

Differential Effects of Cod Proteins and Tuna Proteins on Serum and Liver Lipid Profiles in Rats Fed Non-Cholesterol- and Cholesterol-Containing Diets

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ABSTRACT: Fish muscles are classified into white and red muscles, and the chemical composition of the two fish muscles have many differences. Few reports have assessed the health-promoting functions of white fish muscle proteins (WFP) and red fish muscle proteins (RFP). We therefore evaluated the mechanisms underlying the alteration of lipid profiles and cholesterol metabolism following the intake of WFP prepared from cod and RFP prepared from light muscles of tuna. Male Wistar rats were divided into six dietary groups: casein (23%), WFP (23%), and RFP (23%), with or without 0.5% cholesterol and 0.1% sodium cholate. Compared to the WFP-containing diet, the RFP-containing diet supplemented with cholesterol and sodium cholate significantly increased serum and liver cholesterol contents. However, in the RFP groups, an alteration in cholesterol metabolism including an increased tendency to excrete fecal sterols and hepatic cholesterol 7 α -hydroxylase was related to the reduction of hepatic cholesterol contents. This phenomenon might be related to the tendency of an increased food intake in RFP-containing diets. These results highlight the differential effects of WFP and RFP on serum and liver lipid profiles of Wistar rats fed non-cholesterol- or cholesterol-containing diets under no fasting condition.

Keywords: cod protein, tuna protein, lipid metabolism, cholesterol, rat

INTRODUCTION

In previous epidemiological studies, high fish and seafood consumption had been associated with a reduced risk of lifestyle-related diseases, including type-2 diabetes (1). Fish and seafood contain *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) including eicosapentaenoic acid (EPA, C20:5*n*-3) and docosahexaenoic acid (DHA, C22:6*n*-3). *n*-3 PUFAs are known to exhibit beneficial effects against various diseases, such as insulin resistance and dyslipidemia, and a number of studies have reported that the physiological effects of fish consumption are related to EPA and DHA consumption (2). However, the dietary habits of most countries include consumption of not only EPA and DHA from fish oil, but whole fish, which contains nutrients such as proteins, minerals, vitamins, and fat. The health-promoting effects of the proteins contained in fish meat may have been underestimated because of the potent physiological effects of EPA and DHA in fish and seafood intake studies. The consumption of

lean fish is reported to provide health-promoting effects, including the reduction of serum triacylglycerol (TAG) and the fatty acid desaturase index (C18:1*n*-9/C18:0) in humans (3). In addition, our and other groups have observed in animal models that fish proteins prepared from cod or salmon affect the cholesterol content of serum (4-7), counteract hypertension (8), stimulate fibrinolysis (9), and have anti-obesity functions (10,11).

Fish muscles are roughly classified into white, such as the ones of demersal fish, and red muscles, such as the ones of migratory fish. There are many differences in the chemical composition of the two fish muscle types, the most striking being the myoglobin (Mb) and free L-histidine content of red fish muscles (12). Although the differential effects of dietary red muscle (beef and pork) and white muscle (poultry and lean fish) on serum and liver lipid contents in humans have been reported, researchers did not focus on the ameliorating effects on lipid metabolism induced by differential fish muscles, including white muscle fish proteins (WFP) and red

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muscle fish proteins (RFP) (13,14). Besides other animal proteins such as beef, pork, and poultry proteins, WFP and RFP play an important role in human nutrition worldwide; however, few reports have focused on the effects of dietary WFP and RFP on lipid parameters and mechanisms underlying lipid metabolism. Rodents fed diets containing cholesterol accumulated serum and liver TAG and cholesterol (15). The aim of this research was to investigate the different effects of dietary WFP and RFP on the lipid profiles of serum and liver in rats fed non-cholesterol- and cholesterol-containing diets and to examine the mechanisms underlying these effects. WFP and RFP were prepared from cod (*Theragra chalcogramma*) and tuna (*Thunnus orientalis*) light muscle, respectively.

MATERIALS AND METHODS

Materials and reagents

Fish fillets of cod and tuna, provided by Yashima Shiyoji Co., Ltd. (Shizuoka, Japan), were chopped into small pieces. Muscles were freeze-dried and then washed with *n*-hexane/ethanol (1:1) to remove fats. The residues were air-dried, ground using a Waring blender (GM200; Retsch Technology GmbH, Haan, Germany), and then stored at -35°C . The resulting products were referred to as WFP and RFP. The yield rates for WFP and RFP from wet fillets were 12.1 and 13.5% (w/w), respectively. All other reagents (reagent grade) were purchased from commercial sources.

