13 ± 0.04
Perirenal and retroperitoneal WAT weight	0.13 ± 0.02	0.14 ± 0.06	0.13 ± 0.03
Total WAT weight*	0.50 ± 0.05	0.51 ± 0.14	0.50 ± 0.10

Data are means ± SD (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test. Rats were fed the experimental diets for 4 weeks. Food consumption and body weight were recorded every two days. The liver and WAT weights were obtained after sacrifice.

BW, body weight; WAT, white adipose tissue.

*Total WAT = epididymal WAT + mesentery WAT + perirenal and retroperitoneal WAT.

Table 15. Lipid content in the serum and liver of rats fed the experimental diets for 4 weeks.

	Diet group		
	Control	ProtL	ProtH
Serum (mg/dl)			
TL	219 ± 33 a	186 ± 16 b	169 ± 15 b
TG	35.3 ± 4.5 a	31.3 ± 3.6 a	24.0 ± 4.3 b
CHOL	68.3 ± 12.3 a	62.7 ± 5.8 ab	54.3 ± 9.7 b
PL	95.6 ± 10.6	89.7 ± 18.9	96.6 ± 14.6
HDL-C	58.6 ± 7.6	56.4 ± 7.0	51.4 ± 4.9
LDL-C	7.3 ± 1.1 a	6.9 ± 0.6 a	5.4 ± 0.8 b
VLDL-TG	13.7 ± 1.8 a	6.4 ± 1.9 b	4.2 ± 1.3 b
VLDL-C	1.8 ± 0.3 a	0.7 ± 0.3 b	0.5 ± 0.3 b
Liver (mg/g protein)			
TL	378 ± 47 a	268 ± 51 b	270 ± 23 b
TG	215 ± 46 a	129 ± 52 b	127 ± 22 b
CHOL	21.7 ± 3.9 a	13.8 ± 3.6 b	10.1 ± 1.4 b
PL	139 ± 24	135 ± 23	124 ± 15

Data are means ± SD (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test.

TL, total lipid; TG, triacylglycerol; CHOL, cholesterol; PL, phospholipid; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; VLDL-TG, very low density lipoprotein-triacylglycerol; VLDL-C, very low density lipoprotein-cholesterol.

Table 16. Activities of enzymes related to the fatty acid metabolic pathways in the liver of Wistar rats (nmol/min mg protein).

Enzyme	Diet group		
	Control	ProtL	ProtH
FAS	3.2 ± 0.6 a	4.0 ± 0.6 ab	4.8 ± 0.8 b
ACC	159 ± 20	177 ± 20	170 ± 36
G6PDH	20.3 ± 4.9 a	16.4 ± 3.0 ab	13.0 ± 5.3 b
PAP	9.3 ± 0.6	8.9 ± 0.9	9.3 ± 0.6
CPT	7.5 ± 1.0 a	9.0 ± 1.7 ab	9.7 ± 1.6 b
ACOX	1.5 ± 0.2 a	2.1 ± 0.1 b	2.2 ± 0.2 b

Data are means ± SD (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test. The activities of enzymes related to the fatty acid metabolism were analyzed by absorption spectrometer.

FAS, fatty acid synthase; ACC, acetyl-coenzyme A carboxylase; G6PDH, glucose-6-phosphate dehydrogenase; PAP, phosphatidate phosphatase; CPT, carnitine palmitoyl transferase-2; ACOX, acyl-coenzyme A oxidase.

Table 17. Fecal fatty acid, cholesterol, bile acid, nitrogen contents, and protein digestibility in rats fed the experimental diets for 4weeks.

	Diet group		
	Control	Pro L	ProtH
Fatty acid (mg/day)	40.1 ± 6.5 a	51.7 ± 9.4 a	87.8 ± 12.5 b
Cholesterol (mg/day)	5.8 ± 1.1 a	7.0 ± 1.1 ab	8.1 ± 0.8 b
Bile acid (μmol/day)	3.2 ± 0.8 a	6.0 ± 1.5 b	5.9 ± 0.9 b
Food intake (g/day)	121 ± 9	118 ± 14	126 ± 14
Fecal dry weight (g/day)	11.7 ± 2.2	10.9 ± 2.2	13.7 ± 2.0
Nitrogen (mg/day)	161 ± 23	179 ± 35	165 ± 28
Fecal protein (g/day)	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
Intake of dietary protein (g/day)	22.6 ± 1.4	21.1 ± 3.1	24.1 ± 2.4
Apparent protein digestibility (%)*	95.6 ± 0.7	94.6 ± 1.2	95.6 ± 1.1

Data are means ± SD (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test.

Feces were collected from each group every 24 hours for seven days prior to sacrifice.

*Apparent protein digestibility (%) = (intake of dietary protein – fecal protein) / intake of dietary protein × 100

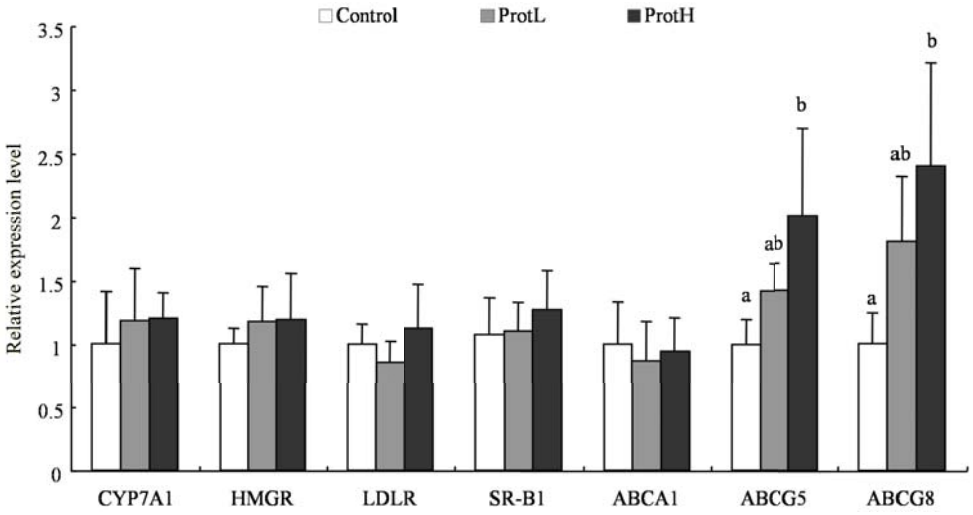


Figure 6. The expression levels of enzymes related to cholesterol metabolism in the liver of Wistar rat fed diets of the control (□), ProtL (■), and ProtH (■) diets.

Data are means ± SD (n=7). Relative values are presented as the ratio of each mRNA to GAPDH mRNA. Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test.

CYP7A1, cholesterol 7α-hydroxylase; HMGR, 3-hydroxy-3methylglutaryl-coenzyme A reductase; LDL-R, low density lipoprotein receptor; SR-B1, scavenger receptor class B type 1; ABCA1, ATP-binding cassette A1; ABCG5, ATP-binding cassette G5; ABCG8, ATP-binding cassette G8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

In this study, we demonstrated that dietary protamine significantly lowered the serum and liver TG contents compared with dietary casein. The reasons for the decrease in serum and liver TG contents were thought to be related to the absorption of lipid from the small intestine [58,64], and we analyzed the effect of dietary protamine on fat absorption by determining the total amount of fatty acids in feces. The fecal fatty acids content was higher in the ProtH group than in the control group. Tsujita *et al.* suggested that a basic protein or protamine strongly inhibited the hydrolysis of trioleoylglycerol emulsified with PC [58]. Moreover, the basic peptide polylysine is a candidate agent for the inhibition of intestinal lipid absorption while resisting proteolysis [64]. Thus, we hypothesized that the inhibition of fat absorption by basic peptides produced during the digestion of protamine, which contains large amounts of arginine corresponding to about 68mol% of its total amino acids. A previous study suggested that protamine reduced the plasma TG concentration through the inhibition of dietary fat absorption [59]. However, in this study, daily lipid (TG) intake was about 8.5 g, and daily fatty acids intake was about 8.0 g when converting TG into fatty acids. The daily excretion of fecal fatty acids was 40.1±6.5 mg and 87.8±12.5 mg for the control and ProtH groups, respectively. Consequently, the apparent lipid digestibility was about 99.5% and 99.0% for the control and ProtH groups, respectively. Therefore, we speculated that the dietary protamine increased fecal fatty acid excretion but did not extremely decrease the serum and liver TG contents.

Another reason considered for the decrease in serum and liver TG contents was the alteration of enzyme activity related to TG metabolism in the liver, and we examined the effect of dietary protamine on enzyme activity related to fatty acid β-oxidation and fatty acid synthesis. As shown in Table 16, CPT-2 and ACOX activities, which are key enzymes of fatty acid β-oxidation in mitochondria and peroxisomes, in the liver were higher in rats fed protamine than in rats fed casein. FAS activity, a rate limiting enzyme in fatty acid synthesis, was higher in rats fed protamine than in rats fed casein. Moreover, PAP activity, a key enzyme in the regulation of de novo TG synthesis, was not different among the groups. Therefore, we hypothesized that the decrease in liver TG content with the protamine-containing diets were attributed to the enhancement of fatty acid β-oxidation and not to the alteration of de

novo TG synthesis. The increased FAS activity due to dietary protamine may be prevented by the enhancement of fatty acid β-oxidation.

In this study, the serum VLDL-TG and VLDL-C contents in rats fed protamine were significantly lower than in rats fed casein. VLDL, which is a TG-rich lipoprotein, is assembled and stored in the liver by microsomal triglyceride transfer protein and apolipoprotein B. In addition, VLDL hydrolyzes TG to fatty acid and monoacylglycerol by the action of lipoprotein lipase in a number of peripheral tissues, including adipose tissue, skeletal and cardiac muscle, and the mammary gland [65]. Further studies are necessary to clarify the effect of dietary protamine on lipoprotein metabolism of VLDL hydrolysis, assembly, and secretion in peripheral tissue and the liver.