Chemical analyses

The moisture content was considered as the loss of weight upon drying at 105°C for 24 h, and the ash content was estimated after direct incubation at 550°C for 24 h. The crude fat content was analyzed according to the chloroform/methanol/water method (16). The EPA and DHA contents of WFP and RFP were measured using gas chromatography (GC) as described previously (17). The cholesterol contents of WFP and RFP were measured by GC (GC-2014; Shimadzu Corporation, Kyoto, Japan) equipped with a DB-5 capillary column (Agilent Technologies Japan Ltd., Tokyo, Japan) with an internal standard of 5α -cholestane. The crude protein content was measured with the Micro-Kjeldahl method using 6.25 as the N-to-protein conversion factor. Amino acid compositions of the investigated proteins were determined using high-performance liquid chromatography (HPLC) with an internal standard of L- α -amino adipic acid as described previously (18). Briefly, proteins were hydrolyzed at a high temperature and under acidic conditions (110°C for 24 h, 6 M HCl) before HPLC analysis. The Mb content of the investigated proteins was measured by spectrophotometry using horse muscle Mb as a

standard (19).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

SDS-PAGE was conducted using 12.5% polyacrylamide gels and a molecular weight standard marker (Bio-Rad Laboratories Inc., Hercules, CA, USA) in accordance with a previous study (20). Protein gels were stained with a 0.25% (w/v) Coomassie brilliant blue solution (water : methanol : acetic acid = 5:4:1, v/v/v).

Animal diet and care

The experimental protocols used in the present research followed the "Guide for the Care and Use of Experimental Animals" issued by the Prime Minister's Office of Japan, and were reviewed and approved by the Animal Ethics Committee of Kansai University (Approval No. 1407). The breeding room was maintained under the following conditions: temperature, $21\sim 23^{\circ}\text{C}$; humidity, $55\sim 65\%$; light period, 08:00 AM~08:00 PM. Four-week-old male Wistar rats were obtained from Japan SLC Inc. (Shizuoka, Japan). The rats were separated into three groups of six rats each in experiments 1 and 2: CAS, rats fed casein; WFP, rats fed WFP; RFP, rats fed RFP; CAS+C, rats fed casein with cholesterol; WFP+C, rats fed WFP with cholesterol; RFP+C, rats fed RFP with cholesterol. Table 1 shows the ingredients of the experimental diets prepared in accordance with the AIN93G formula (21). The rats had free access to tap water and experimental diets.

In both experiments, body weight (BW) and food consumption were measured every 2 days. Before rats were sacrificed, feces were gathered from each rat daily for 3 days, freeze-dried, weighed, and ground using a conventional mill. After breeding for 28 days, BWs of rats were measured under non-fasting conditions and the animals sacrificed with isoflurane (Intervet K.K., Osaka, Japan) anesthesia between 09:00 AM~11:00 AM in each experiment. Blood samples were collected from the ventral aorta, and sera were separated by centrifugation ($2,000\text{ g}$ at 4°C for 15 min). Livers and abdominal white adipose tissues (WATs) from the perinephria, mesenteries, and epididymes were rapidly removed and weighed. Aliquots of liver tissue were stored in RNAlater[®] solution (Sigma-Aldrich Japan K.K., Tokyo, Japan) for quantitative polymerase chain reaction (PCR). Samples were frozen in liquid nitrogen and stored at -80°C until further analysis.

Biochemical analyses

The biochemical parameters of sera were analyzed using a commercial service (Japan Medical Laboratory, Osaka, Japan). The total lipid of liver samples were extracted using the chloroform/methanol/water method (16). Each extracted total lipid fraction was dissolved in 10 volumes

Table 1. Composition of the experimental diets

(unit: g/kg)

	Experiment 1			Experiment 2		
	CAS	WFP	RFP	CAS+C	WFP+C	RFP+C
Casein	230	—	—	230	—	—
WFP	—	230	—	—	230	—
RFP	—	—	230	—	—	230
Dextrinized corn starch	92.1	92.1	92.1	92.1	92.1	92.1
Corn starch	277.386	277.386	277.386	271.386	271.386	271.386
Sucrose	100	100	100	100	100	100
Cellulose	50	50	50	50	50	50
AIN-93G mineral mixture	35	35	35	35	35	35
AIN-93 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
Lard	130	130	130	130	130	130
Cholesterol	—	—	—	5	5	5
Sodium cholate	—	—	—	1	1	1
<i>tert</i> -Butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014

CAS, casein; WFP, white muscle fish protein; RFP, red muscle fish protein; CAS+C, casein with cholesterol; WFP+C, WFP with cholesterol; RFP+C, RFP with cholesterol; AIN, American Institute of Nutrition.

of ethanol, and cholesterol and TAG contents were measured using commercial kits (Cholesterol E-test and Triglyceride E-test, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Phospholipid contents in extracted liver lipids were estimated by phosphorus analyses (22).

Fatty acids content in feces samples was analyzed in accordance with a previous study (23). The neutral sterol (sum of coprostanol and cholesterol) content in feces samples was analyzed by GC (GC-2014, Shimadzu Corporation) as described above. The acidic sterol content in feces was measured with a commercial kit (Total Bile Acid Test Wako kit, Wako Pure Chemical Industries, Ltd.) according to the manufacturer's protocol. Nitrogen contents in feces were determined by the Micro-Kjeldahl method as described above.

Analysis of enzyme activities

The hepatic fractions (crude, cytosol, and mitochondria) were collected by ultracentrifugation as described previously (17). The activities of fatty acid synthase (FAS) (24), acetyl-CoA carboxylase (ACC) (25), glucose-6-phosphate dehydrogenase (G6PDH) (26), and malic enzyme (ME) (27) in the cytosolic fractions, carnitine palmitoyltransferase-2 (CPT-2) (28) in the mitochondrial fractions, and acetyl-coenzyme A oxidase (ACOX) (29) in the crude fractions were assayed spectrophotometrically. Protein content in the three hepatic fractions was measured with the Pierce™ Modified Lowry Protein Assay Kit (Thermo Fisher Scientific K.K., Kanagawa, Japan) according to the manufacturer's instructions.