Another interesting result was the effect of protamine on CHOL metabolism. The serum CHOL and LDL-C contents were significantly decreased in the ProtH group compared with the control group. In addition, the liver CHOL contents in rats fed protamine were significantly lower than in rats fed casein (Table 15). Nagata *et al.* found that the degree of serum CHOL lowering depended on the extent fecal steroid excretion [25]. In this study, the fecal excretion of CHOL and bile acid were higher in rats fed protamine than in rats fed casein (Table 17). Higaki *et al.* suggested that feeding soy protein stimulated the fecal excretion of bile acid and demonstrated that the increase in fecal bile acid was accompanied by an increase in fecal nitrogen excretion [66]. The fecal excretion of bile acid suggested that hydrophobic peptides were produced by intestinal digestion of protein bound bile acid by hydrophobic interaction [67]. Moreover, Kayashita *et al.* reported that a dietary buckwheat protein product had lower plasma CHOL content and enhanced excretion of fecal CHOL and nitrogen compared with rats fed casein, and there was a significant correlation between fecal CHOL and nitrogen [68]. Thus, dietary proteins such as soy and buckwheat protein increased the fecal excretion of steroids concomitant with increased fecal nitrogen due to its low digestibility. However, dietary protamine increased the fecal excretion of CHOL and bile acid, although there was no influence on the fecal excretion of nitrogen. This result indicated the possible effect of dietary protamine on the fecal excretion of CHOL and bile acid by a new mechanism. The mechanism of fecal CHOL and bile acid excretion in rats fed protamine is not clear at present, and further experiments will be necessary to clarify this mechanism and the effects on the

binding capacities of bile acid and the micellar solubility of CHOL and bile acid.

It is well known that serum and liver CHOL levels are maintained by a balance between CHOL biosynthesis and catabolism in the liver, uptake into the liver, and secretion and excretion from the liver. The decreased liver CHOL content found in rats fed protamine could be expected to arise from a decrease in CHOL synthesis, an increase in CHOL catabolism to bile acid, an increase in CHOL efflux, or a decrease in CHOL uptake. Therefore, we evaluate the mRNA expression levels of enzymes and receptors related to CHOL metabolism in the liver using quantitative real-time PCR. CYP7A1, a rate limiting enzyme of bile acid synthesis, HMGR, a rate limiting enzyme of CHOL synthesis, and LDLR and SR-B1, uptake serum lipoproteins, showed differences in rats fed protamine compared with in rats fed casein (Figure 6). The CHOL lowering effect of dietary protamine was not attributable to the decrease in CHOL biosynthesis, catabolism, or uptake.

Much attention has been focused on the finding that control of the delivery and disposal of CHOL are regulated by membrane transporters of the ABC superfamily [69]. The liver mRNA expression levels of ABCA1, a transporter involved in the production of HDL, ABCG5 and ABCG8, which form heterodimers and play a major role in the secretion of CHOL into bile, were analyzed. The expression levels of ABCA1 were not different among the groups. Surprisingly, dietary protamine increased the expression levels of ABCG5 and ABCG8 in the liver. This results in decreased liver CHOL through the enhancement of the secretion of CHOL into bile. In addition, the increased expression levels of ABCG5 and ABCG8 also influenced the excretion of fecal CHOL. Hence, we suspected that the hypocholesterolemic effect of dietary protamine was attributable to the enhanced excretion of fecal CHOL and bile acid and the secretion of CHOL into bile.

These results showed that dietary protamine enhanced β-oxidation activities and the expression levels of ABCG5 and ABCG8 in the liver. We predicted that the alteration in lipid metabolism in rats fed on the protamine diet were possibly due to arginine, a large amount of which is present in protamine. Dietary L-arginine produces nitric oxide with nitric oxide synthase in virtually all mammalian cells and plays an important role in lipid metabolism [70]. Future studies are necessary to test this hypothesis.

Summary

Protamine has been widely used as a pharmaceutical product and natural food preservative. However, few studies have been conducted to assess the beneficial function of dietary protamine. This study examined the effects of dietary salmon protamine on serum and liver lipid levels and the expression levels of genes encoding proteins involved in lipid homeostasis in the liver of rats. Groups of male Wistar rats were fed AIN93G diet containing 2% or 5% protamine. After 4 weeks of feeding these diets, markedly decreased serum and liver cholesterol and triacylglycerol levels were noted. Increased activity of liver carnitine palmitoyltransferase-2 and acyl-CoA oxidase, which are key enzymes of fatty acid β-oxidation in the mitochondria and peroxisomes, was found in rats fed on protamine. Furthermore, rats fed protamine showed enhanced fecal excretion of cholesterol and bile acid and increased liver mRNA expression levels of ATP-binding cassette (ABC) G5 and ABCG8, which form heterodimers and play a major role in the secretion of cholesterol into bile. The decrease in triacylglycerol levels in protamine-fed rats was due to the enhancement of liver β-oxidation. Furthermore, rats fed protamine exhibited decreased cholesterol levels through the suppression of cholesterol and bile acid absorption and the enhancement of cholesterol secretion into bile. These results suggest that dietary protamine has beneficial effects that may aid in the prevention of lifestyle-related diseases such as hyperlipidemia and atherosclerosis.

Chapter 4

Fish protein inhibition of cholesterol and bile acid absorption *in vitro* study

Introduction

Previous studies showed that dietary fish protein decreased blood CHOL levels in laboratory animals when compared with casein [39,40,71-73]. In addition, dietary fish protein also has beneficial effects, such as antihypertensive and anti-obesity properties [74,75].

The effects of various nutrients on plasma CHOL have been reported: initially with dietary CHOL and later with amounts of dietary fat. In addition, dietary proteins, such as plant and animal proteins, have also been found to influence CHOL metabolism in human subjects and animals [36,68,76]. Protein digestion products are often argued to interrupt the intestinal absorption of CHOL and bile acid. Several reports have shown that plant proteins have hypocholsterolemic activity in serum, which may be due to the increased excretion of fecal CHOL and bile acid [68,76]. Furthermore, Nagaoka *et al.* suggested that soy protein peptic hydrolysate had greater binding bile acid capacity and lower micellar solubility of CHOL than casein peptic hydrolysates [77]. Our previous study showed that dietary fish protein decreased serum CHOL concentration through fecal CHOL and bile acid excretion [71]. However, peptides prepared by *in vitro* digestion of fish protein were not examined for their effect on bile acid binding capacity and micellar solubility of CHOL. In addition, Higaki *et al.* suggested that feeding soy protein stimulated the fecal excretion of bile acid and demonstrated that the increase in fecal bile acid was accompanied by an increase in fecal nitrogen excretion [66]. Therefore, to obtain detailed information, this study examined the effects of fish protein from Alaska Pollock fillets, which is widely used for human nutrition, on levels of fecal sterol and nitrogen excretion. Furthermore, peptides prepared by *in vitro* digestion of bile acid binding capacity and micellar solubility of CHOL were also confirmed. This study contributes to the development dietary therapy for hyperlipidemia patients by clarifying the health functionality of fish

protein.

Materials and methods

Materials

Fish protein was produced from Alaska Pollock fillets, as described above. AIN-93 vitamin mix, AIN-93G mineral mix, dextrinized cornstarch, cornstarch, cellulose, sucrose, and casein were purchased from Oriental Yeast Co., Ltd. Choline bitartrate, L-cystine, and soybean oil were purchased from Wako Pure Chemical Industries, Ltd.. All other chemicals were obtained from common commercial sources and were of reagent grade.

Animal care

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the "Guide for the Care and Use of Experimental Animals" of the Prime Minister's Office of Japan. Five-week-old male Wistar rats (Shimizu Laboratory Supplies Co., Ltd.) were housed in plastic cages in an air-conditioned room (temperature, 21-22 °C; humidity, 55-65%; lights on, 08:00-20:00 h). After acclimation for 1 week by feeding a diet prepared according to the recommendation of AIN-93G [11], rats were divided into two groups of seven rats each with similar mean body weight, given free access to drinking tap water, and fed the experimental diets. Table 18 shows the composition of the experimental diets prepared according to a modified AIN-93G hypercholesterolemic diet. Nitrogen content from each protein was adjusted to the same between the diets. Table 19 shows the amino acid composition of the casein and fish protein used in the diets as determined by a commercial service (Japan Food Research Laboratories, Tokyo, Japan).

Food consumption and body weight were recorded every 2 days. Feces were collected from each group every 24 hours for 7 days prior to sacrifice. After treatment with the experimental diets for 4 weeks, rats were weighed and sacrificed under pentobarbital (Nembutal®, Dainippon Sumitomo Pharma Co., Ltd.) anesthesia. Rats were not fasted before being sacrificed because food deprivation prior to sacrifice leads to a significant down regulation of the genes involved in fatty acid synthesis and CHOL

metabolism [13]. Blood was collected and serum was obtained by centrifugation at 1,500 × g for 15 min before being stored at –80°C until analysis. The liver was excised, then weighed, rinsed with ice-cold saline, frozen in liquid nitrogen, and stored at –80°C until analysis. In addition, the small intestine was excided, rinsed with ice-cold saline, divided into two equal lengths (jejunum and ileum regions), and the intestinal mucosa was scraped off. The jejunal and ileal mucosa was taken for mRNA expression analysis and stored in RNA-Later Storage Solution (Sigma Chemical Co.).

Table 18. Composition of the experimental (g/kg).

Components	Casein	Fish protein
Dextrinized corn starch	132	132
Corn starch	391.5	391.5
Casein	200	100
Fish protein		100
Sucrose	100	100
Cellulose powder	50	50
AIN93G mineral mixture	35	35
AIN93 vitamin mixture	10	10
L-Cystine	3	3
Choline bitartrate	2.5	2.5
Soybean oil	70	70
Cholesterol	5	5
Cholic acid	1	1

Biochemical analysis

Serum CHOL, HDL-C, LDL-C and TG were measured using an Olympus AU5431 automatic analyzer with AU regent (Beckman Coulter Inc. Brea, CA, USA). Liver lipid was extracted by the method of Bligh and Dyer [14]. Each total lipid sample was dissolved in an equal volume of DMSO, and the content of TG was determined using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries, Ltd.). CHOL contents in liver and feces were analyzed using a GC system (GC-14B, SHIMAZU Co.) with an SE-30 column (Shinwa Chemical Industries LTD.), in which 5α-cholestane was used as an internal standard. Fecal bile acid content was measured in accordance with the method of Bruusgaard *et al.* [17], and nitrogen content was determined by the Kjeldahl method.

Analysis of mRNA expression

Total RNA was extracted from 100 mg of jejunal and ileal mucosa using TRIZOL Reagent (Invitrogen). cDNA was then synthesized from total RNA using ReverTraAce (TOYOBO Co., Ltd.). Real-time quantitative PCR analysis was performed using an automated sequence detection system (DNA Engine Opticon 2, Bio-Rad Laboratories) using SYBR®GreenER™ qPCR SuperMix Universal (Invitrogen). The primer sequences used for the detection of ABCG5, ABCG8, niemann-pick C1 like 1 protein 1 (NPC1L1), intestinal bile acid transporter (IBAT), and GAPDH were as follows: Forward: 5'-ATGGCCTGTACCAGAAGTGG-3' and 5'-GGATACAAGCCCAGAGTCCA-3' for ABCG5; Forward: 5'-TTCTGCTGCAACGCTCTCTA-3' and 5'-GGAGGAACGACATCTTGGAA-3' for ABCG8; Forward: 5'-AAGAAGGCCTCTTACTCCGC-3' and 5'-GTCTAGCCCCACGTTGATGT-3' for NPC1L1; Forward: 5'-GTTTTCCAGCTCGTCTTTGC-3' and 5'-CCCTTGTTTGTCTCCTGGAA-3' for IBAT; and Forward: 5'-ATGACTCTACCCACGGCAAG-3' and 5'-TACTCAGCACCAGCATCACC -3' for GAPDH. Results were quantified using a comparative method and were expressed as a relative level after normalization to the GAPDH expression level.

In vitro digestion

Casein and fish protein were hydrolyzed by the method of Iwami *et al.* [67] with some modifications. Pepsin hydrolysis parameters were as follows: protein isolate concentration, 10% (w/v); enzyme/substrate ratio, 1:100 (w/w); adjusted to pH 2 with HCl at 37°C for pepsin (Sigma Chemical Co.). After 180 min of incubation, the pepsin was inactivated by neutralization with NaOH, then 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°C for pancreatin. After 180 min of incubation, digestion was stopped by heating to 80°C for 20 min. The digest was centrifuged at 4500 × *g* for 20 min. The supernatant was dialyzed at 4°C in Molecularporous membrane tubing (Spectra/POR, MW cutoff 100-500, Spectrum Medical Ind., Los Angeles, CA, USA) against the distilled water for

3 days to remove the NaCl and amino acid or dipeptide. Subsequently, it was freeze-dried, then were weighed and identified as the soluble fraction of casein hydrolysates and fish protein hydrolyates. The supernatant was dialyzed before freeze-drying because amino acids and dipeptides are absorbed in the intestine and the hypocholesterolemic peptides derived from soybean protein consisted of peptides of molecular weights more than 1 K [76]. The sediment was washed with water three times and centrifuged at 4500 × *g* for 20 min, freeze-dried, then weighed and identified as the insoluble fraction of casein hydrolysates and fish protein hydrolyates.

Bile acid binding capacity

Bile acid binding capacity of the insoluble fraction of casein hydrolysates and fish protein hydrolyates was measured in accordance with the method of Higaki *et al.* [66] and that of the soluble fraction of casein hydrolysates and fish protein hydrolyates was measured in accordance with the method of Sugano *et al.* [76] with some modifications. Samples of the soluble casein hydrolysates and fish protein hydrolyates (10 mg) fractions were incubated by shaking vigorously in 5.0 mL of 0.1 mol/L Tris-HCl-0.1 mol/L NaN_3 buffer, pH 7.4, containing conjugated sodium taurocholate at 37°C for 2 hours. An aliquot of the reaction mixture was dialyzed at room temperature in Molecularporous membrane tubing (Spectra/POR, MW cutoff 3000-5000, Spectrum Medical Ind.) against the same buffer for 3 to 4 days, and the content of sodium taurocholate in the dialyzate was measured as described above.

Micellar solubility of cholesterol

Micellar solubility of CHOL in the presence of the soluble and insoluble fractions of casein hydrolysates and fish protein hydrolyates was measured in accordance with the method of Nagaoka *et al.* [77]. The CHOL content was measured using an enzymatic assay kit (Cholesterol-E-Test Wako, Wako Pure Chemical Industries, Ltd.).

Statistical analysis

Values are means ± SD. The statistical significance of differences was evaluated using Student's *t*-test. Means were considered significantly different at $p <$

0.01 or $p < 0.05$.

Table 19. Amino acid composition of casein and fish protein (g/100g Protein).

Amino acid	Casein	Fish protein
Alanine	2.9	5.8
Arginine	3.5	6.6
Asparic acid [a]	6.7	10.6
Cysteine	0.5	1.0
Glutamic acid [b]	20.2	17.2
Glycine	1.7	3.6
Histidine	2.9	2.2
Isoleucine	5.2	4.7
Leucine	8.9	8.6
Lysine	7.6	10.3
Methionine	2.8	3.3
Phenylalanine	4.8	3.7
Proline	10.6	3.3
Serine	4.9	4.3
Threonine	3.9	4.7
Tryptophan	1.2	1.1
Tyrosine	5.3	3.9
Valine	6.4	5.1

[a] Aspartic acid: Aspartic acid + Asparagine

[b] Glutamic acid: Glutamic acid + Glutamine

Results

Growth parameters and organs weights

The growth parameters of initial body weight, final body weight, body weight gain, food comsumption, food efficiency, and organ weights of relative liver and epididymal WAT weights between the casein and fish protein groups (date not shown) did not differ.

Lipid contents of serum, liver and feces

Table 20 shows lipid indexes in the serum and liver of rats fed diets containing casein and fish protein. Rats fed fish protein had significantly lower serum CHOL, LDL-C, and liver CHOL contents than rats fed casein. Serum HDL-C and liver TG contents did not differ between the two groups of rats.

Table 20. Lipid indexes in the serum and liver of rats fed diets containing casein and fish protein.

	Casein	Fish protein
Serum (mg/dl)		
Cholesterol	73.3 ± 8.2	64.8 ± 7.4 *
HDL-C	52.7 ± 5.6	57.0 ± 5.6
LDL-C	8.2 ± 0.8	7.0 ± 0.8 *
Triacylglycerol	40.3 ± 5.6	39.2 ± 5.8
Liver (mg/g Protein)		
Cholesterol	75.0 ± 16.4	45.7 ± 7.9 **
Triacylglycerol	808 ± 30	833 ± 87

Values are means with SD for seven determinations.

Means values were significantly different from those of rats fed the casein diet: Student's t test, * $p < 0.05$ ** $p < 0.01$

HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

Figure 7 shows fecal CHOL, bile acid and nitrogen excretion of rats fed diets containing casein and fish protein. Rats fed on the fish protein had significantly higher fecal CHOL, bile acid, and nitrogen excretion levels than rats fed casein.

Micellar solubility of cholesterol

Figure 8 shows effects of the soluble and insoluble fractions of casein and fish protein hydrolysates on the micellar solubility of CHOL *in vitro*. The micellar solubility of CHOL in the presence of the soluble fraction of fish protein hydrolyates was significantly lower than the soluble fraction of casein hydrolysates, and that in the presence of the insoluble fraction of fish protein hydrolyates was significantly lower than the insoluble fraction of casein hydrolysates.

Bile acid binding

Table 21 shows production rate and bile acid binding capacity of the soluble and insoluble fractions of casein and fish protein hydrolyzates. The soluble fraction production rate of fish protein hydrolysates was lower than that of casein hydrolysates, whereas the insoluble fraction production rate of fish protein hydrolyates was higher than that of casein hydrolysates.

mRNA expression of intestine

Table 22 shows the mRNA expression levels of CHOL absorption transporters in the jejunum and bile acid absorption transporter in the ileum. There were no significant differences in any of the transporter expression levels between the two groups of rats.

Table 21. Production rate and bile acid binding capacity of the soluble and insoluble fractions of casein and fish protein hydrolysates.

	Casein	Fish protein
Soluble fraction		
Production rate (%)	38.6 ± 0.7	29.0 ± 0.4 **
Binding bile acid (mmol/g)	3.9 ± 0.3	14.1 ± 0.6 **
Insoluble fraction		
Production rate (%)	1.2 ± 0.1	7.1 ± 0.1 **
Binding bile acid (mmol/g)	60.3 ± 4.3	93.6 ± 6.5 **

Values are means with SD for three determinations.

Means values were significantly different from those of casein hydrolyzate: Student's *t*-test, * $p < 0.05$ ** $p < 0.01$

[a] Production rate (%) = digestion product weight / intact protein weight of the initial reaction × 100

CH, casein hydrolyzate; FPH, fish protein hydrolyzate.

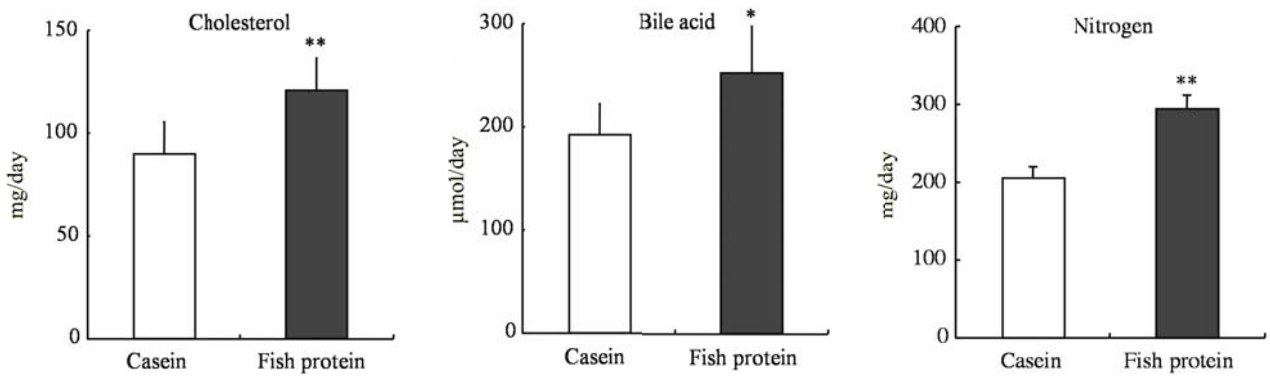


Figure 7. Fecal cholesterol, bile acid and nitrogen excretion of rats fed diets containing casein and fish protein.

Values are means with SD shown by vertical bars from seven determinations.

Means values were significantly different from those of rats fed the casein diet: Student's *t*-test, * $p < 0.05$ ** $p < 0.01$

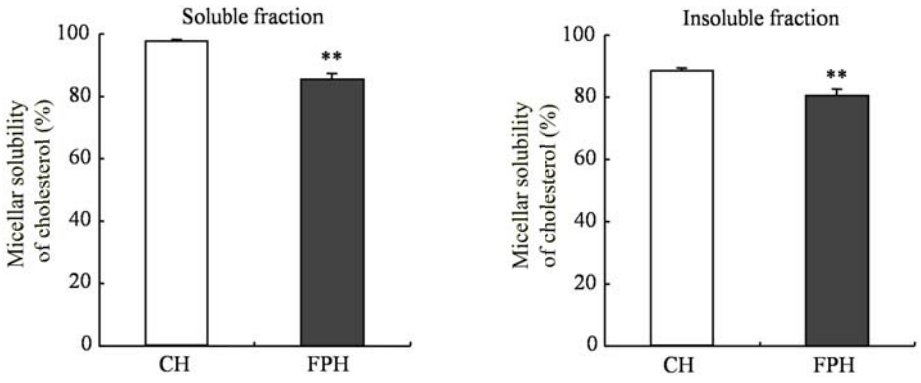


Figure 8. Effects of the soluble and insoluble fractions of casein and fish protein hydrolyzates on the micellar solubility of cholesterol *in vitro*.