Quantitative real-time PCR analysis

Total RNA was isolated from livers using the TRIzol™

reagent (Life Technologies Corporation, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The content and purity of each total RNA were determined at 260 and 280 nm by UV spectroscopy (UV-1800, Shimadzu Corporation) using TrayCell (Helma Analytics, Müllheim, Germany). Subsequently, cDNA synthesis reactions were performed with the RevaTraAce® qPCR RT kit (TOYOBO Co., Ltd., Osaka, Japan) using 1 µg of each total RNA. Gene expressions at the mRNA level were analyzed using a real-time PCR system (Thermal Cycler Dice® Real Time System Single, Takara Bio Inc., Shiga, Japan) and the GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA). The primer sequences for ATP-binding cassette (ABC)A1, ABCG5, ABCG8, acyl-CoA:cholesterol acyltransferase (ACAT)-1, cholesterol 7 α -hydroxylase (CYP7A1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), low-density lipoprotein receptor (LDLR), scavenger receptor class B member 1 (SR-B1), small heterodimer partner (SHP)-1, and sterol regulatory element-binding transcription factor (SREBF)-2 were as follows: forward: 5'-CCC GGC GGA GTA GAA AGG-3' and reverse: 5'-AGG GCG ATG CAA ACA AAG AC-3' for ABCA1; forward: 5'-ATG GCC TGT ACC AGA AGT GG-3' and reverse: 5'-GGA TAC AAG CCC AGA GTC CA-3' for ABCG5; forward: 5'-TTC TGC TGC AAC GCT CTC TA-3' and reverse: 5'-GGA GGA ACG ACA TCT TGG AA-3' for ABCG8; forward: 5'-AAG TAC GCC ATC GGC TCT TA-3' and reverse: 5'-TCA CCA CCA CGT CTG GTT TA-3' for ACAT-1; forward: 5'-CCC AGA CCC TTT GAC TTT CA-3' and reverse: 5'-GAT CCG AAG GGC ATG TAG AA-3' for CYP7A1; forward: 5'-ATG ACT CTA CCC ACG GCA AG-3' and reverse:

5'-TAC TCA GCA CCA GCA TCA CC-3' for GAPDH; forward: 5'-TAC TCC TTG GTG ATG GGA GC-3' and reverse: 5'-CCA TTG GCA CCT GGT ACT CT-3' for HMGCR; forward: 5'-ACC GCC ATG AGG TAC GTA AG-3' and reverse: 5'-CGG CGC TGT AGA TCT TTC TC-3' for LDLR; forward: 5'-GTT CGT TGG GAT GAA CGA CT-3' and reverse: 5'-ATG CCA ATA GTT GAC CTC GC-3' for SR-B1; forward: 5'-CCT TGG ATG TCC TAG GCA AG-3' and reverse: 5'-CAA CCC AAG CAG GAA GAG AG-3' for SHP-1; and forward: 5'-CAC CTG TGG AGC AGT CTC AA-3' and reverse: 5'-TGC CAG AGT GTT GTC CTC AG -3' for SREBF-2. Gene expression levels were expressed relative to the expression level of GAPDH. The primers were designed using a web tool (<http://primer3plus.com/>, Primer3Plus, Michelstadt, Germany).

In vitro protein digestion

The investigated proteins were digested using a simulated gastrointestinal digestion model described in a previous report (30). The sediments of the digestion solutions were washed with distilled water 3 times and centrifuged at 5,000 g at 20°C for 20 min, freeze-dried, and weighed (4). To monitor the degree of hydrolysis (DH) at intervals during the pepsin and pancreatin digestion, aliquots of the digestion reaction were collected after 0, 2, 5, 10, 15, 20, 30, 60, and 120 min of reaction. Aliquots were mixed with equal volumes of 30% (w/w) trichloroacetic acid to inactivate the digestion enzymes and subsequently centrifuged at 12,000 g at 4°C for 15 min. Each DH was calculated using the measurement of free amino groups upon reaction with 2,4,6-trinitrobenzene sulfonic acid; L-leucine was used as a standard (31).

Statistical analysis

Data are shown as the means and standard errors of the means (SEM). Statistical differences between multiple groups were determined by analysis of variance (ANOVA) and then evaluated with the Tukey's multiple comparison test at a $P < 0.05$. For the DH study, two-factor repeated measures analysis of variance was conducted to identify significant effects of time, protein hydrolyzate, and time-protein hydrolyzate interactions. Statistical analysis was performed with the Prism software, version 6.0e (GraphPad Software Inc., La Jolla, CA, USA) or StatView-J software version 5.0 (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

Chemical compositions and molecular weights of the investigated proteins

The chemical compositions of the investigated proteins

are shown in Table 2. The amino acid compositions of WFP and RFP, compared with casein, are rich in alanine, arginine, aspartic acid, and glycine, but poor in lysine, proline, and tyrosine. The histidine and Mb levels in RFP were higher (3.2 and 4.0 times, respectively) than the ones in WFP. WFP and RFP were shown to contain very low amounts of *n*-3 PUFAs (EPA and DHA) and cholesterol.