Values are means with SD shown by vertical bars from three determinations.

Means values were significantly different from those of rats fed the casein diet: Student's *t*-test, * $p < 0.05$ ** $p < 0.01$

CH, casein hydrolyzate; FPH, fish protein hydrolyzate.

Table 22. The mRNA expression levels of CHOL absorption transporters in the jejunum and bile acid absorption transporter in the ileum

	Casein	Fish protein
Intestine	Arbitrary units	
Jejunum		
ABCG5	1.00 ± 0.13	0.97 ± 0.19
ABCG8	1.00 ± 0.13	1.06 ± 0.37
NPC1L1	1.00 ± 0.19	1.12 ± 0.26
Ileum		
IBAT	1.00 ± 0.48	1.05 ± 0.46

Values are means with SD for seven determinations.
Means values were significantly different from those of casein:
Student's *t*-test, * $p < 0.05$ ** $p < 0.01$

ABCG5; ATP-binding cassette G5, ABCG8; ATP-binding cassette G8, NPC1L1; Niemann-Pick C1 like 1 Protein 1, IBAT; intestinal bile acid transporter, GAPDH; glyceraldehyde 3-phosphate dehydrogenase.

Discussion

Table 20 shows the lipid indexes in the serum and liver of rats fed the casein and fish protein diets. Fish protein has been proved to reduce plasma CHOL concentrations in animals when compared with casein [39,40,71-73]. In this study, rats fed fish protein had significantly lower serum CHOL, LDL-C, and liver CHOL contents than rats fed casein. Serum HDL-C and liver TG contents did not differ between the two groups of rats. Many epidemiological and clinical studies have demonstrated that increases in LDL-C level and decreases in HDL-C level in the blood are important risk factors for CHD [78]. This suggests that fish protein might have beneficial health functions to prevent the development of CHD. On the contrary, Wergedahl *et al.* and Shukla *et al.* reported that the HDL-C content was decreased by feeding fish protein from salmon and Alaska pollack in hyperlipidemic obese Zucker rats and Wistar rats, respectively [39,72]. Further studies are needed to resolve these contradictory results on the effect of fish protein intake on HDL-C metabolism.

Regarding the decreased serum and liver CHOL contents related to dietary protein, two main causes have been discussed. One hypothesis is that the amino acid composition of the protein, in particular the ratio of lysine/arginine [79] and the content of specific amino acids that is, methionine [80], cysteine [81], and glycine [82]. However, the amino acid composition of fish protein proved to be similar to that of casein and the difference could not be explained in terms of differences in cysteine, methionine, and glycine content alone (Table 19). The lysine/arginine ratio of the fish protein was 1.55 and that of casein was 2.15. In this study, 10% fish protein was replaced in total 20% dietary protein; therefore, the amino acid composition and the lysine/arginine ratio of casein and fish protein diet were similar. This suggests that the hypocholesterolemic effect of fish protein was not the result of a metabolic effect due to differences in amino acid composition.

The other hypothesis involves an intradigestive tract effect, that is, the digestibility of dietary proteins and the physicochemical properties of digestion products in the digestive tract are related to CHOL metabolism [83]. Nagata *et al.* found that the degree of serum CHOL lowering depends on the extent fecal excretion of steroids [25]. To clarify the mechanism of the decrease in serum and liver CHOL contents related to a fish protein diet, fecal CHOL, bile acid, and nitrogen excretion

were analyzed. Rats fed on the fish protein had significantly higher fecal CHOL, bile acid, and nitrogen excretion levels than rats fed casein. Furthermore, Higaki *et al.* suggested that feeding soy protein stimulated the fecal excretion of bile acid, and they demonstrated that the increase in fecal bile acid was accompanied by an increase in fecal nitrogen excretion [66]. In addition, Kayashita *et al.* suggested that dietary buckwheat protein product enhanced the excretion of fecal neutral sterols and there was a significantly correlation between fecal neutral sterols and nitrogen [68]. The fecal bile acid content correlated positively with the fecal nitrogen content ($r = 0.9447$; $p < 0.01$), and a significant correlation ($r = 0.6472$; $p < 0.01$) between fecal CHOL and nitrogen was also observed. Dietary fish protein may effectively inhibit the absorption of CHOL in the jejunum and bile acid in the ileum. Thus, rats fed fish protein showed decreased serum and liver CHOL contents through the suppression of CHOL and bile acid absorption compared with casein. On the other hand, the enhancement of fecal CHOL and bile acid excretion by fish protein is at least due to the indigestible protein and peptides remaining after digestion because fecal nitrogen excretion was increased.

Previous studies suggested that soy protein [77] and egg ovomucin [84] suppressed the micellar solubility of CHOL and enhanced bile acid binding capacity *in vitro* resulting in increased fecal steroid excretion. However, dietary proteins are hydrolyzed by digestive enzymes in the stomach and intestine; therefore, the use of intact protein to assess the micellar solubility of CHOL and bile acid binding capacity analysis is not appropriate. This study examined digested casein and fish protein using an *in vitro* digestion model [67] by dividing each sample into soluble and insoluble fractions, then the micellar solubility of CHOL and bile acid binding capacity were measured

This study indicated that the micellar solubility of CHOL in the presence of the soluble fraction of fish protein hydrolyates was significantly lower than the soluble fraction of casein hydrolysates, and that in the presence of the insoluble fraction of fish protein hydrolyates was significantly lower than the insoluble fraction of casein hydrolysates (Figure 8). CHOL is rendered soluble in bile salt-mixed micelles and absorbed. Previous studies suggested that soy protein [77], β-lactoglobulin [85], and sunflower protein [86] decreased the micellar solubility of CHOL. We have showed for the first time that dietary fish protein increased fecal CHOL excretion through suppression of the micellar solubility of CHOL by fish protein hydrolyates

The soluble fraction production rate of fish protein hydrolyates was lower than that of casein hydrolysates, whereas the insoluble fraction production rate of fish protein hydrolyates was higher than that of casein hydrolysates (Table 21). The insoluble fraction was termed resistant protein and had dietary fiber-like properties. Resistant protein was reported to stimulate fecal bile acid and nitrogen excretion [67,68] and suppress liver carcinogenesis [87]. The increased fecal nitrogen excretion resulting from the dietary fish protein diet may be due to the increased production rate of fish protein hydrolyates by the insoluble fraction following *in vitro* digestion. Bile acid binding capacity to the soluble fraction of fish protein hydrolyates was higher than that of casein hydrolysates and also of the insoluble fraction of fish protein hydrolyates was higher than that of casein hydrolysates. Iwami *et al.* showed a correlation between the hydrophobicity of a protein hydrolysate and its binding capacity of bile acids and suggested that a peptide with a high bile acid binding capacity could inhibit the reabsorption of bile acid in the ileum resulting in decreased the blood CHOL levels [67]. In this study, the soluble and insoluble fraction of fish protein hydrolyates had a significantly higher bile acid binding capacity than that of casein hydrolysates. Therefore, dietary fish protein may produce a peptide with high hydrophobicity with strong bile acid binding capacity through digestion in the gastrointestinal tract compared with casein. Comparing the casein and fish protein for hydrophilic amino acids, such as glutamic acid, glutamine and serine, and hydrophobic amino acids, such as valine, leucine, and isoleucine, the contents were almost the same (Table 19). The reason for the difference in bile acid binding capacity due to the digestion products of casein and fish protein was not clear at present. We have suggested that the decrease in serum and liver CHOL contents in rats fed the fish protein diet was attributable to the suppression of CHOL and bile acid absorption through the enhancement of fecal CHOL and bile acid excretion.

In previous study, dietary fish protein altered the expression of genes related the endogenous CHOL metabolism of rats [39,71,73]. Our previous study suggested that dietary fish protein enhanced the liver CYP7A1 expression level through regulation by the FXR/SHP-1 pathway resulting from the inhibition of bile acid reabsorption [71]. Much attention has been focused on the finding that control of the absorption and disposal of CHOL are regulated by NPC1L1, ABCG5, and ABCG8 heterodimers [88]. For example, Mathur *et al.* suggested that a diet enriched in n-3

fatty acids, such as DHA, decreases the gene expression of NPC1L1 in the duodenum and jejunum of hamsters [89]. However, there has not been a study focused on the influence of dietary fish protein on CHOL and bile acid absorption-related transporters in of intestine of animals. Therefore, in this study, the mRNA expression levels of CHOL absorption transporters in the jejunum and bile acid absorption transporter in the ileum using quantitative real-time PCR were analyzed. There were no significant differences in any of the transporter expression levels between the two groups of rats (Table 22). This result at least predicts that the decrease in CHOL content resulting from fish protein diets was not related to intestinal CHOL and bile acid absorption transporters.

Summary

Fish protein has been shown to decrease serum cholesterol content through the inhibition of cholesterol and bile acid absorption in laboratory animals, although the mechanism for this effect is not yet fully understood. The purpose of this study was to elucidate the mechanism of inhibition of cholesterol and bile acid absorption following fish protein intake. Male Wistar rats were divided into two dietary groups of seven rats each: casein (20%); fish protein (10%) + casein (10%) each with cholesterol 0.5% cholesterol and 0.1% sodium cholate. After 4 weeks on each diet, the serum and liver cholesterol content and fecal cholesterol, and bile acid and nitrogen excretion levels were measured. Fish protein decreased serum and liver cholesterol content and increased fecal cholesterol and bile acid excretion and simultaneously increased fecal nitrogen excretion. In addition, fish protein hydrolysate prepared by *in vitro* digestion had lower micellar solubility of cholesterol and higher binding capacity for bile acids compared with casein hydrolysate. These results suggested that the hypocholesterolemic effect of fish protein is mediated by increased fecal cholesterol and bile acid excretion, which is due to the digest products of fish protein having reduced micellar solubility of cholesterol and increased bile acid binding capacity.

Chapter 5

Dietary fish peptides improving effect on rat cholesterol metabolism compared with intact fish protein

Introduction

Protein digestion products are often argued to interrupt the intestinal absorption of bile acids and CHOL. Several reports have shown that peptides prepared by in vitro digestion of plant proteins had hypocholesterolemic activity in serum, which may be due to the increased excretion of fecal CHOL and bile acid [90,91]. These reports also indicated that animal peptides prepared by the treatment of pork meat with papain or β-lactoglobulin with trypsin had hypocholesterolemic activity [85,92], although intact animal proteins in beef, pork and turkey had no effect on reducing CHOL in plasma or the liver [36]. On the other hand, dietary fish protein hydrolysates have many beneficial effects such as anti-hypertensive, anti-oxidative, and immunomodulating properties [93-95], while few studies have focused on the hypocholesterolemic effects of dietary fish protein hydrolysates in the serum and livers of experimental animals [72]. Moreover, there have been no studies evaluating mRNA expression levels of liver CHOL metabolism-associated genes in response to the intake of fish protein or peptides.

It has also been found that the peptides prepared from pork meat by papain hydrolysis show hypocholesterolemic activity in animals [92]. A previous study suggests that products artificially digested using pepsin- and trypsin-hydrolysed pork meat had no plasma hypocholesterolemic effect [96]. Therefore, I hypothesized that dietary fish peptides prepared by papain alter blood lipid indexes compared with dietary fish protein. In this study, I evaluated the effects of dietary fish peptides and fish protein on the lipid metabolism, especially serum and liver CHOL levels, along with expression levels of genes related to CHOL metabolism and CHOL excretion in rats fed experimental diets with and without CHOL.

Materials and methods

Preparation of fish protein and fish peptides

Fish fillets of Alaska Pollock (*Theragra chalcogramma*) were obtained from Suzuhiro Co., Ltd. (Odawara, Japan). The minced fillets were washed three times with cold distilled water and then dried. The resulting meat was treated with cold acetone, ethyl acetate, and *n*-hexane to remove lipids. The solvents in the meat were evaporated under N_2 gas, and then the meat was stored at −30°C before use as fish protein.

Fish protein was homogenized with 10 volumes of 0.02% (w/w) papain (W-40 Amano Enzyme, Inc., Nagoya, Japan) at pH 7.0 and incubated at 60°C for 1 hour. The digests were heated to 95°C for 30 min. to inactivate the papain, and the reaction mixture was then dried using a drum dryer. This fish protein hydrolysates was termed fish peptides.

Table 23 shows the compositions of casein, fish protein, and fish peptides. The crude protein content was determined by the Kjeldahl method. The crude fat content was measured by the Soxhlet method. The moisture content was estimated as the loss in weight after drying at 105°C for 24 hour. The ash amount was analyzed by direct ignition at 550°C for 24 hour.

The amino acid composition of the dietary proteins for the experimental diets was measured by HPLC as follows. The dietary proteins were hydrolysed using 6 M HCl for 24 hours. The hydrolysed samples were subjected to HPLC, in which the samples were automatically derivatized using *o*-phthalaldehyde and separated on a reversed-phase column (GL Sciences Inertsil ODS-3, 5 μm, 250 × 4.6 mm) at 40°C with 25 mM sodium phosphate buffer (pH 7.0) and acetonitrile using a gradient program at a flow rate of 1.0 mL/min. The eluate was monitored by fluorescence detection with an excitation wavelength of 340 nm and emission wavelength of 450 nm. The identification and quantitation of each amino acid were carried out using commercially available authentic standard mixtures and a CR-6A Chromatopac integrator (SHIMAZU Co.).

The MW distribution of the dietary proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel (20%) electrophoresis with polypeptide standards (Bio-Rad Laboratories).

Table 23. Nutrients in dietary proteins (g/100g)

	Dietary protein		
	Casein	Fish protein	Fish peptides
Crude protein	88.2	88.6	88.1
Crude fat	1.5	1.2	0.3
Moisture	7.2	7.3	8.0
Ash	1.7	1.5	2.3

Animal care

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University and followed the 'Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions' (Ministry of Education, Culture, Sports, Science and Technology, Notice No. 71, June 1, 2006). Five-week-old male Wistar rats obtained from Shimizu Laboratory Supplies Co., Ltd. were housed in plastic cages in an air-conditioned room (temperature, 20–22°C; humidity, 55–60%; lights on 8:00–20:00). After acclimation for 3 days on the recommended American Institute of Nutrition (AIN-93G) diet [11], the rats were then divided into 6 dietary groups as shown in Table 24, where CAS, FPro, and FPep indicate the groups receiving diets of casein, fish protein, and fish peptides, respectively. The experimental diets were prepared according to the AIN-93G formula, with CHOL diet containing 5g/kg CHOL and 1g/kg cholic acid. Rats were given free access to the experimental diets and drinking tap water. All of the diet components were products of Oriental Yeast Co., Ltd..

Feces were collected from each group every 24 hours for 7 days prior to sacrifice. After feeding with the experimental diets for 4 weeks, the rats were weighed and sacrificed under pentobarbital (Nembutal®, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) anesthesia. Rats were not fasted before being killed because food deprivation before killing leads to a significant down-regulation of the genes involved in fatty acid synthesis and CHOL metabolism [13]. Blood was collected from the abdominal descending aorta without anticoagulants, and then serum was obtained by centrifugation of the blood at 1,500 × g for 15 min. Liver and abdominal WAT located in the epididymis, mesentery, and perinephria were excised, weighed, and perfused with cold saline. An aliquot of the liver was taken for mRNA expression

analysis and stored in RNA-Later Storage Solution (Sigma Chemical Co.). All other samples were frozen rapidly in liquid nitrogen and stored at −80°C until analysis.

Analysis of the serum, liver, and feces lipid indexes

CHOL, TG, PL, HDL-C, and LDL-C contents in serum were measured in triplicate using an Olympus AU5431 automatic analyzer.

Total liver lipids were extracted by the Bligh and Dyer method [14]. Liver CHOL contents were determined by GC (GC-14B, SHIMAZU Co.) using 5α-cholestane as an internal standard. Liver TG contents were determined using an enzymatic assay kit (Triglyceride-E-Test Wako, Wako Pure Chemical Industries, Ltd.) after the total liver lipid was dissolved in an equal volume of DMSO. Liver PL contents were determined by separation using silica gel column chromatography with chloroform and methanol as elution solvents [15]. Liver protein contents were determined according to the method of Lowry *et al.* using BSA as a standard [61].

Fecal CHOL content was determined by GC [97]. Fecal bile acid content was measured as 3α-hydroxysteroid equivalent based on the molar extinction coefficient of NADH at 340 nm [44].

Analysis of liver mRNA expression levels

Total RNA was extracted from livers using an RNeasy Mini Kit (Qiagen), and cDNA was synthesized from the total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd.). Real-time quantitative PCR analysis was performed using an automated sequence detection system (ABI Prism 7000; Applied Biosystems Japan Ltd.). PCR cycling conditions were as follows: 50°C for 2 min., 95°C for 10 min., followed by 40 cycles of 95°C for 15s and 60°C for 1 min. The mRNA expression levels of HMGR, CYP7A1, ACAT-1, SREBP-2 and GAPDH were measured using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd.). PCR primers (HMGR: Rn00565598_m1; CYP7A1: Rn00564065_m1; ACAT-1: Rn00567139_m1; SREBP-2: Rn01502638_m1; GAPDH: Rn99999916_s1) were purchased from Applied Biosystems Japan Ltd.. The expression signal of GAPDH, a housekeeping gene, served as an internal control for normalization. The mRNA expression levels of LDLR, SHP-1, FXRα, LRH-1, and GAPDH were measured using

SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd.). The PCR solution (25 μL) was composed of 12.5 μL SYBR Green PCR Master Mix (Applied Biosystems Japan Ltd.) solution, 5 μL template cDNA, 1 μL forward primer, 1 μL reverse primer, and 5.5 μL RNase free water. The primer sequences used for detecting LDLR, SHP-1, FXRα, LRH-1 and GAPDH were as follows: LDLR = forward: 5 ' CACCCCCTCGTTGAAAACCT 3 ' and reverse: 5 ' CCTTAGCCAGCTCTTCCAGATC 3 ' ; SHP-1 = forward: 5 ' CGCCTGGCCCGAATC 3 ' and reverse: 5 ' GAAGGGTACAGGAGATGTTCTTGAG 3 ' ; FXRα = forward: 5 ' GGGCCTTGGACGTCTCTGA 3 ' and reverse: 5 ' CTGGGATGGTGGTCTTCAAATAA 3 ' ; LRH-1 = forward: 5'TCCGGGCAATCAGCAAA3' and reverse: 5' CCCCATTCACGTGCTTGTAGT 3'; GAPDH = forward: 5' GAAGACACCAGTAGACTCCACGACATA 3' and reverse: 5' GAAGGTCGGTGTGAACGGATT 3'.

***In vitro* digestion of protein**

Casein, fish protein, and fish peptides were digested by the method of Iwami *et al.* [67] with some modifications. Pepsin hydrolysis parameters were as follows: protein concentration, 10% (w/v); enzyme/substrate ratio, 1:100 (w/w); adjusted to pH 2 with HCl at 37°C for pepsin (Sigma Chemical Co.). After 180 min of incubation, the pepsin was inactivated by neutralization with NaOH and porcine pancreatin (Sigma Chemical Co.) was then added. Pancreatin hydrolysis parameters were as follows: enzyme/substrate ratio, 1:30 (w/w); adjusted to pH 7.4 with NaOH at 37°C for pancreatin. After 180 min of incubation, digestion was stopped by heating to 80°C for 20 min. The digest was centrifuged at 4,500 *g* for 20 min. The sediment was washed with distilled water three times and centrifuged at 4,500 *g* for 20 min, freeze-dried, then weighed and identified as the insoluble digestion products of casein, fish protein, and fish peptides.

Bile acid binding capacity

Taurocholate and deoxycholate binding capacities of the insoluble digestion products of casein, fish protein, and fish peptides were measured in accordance with the method of Higaki *et al.* [66].

Micellar solubility of cholesterol

Micellar solubilities of CHOL in the presence of the insoluble digestion products of casein, fish protein, and fish peptides were measured in accordance with the method of Nagaoka *et al.* [77] with some modifications. CHOL content was measured using an enzymatic assay kit (Cholesterol-E-Test Wako, Wako Pure Chemical Industries, Ltd.).

Statistical analysis

Data are expressed as means ± SE of 7 rats. The statistical differences were determined divided with non-CHOL diet and CHOL diet. Statistical differences between multiple groups were determined by one-way ANOVA. Statistical comparisons were made using the Tukey-Kramer test. The difference was considered significant at $p < 0.05$. The analyses were performed using StatView-J version 5.0 software (Abacus Concepts).

Table 24. Composition of experimental diets (g/kg diet).

Composition	Non-cholesterol diet			High-cholesterol diet		
	CAS	FPro	FPep	CAS	FPro	FPep
Dextrinized corn starch	132	132	132	132	132	132
Corn starch	397.5	397.5	397.5	391.5	391.5	391.5
Casein	200	100	100	200	100	100
Fish protein		100			100	
Fish peptides			100			100
Sucrose	100	100	100	100	100	100
Cellulose powder	50	50	50	50	50	50
AIN93G mineral mixture	35	35	35	35	35	35
AIN93 vitamin mixture	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Soybean oil	70	70	70	70	70	70
Cholesterol				5	5	5
Cholic acid				1	1	1

Table 25. Amino acid composition in the experimental diets (g/100g protein).