The SDS-PAGE patterns of casein, WFP, and RFP are presented in Fig. 1. In the casein pattern, the two prominent bands around 31 kDa likely contained α -casein and β -casein. WFP and RFP contained large amounts of myofibrillar proteins including myosin (heavy chain, approximately around 116 kDa) and actin (approximately around 45 kDa). The arrows in Fig. 1 indicate strong bands in the RFP sample not present in the WFP sample.

Growth parameters and organ weights

The growth variables and organ weights of experiments 1 and 2 are presented in Table 3. No significant differences were noted in growth variables including the initial BW, food efficiency, and water intake among experimental groups, whereas the final BW, BW gain, and food intake in rats fed RFP-containing diets (RFP and RFP+C groups) tended to be higher than those of rats fed ca-

Table 2. Chemical composition of casein, WFP, and RFP

Component	Casein	WFP	RFP
Crude protein (g/100 g)	85.9	85.4	85.7
Amino acid compositions (wt %)			
Alanine	2.3	6.3	6.6
Arginine	3.6	8.0	7.2
Aspartic acid ¹⁾	4.9	10.1	9.8
Glutamic acid ²⁾	18.5	16.6	14.5
Glycine	1.5	4.5	4.2
Histidine	2.5	2.6	8.2
Isoleucine	4.4	4.1	4.3
Leucine	8.3	7.9	7.5
Lysine	12.0	9.4	7.1
Methionine	2.3	3.2	3.3
Phenylalanine	5.5	4.1	3.8
Proline	13.6	4.6	3.5
Serine	4.4	4.5	3.9
Threonine	3.9	4.7	4.8
Tyrosine	6.5	3.7	3.5
Valine	5.7	4.5	4.4
Mb (g/100 g)	ND	0.1	0.4
Crude fat (g/100 g)	0.7	1.0	0.7
EPA+DHA (g/100 g)	ND	0.2	0.1
Cholesterol (mg/100 g)	20.0	10.0	13.0
Moisture (g/100 g)	5.6	5.9	5.3
Ash (g/100 g)	1.8	6.3	5.7

WFP, white muscle fish protein; RFP, red muscle fish protein.

¹⁾Aspartic acid+asparagine.

²⁾Glutamic acid+glutamine.

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Mb, myoglobin; ND, not detected.

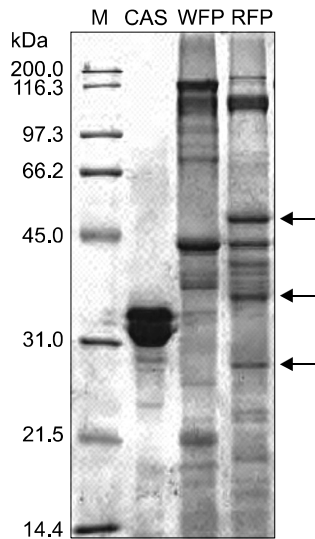


Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of casein, WFP, and RFP. SDS-PAGE was performed using 12.5% polyacrylamide gels. M, molecular weight marker; CAS, casein; WFP, white muscle fish protein; RFP, red muscle fish protein.

sein- ($P=0.13$ and 0.15 , respectively) and WFP-containing diets ($P=0.13$ and 0.12 , respectively).

When rats were fed a non-cholesterol-containing diet, no significant differences were observed in the relative weights of liver, perirenal WAT, mesentery WAT, and epididymal WAT among the experimental groups. When rats were fed a cholesterol-containing diet, relative liver weights in the WFP+C group were significantly decreased compared with the CAS+C and RFP+C groups. In addition, no influence of cholesterol-containing diets (experiment 2) on the relative WAT weights were observed.

Biochemical parameters of sera, livers, and feces

The serum, liver, and fecal biochemical parameters obtained in experiments 1 and 2 are shown in Table 4. When rats were fed a non-cholesterol-containing diet, the serum biochemical parameters, including aspartate aminotransferase, alanine aminotransferase, TAG, cholesterol, high-density lipoprotein (HDL)-cholesterol, non-HDL-cholesterol, phospholipid (PL), and non-esterified fatty acid (NEFA) did not display significant differences among the experimental groups. On the other hand, when rats were fed a cholesterol-containing diet, the serum contents of cholesterol, HDL-cholesterol, non-HDL-cholesterol, PL, and NEFA in the WFP+C group were significantly decreased compared with the ones in the RFP+C group.

The liver cholesterol contents in rats fed WFP-containing diets (WFP and WFP+C) were significantly decreased compared with the ones in rats fed RFP-containing diets (RFP and RFP+C). The liver TAG content in the WFP group was lower than the one in the RFP group when cholesterol was not administered. There were no significant differences in the liver PL contents among the experimental groups in each experiment.