Amino acid	Dietary protein		
	Casein	Fish protein	Fish peptides
Alanine	4.3	8.6	8.4
Arginine	2.7	5.0	5.0
Aspartamic acid	6.7	10.5	10.5
Cystine	0.3	0.6	0.5
Glutamic acid	18.3	15.4	15.7
Glycine	3.0	6.2	6.4
Histidine	2.5	1.9	1.8
Isoleucine	5.3	4.8	4.7
Leucine	9.1	8.6	8.5
Lysine	6.9	9.2	9.1
Methionine	2.5	2.9	2.9
Phenylalanine	3.9	2.9	2.9
Proline	12.3	3.7	3.7
Serine	6.2	5.4	5.7
Threonine	4.4	5.2	5.3
Tryptophan	0.8	0.7	0.7
Tyrosine	3.9	2.8	2.8
Valine	7.2	5.7	5.5

Results

Amino acid composition and molecular weight of fish protein hydrolysates

Table 25 shows amino acid composition of casein, fish protein, and fish peptides. It was found that in fish protein and fish peptides, the levels of alanine, arginine, aspartic acid, glycine, and lysine were high, while the level of proline was low. However, the levels of branched-chain amino acids, such as valine, leucine, and isoleucine, were nearly identical, and the polarity of amino acids did not reveal any significant differences. The amino acid composition of casein, fish protein, and fish peptides were almost the same.

Figure 9 shows the MW of dietary proteins. The fish protein was detected mostly as a two band (ca. 200 and 45 kDa), which may be derived from myofibrillar protein, while the fish peptides were detected at a MW < 5kDa.

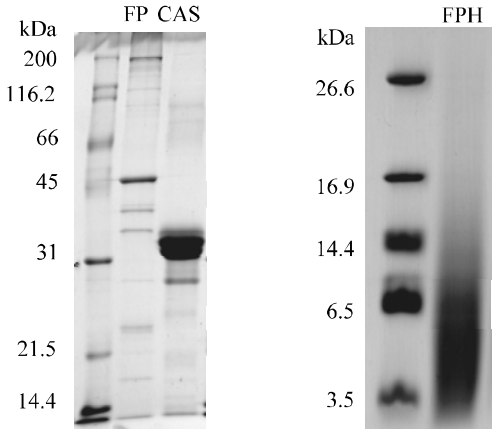


Figure 9 Coomassie blue stained SDS-PAGE of casein (CAS), fish protein (FP) and fish peptides (FPH).

Growth indexes and organ weights

Table 26 shows initial and final body weights, body weight gain, energy intake, food efficiency ratio, and relative weights of liver and WAT (the sum of epididymal, mesentery, and perirenal WAT). In the case of non-CHOL diets, there were no

significant differences in any indexes between the CAS, FPro, and FPep groups except that the liver weight of the FPro group was significantly lower than that of the CAS group. In the case of CHOL diets, there were no significant differences in any indexes among all groups.

Serum, liver, and fecal lipid indexes

Table 27 shows the lipid indexes in serum, liver and feces. In the case of non-CHOL diets, serum CHOL, TG, and LDL-C contents in the FPep group were significantly lower than those in the CAS group. Serum CHOL, TG, and LDL-C contents in the FPep group also tended to be lower than those in the FPro group. The liver CHOL and TG contents in the FPro group tended to be lower than those in both the CAS and FPep groups, while liver CHOL and TG contents in the FPep group was similar to that in the CAS group. Fecal CHOL and bile acid contents in both the FPro and FPep groups were significantly higher than in the CAS group.

In the case of CHOL diets, serum CHOL and LDL-C contents in the FPep group were significantly lower than those in the CAS group. Serum CHOL and LDL-C contents in the FPep group also tended to be lower than those in the FPro group. Liver CHOL content in both the FPro and FPep groups was significantly lower than in the CAS group. Liver CHOL content in the FPro group was also significantly lower compared to the FPep group. Fecal CHOL content in both the FPro and FPep groups were significantly higher than in the CAS group. Fecal bile acid content in the FPep group was significantly higher than those in both the CAS and FPro groups. Fecal CHOL content in the FPep group was significantly lower than that of the FPro group, whereas fecal bile acid content in the FPep group was higher than that in the FPro group. No significant differences in HDL-C contents were observed among any of the groups in both the non-CHOL diet and high-CHOL diets.

mRNA expression levels

Figure 10 shows the relative mRNA expression levels related to CHOL metabolism in the liver. When rats were fed the non-CHOL diets (A), all the relative expression levels of mRNAs were not significantly different among all groups. CYP7A1 expression levels tended to be higher in the FPro group compared with the

CAS group, while SHP-1 expression levels in the FPro and FPep groups tended to be lower than in the CAS group. When rats were fed the CHOL diets (B), ACAT-1 expression levels in the FPro group were significantly lower than those in the CAS and FPep groups. All other relative mRNA expression levels were not significantly different among any of the groups. CYP7A1 expression levels in the FPro group tended to be higher than in the CAS group. SHP-1 expression levels tended to be lower in both the FPro and FPep groups than in the CAS group.

Insoluble fraction products rate and bile acid binding

Figure 11 illustrates the production rates of the insoluble digestion products of casein, fish protein, and peptides. Fish protein had a significantly higher production rate of the insoluble digestion products compared with casein. Fish peptides also had a significantly higher production rate of the insoluble digestion products than casein, and were significantly higher than fish protein as well.

Bile acid binding capacity

Figure 12 illustrates the taurocholate and deoxycholate binding capacities of the insoluble digestion products of casein, fish protein, and fish peptides. The insoluble digestion products of fish protein were significantly higher in term of the taurocholate and deoxycholate binding capacities compared with the insoluble digestion products of casein. In addition, Fish peptides also had a significantly higher taurocholate and deoxycholate binding capacities than casein, and were significantly higher than fish protein.

Micellar solubility of cholesterol

Figure 13 illustrates the micellar solubilities of CHOL in the presence of the insoluble digestion products of casein, fish protein, and fish peptides by *in vitro* digestion. The insoluble digestion products of fish protein and peptides were significantly lowered the micellar solubility of CHOL compared with the insoluble digestion products of casein.

Table 26. Initial body weight, final body weight, body weight gain, energy intake, energy efficiency ratio, relative liver weight, and various WAT weights of rats fed the experimental diets for 4weeks

	CAS	FPro	FPep
Non-cholesterol diet			
Growth parameters			
Initial BW (g)	101 ± 4	101 ± 1	110 ± 2
Final BW (g)	307 ± 10	311 ± 6	319 ± 5
BW gain (g/day)	7.3 ± 0.3	7.5 ± 0.2	7.5 ± 0.1
Energy intake (kcal/day)	69.3 ± 2.7	72.1 ± 2.9	72.9 ± 3.2
Food efficiency (g/kcal)*	0.106 ± 0.004	0.104 ± 0.003	0.102 ± 0.002
Relative organ weights			
Liver weight (g/kg BW)	39.0 ± 1.1 a	35.8 ± 1.4 b	37.9 ± 1.0 ab
WAT weight (g/kg BW)	45.1 ± 1.8	50.4 ± 2.8	50.0 ± 1.4
Cholesterol diet			
Growth parameters			
Initial BW (g)	109 ± 3	103 ± 2	106 ± 3
Final BW (g)	314 ± 6	303 ± 6	317 ± 8
BW gain (g/day)	7.3 ± 0.3	7.1 ± 0.2	7.6 ± 0.3
Energy intake (kcal/day)	67.0 ± 1.4	70.1 ± 2.2	71.4 ± 1.6
Food efficiency (g/kcal)*	0.110 ± 0.003	0.102 ± 0.003	0.106 ± 0.003
Relative organ weights			
Liver weight (g/kg BW)	53.9 ± 2.0	50.4 ± 1.4	52.8 ± 1.3
WAT weight (g/kg BW)	44.0 ± 1.9	41.3 ± 1.4	39.2 ± 1.2

Data are means ± SE (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ by Turkey-Kramer test. Rats were fed the experimental diets for 4 weeks. Food consumption and body weight were recorded every two days. The liver and WAT weights were obtained after sacrifice.
BW, body weight; WAT, white adipose tissue.
*WAT = epididymal WAT + mesentery WAT + perirenal and retroperitoneal WAT.

Table 27. Serum, liver and feces lipid contents of rats fed experimental diets for 4weeks.

	CAS	FPro	FPep
Non-cholesterol diet			
Serum (mg/dl)			
Cholesterol	77.6 ± 2.5 a	70.7 ± 2.1 ab	66.0 ± 2.9 b
TG	33.0 ± 1.2 a	30.6 ± 1.5 ab	26.4 ± 1.4 b
HDL-C	67.9 ± 3.0	74.3 ± 2.2	71.4 ± 2.6
LDL-C	5.3 ± 0.3 a	4.8 ± 0.3 ab	4.1 ± 0.2 b
Liver (mg/g protein)			
Cholesterol	35.4 ± 5.7	28.7 ± 2.6	36.0 ± 2.1
TG	266 ± 27	189 ± 19	234 ± 26
Feces			
Cholesterol (mg/day)	30.0 ± 0.8 a	46.8 ± 0.7 b	40.8 ± 1.0 b
Bile acid (μmol/day)	41.0 ± 1.7 a	85.9 ± 1.9 b	98.2 ± 1.3 b
Cholesterol diet			
Serum (mg/dl)			
Cholesterol	84.9 ± 3.2 a	77.7 ± 1.9 ab	72.4 ± 2.1 b
TG	34.7 ± 1.5	32.9 ± 2.3	29.6 ± 1.3
HDL-C	63.5 ± 1.1	70.0 ± 3.0	70.5 ± 1.7
LDL-C	5.7 ± 0.1 a	5.0 ± 0.2 ab	4.2 ± 0.3 b
Liver (mg/g protein)			
Cholesterol	478 ± 33 a	270 ± 32 c	348 ± 38 b
TG	1177 ± 65 a	801 ± 58 b	1250 ± 87 a
Feces			
Cholesterol (mg/day)	136 ± 4.3 a	388 ± 9.7 c	274 ± 8 b
Bile acid (μmol/day)	106 ± 4.3 a	153 ± 5.8 a	214 ± 6 b

Data represent means ± SE (n=7). Values in the same row not sharing a common letter are significantly different at $p < 0.05$ according to the Tukey–Kramer test.
TG, triacylglycerol; HDL-C, high–density lipoprotein–cholesterol; LDL-C, low–density lipoprotein–cholesterol.

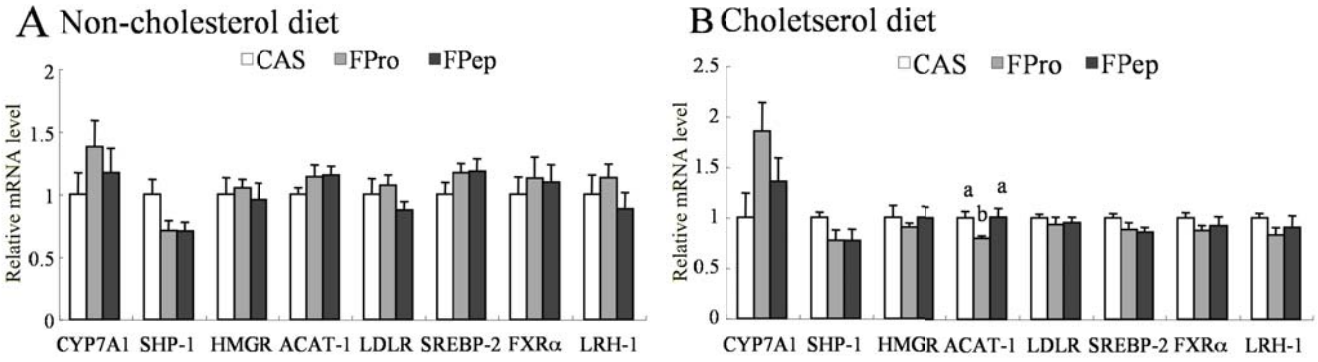


Figure 10. Effects of dietary casein, fish protein, and fish peptides on mRNA expression levels of cholesterol metabolism genes in rats fed the experimental diets for 4 weeks.

(A) non-cholesterol diet group, (B) cholesterol diet group

Data are means ± SEM (n=7). Relative values are represented as the ratio of mRNA to GAPDH mRNA. Values not sharing a common letter are significantly different at $p < 0.05$. The data were analyzed using the Tukey-Kramer test.

CYP7A1: cholesterol 7α-hydroxylase; SHP-1: small heterodimer partner-1; HMGR: 3-hydroxy-3methylglutaryl-coenzyme A reductase; ACAT-1: acyl-coenzyme:cholesterol acyltransferase-1; LDLR: low density lipoprotein receptor; SREBP-2: sterol regulatory element-binding protein-2; FXRα: farnesoid X receptor-α; LRH-1: liver receptor homolog-1.

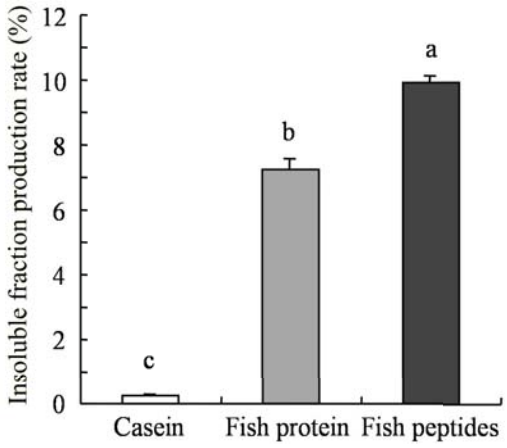


Figure 11. Production rate of the insoluble digestion products of casein, fish protein, and fish peptides hydrolysates.

Data represent means ± SE (n=3). Values not sharing a common letter are significantly different at $p < 0.05$ according to the Tukey–Kramer test.

Production rate (%) = the insoluble digestion product weight / intact protein weight of the initial reaction × 100

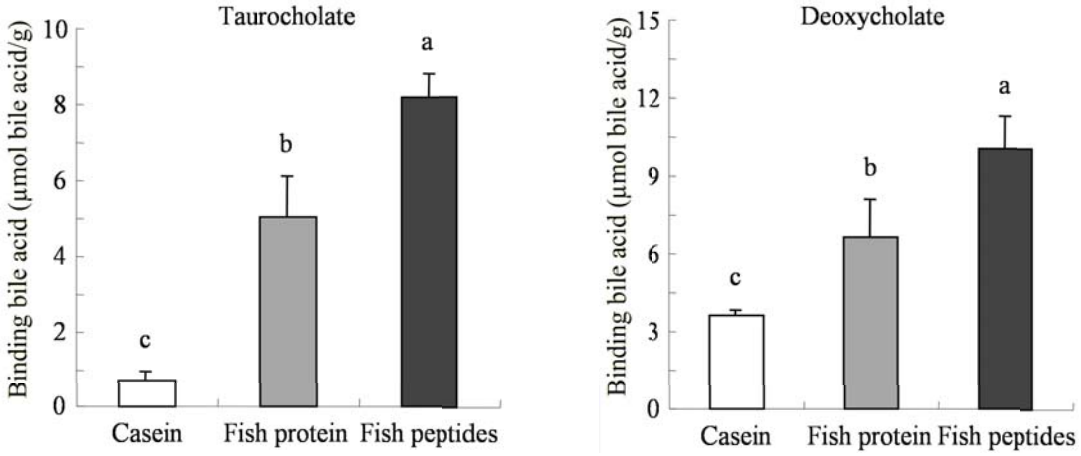


Figure 12. Taurocholate and deoxycholate binding capacities of the insoluble digestion products of casein, fish protein, and fish peptides hydrolysates.

Data represent means ± SE (n=3). Values not sharing a common letter are significantly different at $p < 0.05$ according to the Tukey–Kramer test.

Bile acid binding capacity of protein (mmol/g) = (production rate/100) × bile acid binding capacity

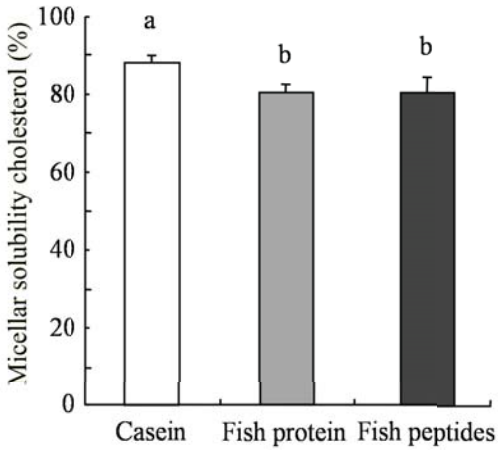


Figure 13. Effects of the insoluble digestion products of casein, fish protein, and fish peptides hydrolysates on the micellar solubility of cholesterol.

Data represent means ± SE (n=3). Values not sharing a common letter are significantly different at $p < 0.05$ according to the Tukey–Kramer test.

Discussion

The hypocholesterolemic effect of dietary proteins has been studied in both experimental animals and human subjects. For example, rats fed soy protein had a lower plasma CHOL concentration accompanied by increased fecal steroid concentrations when compared with rats fed casein [98]. Moreover, soy peptides produced by microbial protease showed a stronger hypocholesterolemic effect than soy protein in rats fed diets enriched with CHOL and cholic acid [76]. Several researchers have suggested that dietary fish protein reduces plasma CHOL concentrations in laboratory animals compared with dietary casein [39,40,71,72]. It has also been found that peptides prepared from pork meat by papain hydrolysis shows hypocholesterolemic activity in animals [92]. We hypothesized that dietary fish peptides prepared by papain alter CHOL metabolism in rats compared with intact fish protein. Therefore, this study examined the effects of fish peptides on serum and liver CHOL contents compared with the effects of casein and fish protein in both non-CHOL and CHOL diets. Furthermore, the effects of fish protein and fish peptides on mRNA expression levels of liver CHOL metabolism were examined.

The important findings in the present study were that serum CHOL and LDL-C contents decreased and HDL-C contents remained unchanged in rats fed the fish peptides-containing diet compared with rats fed the casein-containing diet (Table 5). Furthermore, when rats fed fish peptides were compared with rats fed fish protein, there was a tendency toward decreased serum CHOL and LDL-C contents. It was suggested that dietary fish peptides prepared as a fish protein hydrolysates by papain were more effective in decreasing serum CHOL and LDL-C contents compared with dietary intact fish protein.

Energy intake and food efficiency of rats fed fish peptides were similar to those of rats fed casein (Table 26). Fish peptides molecules were < 5 kDa and had amino acid compositions similar to those of intact fish protein. Therefore, the effects of fish peptides on serum CHOL and LDL-C contents resulted from peptides produced during gastric and intestinal digestion of fish peptides. We therefore analyzed the effects of fish peptides on fecal CHOL and bile acid excretions. Nagata *et al.* found that the degree of decrease in serum CHOL concentrations depended on the extent fecal steroid excretion [25]. Fecal CHOL and bile acid contents in rats fed the fish peptides diet

were higher by 1.36- and 2.39-fold in the non-CHOL diet and 2.02- and 2.01-fold in the CHOL diet, respectively, than rats fed the casein diet (Table 27). These results suggest that dietary fish peptides may effectively inhibit the absorption of CHOL across the jejunal epithelium and bile acids in the ileum, which is responsible for the micellar solubility of CHOL and capture of bile acids by peptides produced during digestion of the dietary fish peptides.

On the other hand, fecal CHOL and bile acid contents of rats fed the fish protein diet were higher by 1.56- and 2.09-fold in the non-CHOL diet and 2.86- and 1.44-fold in the CHOL diet, respectively, than rats in the casein diet group (Table 27). When rats fed fish protein diets were compared with rats fed fish peptides diets, the fecal CHOL contents were higher but the fecal bile acid concentrations were lower. Saeki *et al.* suggested that the inhibition of CHOL absorption was not the major factor involved in the differential effects of dietary proteins on serum CHOL [99]. Therefore, we predicted that peptides led to a decrease in serum CHOL contents due to an increase in fecal bile acid amounts. The reason for this difference in fecal CHOL and bile acid contents in rats fed fish protein and fish peptides is not clear at present. It is necessary to perform further experiments in order to clarify the mechanism of the altered binding capacities of bile acid by hydrophobic binding and micellar solubility of CHOL and bile acid.

Liver CHOL and TG contents of rats fed the fish protein diet were lower by 0.72- and 0.71-fold in the non-CHOL diet and 0.56- and 0.68-fold in the CHOL diet than those of rats fed the casein diet (Table 27). Furthermore, it was speculated that the decreased liver weights in rats fed fish protein could be mainly due to the prevention of lipid accumulation. The effects of decreased liver CHOL and TG contents in rats fed the fish peptides diet were weaker compared with rats fed the fish protein diet. Previously, it was suggested that reduction of lipoprotein formation with the subsequent lowering of plasma lipids could lead to an accumulation of lipids in the liver [100]. Moreover, liver TG contents were regulated by fatty acid synthesis and β-oxidation. It is necessary to perform further experiments in order to clarify the mechanism by measuring CHOL and TG secretion from the liver to blood and fatty acid synthesis and β -oxidation activities.