The fecal contents of acidic sterols and nitrogen in rats fed RFP-containing diets (RFP and RFP+C) were significantly higher than the ones in rats fed casein-containing diets (CAS and CAS+C). When rats were fed cholesterol-containing diets, the WFP+C diet tended to result in higher fecal acidic sterol excretions compared with the CAS+C diet ($P=0.10$); however, no significant differences were observed. The fecal neutral sterols in the WFP+C group tended to be higher compared with the ones in the CAS+C group ($P=0.07$). No significant differences in fecal dry weight and fatty acid contents were found

Table 3. Growth parameters and relative organ weights in rats fed experimental diets for 4 weeks

	Experiment 1			Experiment 2		
	CAS	WFP	RFP	CAS+C	WFP+C	RFP+C
Growth parameters						
Initial BW (g)	72.9±1.1	71.9±0.9	71.8±1.1	84.6±2.5	83.5±1.9	84.5±2.3
Final BW (g)	303.6±10.8	305.1±4.5	320.2±3.9	317.1±8.3	322.9±5.4	344.2±10.4
BW gain (g/d)	8.2±0.4	8.3±0.2	8.9±0.1	8.6±0.4	8.9±0.2	9.6±0.3
Food intake (g/d)	17.3±0.8	17.3±0.8	19.6±0.9	17.9±0.7	17.7±0.7	20.0±0.9
Food efficiency (g/g)	0.48±0.02	0.48±0.01	0.45±0.01	0.48±0.02	0.50±0.01	0.48±0.02
Water intake (mL/d)	35.7±2.6	36.0±2.7	37.4±2.8	33.5±2.1	32.5±2.0	34.0±2.3
Organ weight (g/100 g BW)						
Liver weight	4.43±0.32	4.20±0.08	4.23±0.06	6.07±0.11 ^b	5.32±0.13 ^a	6.18±0.21 ^b
Perirenal WAT weight	1.28±0.14	1.32±0.10	1.46±0.19	1.36±0.09	1.35±0.11	1.80±0.26
Mesentery WAT weight	1.26±0.20	1.36±0.08	1.34±0.18	1.51±0.17	1.57±0.06	1.58±0.08
Epididymal WAT weight	1.33±0.15	1.42±0.07	1.41±0.05	1.57±0.15	1.37±0.17	1.54±0.24

Data represent means±SEM (n=6).

Values in the same row not sharing a common letters (a,b) are significantly different at $P<0.05$ using the Tukey's multiple comparisons test.

CAS, casein; WFP, white muscle fish protein; RFP, red muscle fish protein; CAS+C, casein with cholesterol; WFP+C, WFP with cholesterol; RFP+C, RFP with cholesterol; BW, body weight; WAT, white adipose tissue.

Table 4. Biochemical parameters in serum, liver, and feces in rats fed experimental diets for 4 weeks

	Experiment 1			Experiment 2		
	CAS	WFP	RFP	CAS+C	WFP+C	RFP+C
Serum biochemical parameters						
AST (IU/L)	73.7±3.8	69.2±1.0	72.3±1.4	65.7±3.6	65.2±2.5	69.0±3.0
ALT (IU/L)	42.8±3.0	40.8±1.7	38.0±1.3	34.8±3.5	39.5±2.6	34.7±2.8
TAG (mg/dL)	130.8±23.7	116.4±26.0	79.4±12.1	162.5±28.4	164.2±29.9	181.5±29.9
Cholesterol (mg/dL)	82.5±6.0	84.5±5.6	94.3±2.8	94.5±1.3 ^a	85.0±3.5 ^a	114.3±6.1 ^b
HDL-cholesterol (mg/dL)	55.5±5.3	58.8±4.2	67.5±2.5	52.8±2.2 ^{ab}	48.0±2.2 ^a	63.2±3.6 ^b
Non-HDL-cholesterol (mg/dL)	27.0±1.0	25.7±1.4	26.8±0.4	44.0±2.5 ^{ab}	37.0±1.8 ^a	51.2±3.8 ^b
PL (mg/dL)	149.5±9.8	151.7±5.2	168.2±3.5	156.3±7.2 ^a	144.8±2.6 ^a	187.0±7.1 ^b
NEFA (μEq/L)	1,036.3±133.1	926.5±62.0	978.6±197.4	653.8±33.3 ^{ab}	551.0±12.3 ^a	748.2±56.4 ^b
Liver lipid contents (mg/g)						
TAG	78.7±9.5 ^{AB}	52.8±3.8 ^A	111.8±18.9 ^B	195.7±11.3	168.6±13.1	192.2±19.3
Cholesterol	4.11±0.48 ^B	2.51±0.36 ^A	3.26±0.31 ^B	6.79±0.82 ^a	4.76±0.45 ^a	12.93±1.11 ^b
PL	21.2±1.2	20.0±0.6	19.2±1.3	21.7±0.8	22.0±0.4	20.0±0.8
Fecal biochemical parameters						
Dry weight (g/d)	7.77±0.24	8.16±0.14	8.61±0.31	6.10±0.33	8.88±1.4	8.09±0.52
FFA (mg/d)	124.0±8.4	138.1±4.7	141.2±10.5	316.7±32.8	435.4±61.1	414.9±65.7
Neutral sterols (mg/d)	14.2±1.3	17.7±2.7	18.8±1.6	37.5±2.8	72.4±16.0	59.8±8.0
Acidic sterols (mg/d)	13.6±2.3 ^A	16.7±2.0 ^{AB}	21.9±1.6 ^B	22.0±2.2 ^a	35.2±3.8 ^{ab}	39.9±6.0 ^b
Nitrogen content (mg/d)	9.36±0.56 ^A	10.21±0.27 ^A	13.22±0.70 ^B	6.05±0.90 ^a	11.32±2.42 ^{ab}	13.57±1.13 ^b

Data represent means±SEM (n=6).

Values in the same row not sharing a common letters (a,b or A,B) are significantly different at $P<0.05$ using the Tukey's multiple comparisons test.

CAS, casein; WFP, white muscle fish protein; RFP, red muscle fish protein; CAS+C, casein with cholesterol; WFP+C, WFP with cholesterol; RFP+C, RFP with cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TAG, triacylglycerol; HDL, high-density lipoprotein; PL, phospholipid; NEFA, non-esterified fatty acid; FFA, free fatty acid.

among the experimental groups in each experiment.

Enzyme activities and mRNA expression levels in livers

Liver enzyme activities and mRNA expression levels related to lipid metabolism obtained in experiments 1 and 2 are shown in Table 5. When rats were fed a non-cholesterol-containing diet, the RFP group showed significantly increased hepatic FAS activity compared with the CAS group, and hepatic ACC, G6PDH, and ME activities compared with the WFP group. However, the liver CPT-2 and ACOX activities did not differ significantly among the groups in experiment 1. The RFP+C group had a significantly lower ACOX activity and a higher G6PDH activity compared with the CAS+C group. In addition, the RFP+C group tended to display an ACC activity higher than the one of the CAS+C group ($P=0.06$).

When rats were fed a non-cholesterol-containing diet, no significant differences in gene expression levels were identified among the experimental groups. When rats were fed a cholesterol-containing diet, the WFP+C and RFP+C groups tended to have higher CYP7A1 levels ($P=0.09$ and 0.07 , respectively) and lower SHP-1 levels ($P=0.08$ and 0.06 , respectively) compared with the CAS+C group. Expression levels of HMGCR in the WFP+C group tended to be lower than in the CAS+C group ($P=0.07$). No significant differences in the expression levels of other genes were identified among the

investigated groups in experiment 2.

Degree of hydrolysis and insoluble fraction production rate

The time courses of DH and the production rates of insoluble fractions of the investigated proteins are shown in Fig. 2. The DH of the experimental proteins was high for 20 min after pepsin and pancreatin were added; hydrolysis proceeded slowly thereafter. During pepsin digestion, the DH of RFP was higher than that of casein. The insoluble fraction production rates of WFP and RFP were significantly higher than the one of casein.

DISCUSSION

n-3 PUFAs are well known to influence lipid profiles, including blood lipids and lipoproteins in humans and in animal models (2). We therefore tested for EPA and DHA in our WFP and RFP samples. The WFP and RFP samples contained very low amounts of EPA and DHA (0.2 g/100 g and 0.1 g/100 g, respectively). In addition, the cholesterol contents of the WFP and RFP samples were also low (10 mg/100 g and 13 mg/100 g, respectively). Therefore, these results indicate the successful fractionation of WFP and RFP, allowing us to exclude the possible effects of *n-3* PUFAs and cholesterol.

Table 5. Enzymes activities and relative mRNA expression levels in livers from rats fed experimental diets for 4 weeks

	Experiment 1			Experiment 2		
	CAS	WFP	RFP	CAS+C	WFP+C	RFP+C
Enzyme activity (nmol/min/mg protein)						
CPT-2	1.46±0.23	1.38±0.44	1.32±0.22	1.37±0.08	1.15±0.46	0.70±0.16
ACOX	2.17±0.47	3.06±0.32	2.23±0.60	1.90±0.12 ^b	1.72±0.24 ^{ab}	1.08±0.15 ^a
FAS	1.39±0.17 ^{AB}	1.20±0.15 ^A	1.97±0.21 ^B	1.35±0.13	1.26±0.05	1.25±0.07
ACC	56.8±5.2 ^A	55.8±8.2 ^A	91.7±10.1 ^B	66.5±4.8	84.6±10.9	97.6±5.4
G6PDH	10.8±0.9 ^A	12.5±1.7 ^{AB}	24.4±5.4 ^B	4.5±0.2 ^a	3.8±0.2 ^a	7.5±1.1 ^b
ME	9.7±1.1 ^A	16.2±.4 ^{AB}	21.8±2.2 ^B	4.2±0.3	4.4±0.3	4.9±0.2
mRNA expression level (arbitrary units)						
ABCA1	100.0±23.0	96.6±23.8	82.5±20.9	100.0±16.5	85.1±24.1	100.2±48.4
ABCG5	100.0±21.2	102.8±16.6	101.1±17.8	100.0±11.9	89.5±13.9	91.8±16.4
ABCG8	100.0±28.7	153.4±20.5	182.3±66.2	100.0±16.1	55.9±24.1	41.5±14.0
ACAT-1	100.0±2.8	92.2±12.7	70.6±13.6	100.0±18.7	92.7±27.5	106.3±48.3
CYP7A1	100.0±19.0	161.6±45.8	91.7±31.4	100.0±17.9	201.4±36.0	213.9±35.2
HMGCR	100.0±7.3	115.6±29.8	106.2±13.9	100.0±12.5	58.4±17.6	86.6±15.7
LDLR	100.0±19.6	59.6±15.0	61.2±12.1	100.0±22.6	62.3±8.1	51.6±7.2
SRB1	100.0±24.6	66.1±9.3	44.2±12.3	100.0±36.8	54.0±15.1	71.4±19.3
SHP-1	100.0±21.4	158.7±50.0	147.3±72.6	100.0±19.0	50.3±11.0	50.1±9.8
SREBF-2	100.0±22.2	64.5±17.7	59.1±10.4	100.0±8.2	61.1±19.3	75.2±15.3

Data represent means±SEM (n=6).