CHOL homeostasis is maintained by a balance of its uptake, biosynthesis, catabolism, and excretion. Moreover, the maintenance of CHOL homeostasis is also

regulated by transcription factors and nuclear receptors. The beneficial effects of dietary fish peptides on circulating CHOL can be confirmed by examining the expression levels of liver CHOL metabolism enzymes, such as CYP7A1, HMGR, ACAT-1 and LDLR. The present study sought to clarify the effects that fish protein and fish peptides have on liver transcription factors and nuclear receptors involved in CHOL metabolism, such as SREBP-2, SHP-1, FXRα and LRH-1. It was shown that dietary fish protein and fish peptides did not influence the liver gene expression levels of HMGR, LDLR, or SREBP-2, indicating that dietary fish protein and fish peptides cannot alter CHOL uptake and biosynthesis. When rats were fed CHOL diets, the expression level of ACAT-1, which catalyzes the storage of excess CHOL in cells [101], was significantly lower in the FPro group than in the CAS group, as shown in Figure 10, and results from rats in the FPro group showed that liver CHOL concentrations had decreased remarkably. Rats fed the fish protein diet showed a tendency for increased CYP7A1 expression levels in both CHOL and non-CHOL diets. These increased CYP7A1 expression levels may be related to the decreases in liver CHOL. By contrast, CYP7A1 expression levels in rats fed the fish peptides diet did not increase compared with rats fed casein, whereas SHP-1 expression levels tended to decrease in rats fed the fish protein diet in parallel. SHP-1 expression levels were negatively regulated via the inactivation of FXRα by the decreased re-absorption of bile acid [102]. Rats fed fish peptides showed higher fecal bile acid contents than rats fed casein or fish protein. Therefore, it is suspected that CYP7A1 expression levels in rats fed fish peptides diets might be negatively regulated by nuclear receptors beside SHP-1. CYP7A1 expression is negatively regulated by bile acid through SHP-1, while it is positively regulated by hepatocyte nuclear factor-4α (HNF-4α) and liver X receptor-α (LXRα) [103]. HNF-4α and LXRα expression levels should be determined to elucidate how CYP7A1 is down-regulated in rats fed the fish peptides diet.

Two main arguments have been discussed concerning the effects of dietary protein on serum CHOL contents. One hypothesis is the amino acid composition of protein, such as the lysine/arginine ratio or methionine [79,80]. However, the amino acid compositions of fish protein and peptides were almost the same (Table 2), and the difference effects of fish protein and peptides cannot be explained in terms of differences in amino acid composition. The other hypothesis is that there are differences in the structures of peptides produced during digestion. Previous studies

suggest the hypocholesterolemic effects of peptides from papain hydrolysate of pork meat [92] and peptides from microbial proteases hydrolysate of soybean protein compared with intact protein [76]. In this study, dietary fish peptides exerted a stronger hypocholesterolemic effect than intact fish protein. Therefore, it is suggested that these changes appear to be due to differences between the structures of the peptides produced during gastric and intestinal digestion compared to intact fish protein and fish peptides.

Fish protein had a significantly higher production rate of the insoluble digestion products compared with casein (Figure 11). Fish peptides also had a significantly higher production rate of the insoluble digestion products than casein, and were significantly higher than fish protein as well. The insoluble digestion products of fish protein were significantly higher in term of the taurocholate and deoxycholate binding capacities compared with the insoluble digestion products of casein. The insoluble digestion products of fish peptides were significantly higher in term of the taurocholate and deoxycholate binding capacities compared with the insoluble digestion products of casein and fish protein. The insoluble digestion products of fish protein and peptides were significantly lowered the micellar solubility of CHOL compared with the insoluble digestion products of casein. These results suggested that the increased fecal acidic and neutral sterols excretions in rats fed a fish protein and peptides diet were at least partly due to the insoluble digestion products of fish protein and peptides, which have high bile acid binding capacity and low micellar solubility of CHOL.

Summary

Dietary protein affects lipid metabolism in both humans and animals. The digestion products of proteins can interrupt the intestinal absorption of bile acid and cholesterol, and the intake of certain protein hydrolysates is more effective in lowering serum cholesterol than the intact protein. This study examined that dietary fish protein hydrolysates (fish peptides) prepared by papain alter cholesterol metabolism in rats fed with or without cholesterol diet compared with intact fish protein. Male Wistar rats were divided into the following 6 dietary groups of 7 rats each: casein (20%), fish protein (10%) + casein (10%) and fish peptides (10%) + casein (10%) with cholesterol (0.5% cholesterol and 0.1% cholic acid) or without cholesterol. After 4 week on these diets, the serum, liver, and feces lipid indexes were measured. In rats fed the fish peptides diet with or without cholesterol, the indexes of lipid metabolism namely serum cholesterol, triacylglycerol, and low density lipoprotein-cholesterol contents were significantly lower, while fecal cholesterol and bile acid were higher compared with the casein diet. The fish protein diet exerted similar effects as the fish peptides diet with or without cholesterol, although the effects of the fish protein diet were weaker than those of the fish peptides diet. The hypocholesterolemic effects of fish protein and peptides were mediated by increased fecal acidic and neutral sterols excretions, which were due to the digested products of fish protein and peptides having reduced micellar solubility of cholesterol and increased bile acid binding capacity. The results suggest that the intake of fish peptides is effective in improving lipid metabolism, especially of cholesterol, and may have beneficial effects for hypercholesterolemia patients.

Conclusion

Epidemiological evidence from Greenland Inuits and Japanese fishing villages suggests that eating marine animals and fish can prevent CHD [33,34]. In Japan, fish has traditionally formed one of the main food sources. In recent years, however, the intake of livestock food products, such as dairy products and meats, has increased in Japan; this shift could be responsible for the increased incidence of lifestyle-related diseases such as hyperlipidemia, atherosclerosis, diabetes, and hypertension, and, through these, to the increased incidence of CHD [1]. It has been suggested that an increase in total fat, saturated fatty acids, and the ratio of n-6 PUFAs to n-3 PUFAs in the average diet strongly affects the incidence of arteriosclerosis and coronary or cerebral artery disease [1,2].

On the other hand, it is also thought that improving lipid metabolism in the circulatory system can prevent the development of arteriosclerotic disease. Dietary therapy is considered the first-choice treatment for arteriosclerotic disease and is as important as medical treatment. Fish contain n-3 PUFAs, such as EPA and DHA, which are associated with a reduced risk of death due to CHD. Several mechanisms have been proposed to explain how n-3 PUFAs might beneficially influence cardiovascular disease [104], but it is not possible to explain the role of marine foods in health only in terms of EPA and DHA. Marine products contain protein and PLs containing n-3 PUFAs in addition to the TG containing n-3 PUFAs. In this study, I examined the effects of novel components such as PLs containing n-3 PUFA and protein on improving lipid metabolism.

Chapter 1 described the effect of PLs containing n-3 PUFAs extracted from squid mantle muscle on improving lipid metabolism. Dietary PLs containing n-3 PUFAs decreased serum and liver TG contents through the suppression of liver SREBP-1c mRNA expression levels. They also decreased serum and liver CHOL contents through the enhancement of fecal CHOL excretion and the export of CHOL into bile from the liver by increasing ABCG5 and ABCG8 expression levels. As the differences of serum lipid contents between the PL containing n-3 PUFAs and TG containing n-3 PUFAs were the decreasing serum CHOL contents. Dietary PLs containing n-3PUFAs exerted hypotriglyceridemic and hypocholesterolemic effects. Furthermore, PLs containing n-3PUFAs more effectively reduced key factors for

atherosclerosis than TG containing n-3 PUFAs did.

Chapter 2 described the effect of fish protein prepared from Alaska pollock fillets on improving lipid metabolism. Compared with casein, fish protein promoted decreases in liver CHOL and TG and serum CHOL and LDL-C contents. The reason for the decreases in CHOL contents in the serum and liver was the enhancement of fecal CHOL and bile acid excretion and of the mRNA expression level of CYP7A1, a rate-limiting enzyme of bile acid synthesis. In addition, it was found that dietary fish protein affects the FXR/SHP-dependent pathway, which is negatively regulated by the decreased reabsorption of bile acid. Dietary fish protein also increased binding to the promoter of CYP7A1 through activated LRH-1.

Chapter 3 described the effect of Salmon protamine on improving lipid metabolism. Compared with dietary casein, dietary salmon protamine lowered TG in the serum and the liver, at least in part through the enhancement of β-oxidation in the liver. The decreases in serum CHOL, LDL-C, and liver CHOL contents in rats fed protamine have been suggested to be related to enhanced fecal excretion of CHOL and bile acid and increased mRNA expression of ABCG5 and ABCG8 in the liver. In view of the fecal nitrogen results, I suspected that the enhanced fecal excretion of CHOL and bile acid may have been achieved through the different mechanism of the effect of dietary soy protein.

If fish protein is to be used effectively as a health food, it is necessary to clarify the details of the mechanism of lipid absorption. Chapter 4 described the mechanism by which dietary fish protein inhibition of lipid absorption in the intestine. The hypocholesterolemic effect was found to be mediated by indigestible protein and peptides remaining after the digestion of fish protein. Furthermore, fish protein hydrolysates inhibited the micellar solubility of CHOL and increased bile acid binding capacity compared with casein hydrolysates, as observed through an *in vitro* study. Therefore, the increased fecal CHOL and bile acid excretion in rats fed fish protein was due to fish protein hydrolysates, which have low micellar solubility of CHOL and high bile acid binding capacity.

The effects of peptides prepared using protease are known to be different from those of intact protein. In search of a more beneficial new food material, I prepared fish peptides using papain. Fish peptides decreased serum CHOL and LDL-C contents and increased fecal CHOL and bile acid levels compared with casein and intact fish

protein. These results suggested that the intake of fish peptides is more effective in the suppression of lipid absorption than the intake of intact fish protein is. These changes appear to be due to differences between the structures of the peptides produced during gastric and intestinal digestion compared to intact fish protein and fish peptides. Dietary fish peptides prepared using papain might provide health benefits through decreasing CHOL levels in the blood, which would help prevent the development of circulatory system diseases such as arteriosclerosis.

Marine foods, unlike many livestock products, are a source of animal protein that can be continually harvested without causing environmental degradation, as long as marine resources are well managed. They are also important as a source of the lipids and proteins that are required for the maintenance and improvement of health. In the future, marine foods will play an important role in averting food shortages due to global population increase. This study has shown that PLs containing n-3 PUFAs, protein produced from fish fillets, and protamine produced from salmon testes improve lipid metabolism through decreasing serum and liver lipid levels. The beneficial effects of dietary fish should therefore be attributed not only to n-3 PUFA but also to PLs containing n-3 PUFAs and fish protein. Thus fish consumption can aid in the prevention of lifestyle-related diseases through improving lipid metabolism. In addition, fish peptide prepared from fish protein using papain is more effective than intact fish protein at improving lipid metabolism. The results of this study may lead to the creation of a new field of functional food materials, both domestically and internationally. It should also contribute to efforts to find uses for marine waste products.

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