Values in the same row not sharing a common letters (a,b or A,B) are significantly different at $P<0.05$ using the Tukey's multiple comparisons test.

The mRNA expression levels were determined by real-time polymerase chain reaction analysis using the glyceraldehyde 3-phosphate dehydrogenase mRNA expression level for normalization. mRNA expression levels of genes are shown relative to those determined from livers of rats fed the CAS (Experimental 1) and CAS+C (Experimental 2) diets (set at 100).

CAS, casein; WFP, white muscle fish protein; RFP, red muscle fish protein; CAS+C, casein with cholesterol; WFP+C, WFP with cholesterol; RFP+C, RFP with cholesterol; CPT-2, carnitine palmitoyltransferase-2; ACOX, acetyl-CoA oxidase; FAS, fatty acid synthase; ACC, acetyl-CoA carboxylase; G6PDH, glucose-6-phosphate dehydrogenase; ME, malic enzyme; ABCA1, ATP-binding cassette subfamily A1; ABCG, ATP-binding cassette subfamily G; ACAT-1, acetyl-CoA acetyltransferase-1; CYP7A1, cholesterol 7 α -hydroxylase; HMGCR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDLR, low-density lipoprotein receptor; SRB1, scavenger receptor class B member 1; SHP-1, small heterodimer partner-1; SREBF-2, sterol regulatory element binding factor-2.

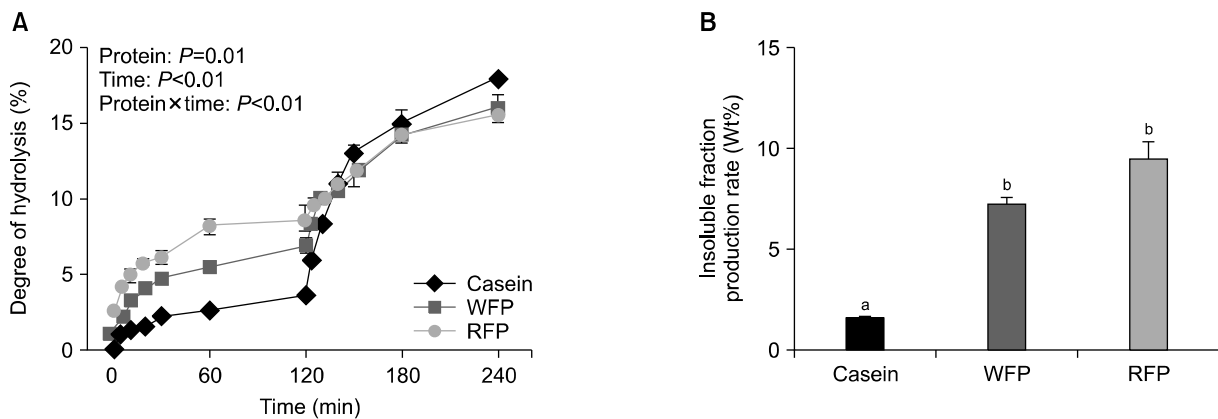


Fig. 2. Time courses of degree of hydrolysis and insoluble fraction production rate in simulated gastrointestinal digestions. Data represent means±SEM (n=4). (A) Significant effects of time, protein, and time-protein interactions were identified by two-factor repeated measure analysis of variance. (B) Values in the same row not sharing a common letters (a,b) are significantly different at $P<0.05$ using the Tukey's multiple comparisons test. RFP, red muscle fish protein; WFP, white muscle fish protein.

In the present study, rats fed a WFP-containing diet for 4 weeks showed a reduction in hepatic cholesterol when their diets did not contain cholesterol. These data support previous findings on the hepatic cholesterol-lowering function of dietary proteins prepared from cod (5,6, 10). However, the RFP+C diet significantly increased the serum and liver biochemical parameters related to cho-

lesterol compared with the WFP+C diet. Only a few reports have addressed the effect of dietary tuna proteins on cholesterol metabolism; however, other fish proteins, including sardine and salmon proteins, also contributed to decrease the serum cholesterol content in type 2 diabetes rats (7,32). To determine how WFP and RFP affected the lipid metabolism in rats, we investigated the expres-

sion levels of genes related to the cholesterol pathway in the liver and lipid excretion in feces.

Several mechanisms may explain the cholesterol-lowering function of dietary proteins. One possibility is that the lowering of the hepatic cholesterol content is the result of an enhanced excretion of fecal sterols. In this study, compared with casein-containing diets (CAS and CAS+C), the RFP-containing diets (RFP and RFP+C) enhanced the fecal acidic sterol excretion, whereas the WFP+C diet tended to increase the neutral and acidic sterols ($P=0.07$ and 0.10 , respectively). These data support the positive effect of dietary WFP on fecal sterol excretion we proposed in our previous study (4). A previous report suggested that the enhancement of fecal neutral and acidic sterol excretions is accompanied by an increase in fecal nitrogen contents (33). Rats fed the RFP-containing diets (RFP and RFP+C) had significantly increased fecal nitrogen contents, and RFP diets were hard to be digested by pancreatin, as the high production rate of insoluble fractions in our *in vitro* experiment suggested (Fig. 2). The enhancement of fecal neutral and acidic sterol excretions by RFP-containing diets might be associated with indigestible proteins and peptides. The relationship between fecal sterols excretion and hepatic cholesterol content in the RFP+C group was unclear.

Another possible explanation for the cholesterol contents in serum and liver could be related to the balance between cholesterol biosynthesis, catabolism, efflux, and incorporation in hepatic tissue. In this study, the WFP+C diet tended to result in higher CYP7A1, a rate-limiting enzyme involved in the conversion of cholesterol to 7α -hydroxylated bile acid, expression levels and lower HMGCR, a rate-limiting enzyme involved in cholesterol synthesis, and expression levels compared with the CAS+C diet. Therefore, WFP had a cholesterol-lowering effect partly due to the enhancement of CYP7A1 expression and suppression of HMGCR when rats were fed a cholesterol-containing diet. This phenomenon was also observed in our previous report (5). In addition, the RFP+C diet also tended to result in higher hepatic CYP7A1 expression levels compared with the CAS+C diet. Bile acids are endogenous ligands for the farnesoid X receptor (FXR), and the activation of FXR indirectly represses CYP7A1 through induction of SHP-1, a transcription factor (34). The SHP-1 expression levels in the WFP+C and RFP+C groups tended to be lower than the one in the CAS+C group. The WFP+C and RFP+C diets might therefore enhance CYP7A1 expression negatively regulating the FXR/SHP-1 pathway by inhibiting reabsorption of bile acids in the ileum. The hypocholesterolemic alteration of the cholesterol metabolism by dietary proteins is associated with amino acid composition or peptides released from the digestive tract. Specific amino acids, such as methionine (35), cysteine (36), and glycine

(37), and indigestible peptides (4) might be responsible for this hypocholesterolemic effect. Saithe (*Pollachius virens*) protein hydrolysates, rich in glycine, increased plasma acidic sterols concomitantly with decreased liver lipids (38). In addition, our previous study reported that dietary cod proteins decrease serum and liver cholesterol contents, suggesting the suppression of neutral and acidic sterol absorption via decreased micellar cholesterol solubility and reflecting the high acidic sterol-binding capacity of insoluble peptides (4). The metabolic alteration, namely the increased tendency to excrete fecal neutral and acidic sterols, in the WFP+C and RFP+C groups could be therefore explained with the difference in glycine contents and production rates of insoluble peptides, but serum and liver cholesterol were not decreased in the RFP+C group.

In the present study, the alteration of the metabolic pathway in the RFP+C group contradicted the elevation of serum and liver cholesterol contents. The reason for this is probably due to the increased food intake in RFP-containing diets (RFP and RFP+C) compared with CAS-containing diets ($P=0.13$ and 0.15 , respectively) and WFP-containing diets ($P=0.13$ and 0.12 , respectively). A previous study reported that rats fed with a 40% reduced calorie intake had significantly lower serum cholesterol contents compared with rats with free access to food (39). We thus hypothesized that the increased serum and liver cholesterol contents induced by the RFP+C diet were attributable to increased food intake and not to enhanced fecal sterol excretion and hepatic CYP7A1 levels. No significant differences in food efficiency were identified among the experimental groups. The increased final BW and BW gains observed in the RFP and RFP+C groups were probably due to the increase in food intake. Several peptides generated from the digestive tract upon protein intake have been known to alter cholecystokinin (CCK) secretion, which is linked to increased appetite (40). The higher food intake by the RFP-fed groups might have been due to a lower secretion of CCK compared with the other two groups. If rodents are sacrificed at the same time, fasting times are different between rodents with free access and restricted access to food (41). A previous study reported that fasting time has a significant impact on lipid metabolism, and especially on enzymes involved in fatty acid and cholesterol biosynthesis in rat livers (42). In order to elucidate the mechanism of lipid lowering induced by dietary food components, rodents should not be fasted in the last day (42). In the present study, we chose the no fasting condition and restricted access to the diets in order to control the equivalent fasting time among the experimental groups. However, there is the possibility that the serum and liver lipid contents in rats fed RFP-containing diets under pair-feeding conditions obtained in this experiment would be different un-

der no fasting conditions. Moreover, a pair-feeding study is necessary in order to investigate the possible effects of a difference in food intake on serum and liver cholesterol contents.

On the other hand, rats fed the RFP diet had increased hepatic TAG contents compared with rats fed the WFP diet. It is possible that the lowering of hepatic TAG is mainly due to the inhibition of the pancreatic lipase activity in the intestine and a reflection of the endogenous fatty acid metabolism in the liver. There were no differences in fecal fatty acid excretions among the experimental groups, reflecting the inhibition of pancreatic lipase activity. The activities of hepatic FAS and ACC, which are rate-determining enzymes in fatty acid biosynthesis, were significantly higher in the RFP group than in the WFP group. The elevation of liver TAG content upon the RFP diet might therefore be in part attributable to an enhancement of fatty acid biosynthesis in the liver. In previous studies, decreased hepatic TAG contents were not exclusively caused by variations in fatty acid oxidation, but also by variations in biosynthesis and absorption in the liver (43) and other metabolic pathways (44). Increased serum NEFA contents are commonly associated with impaired insulin-mediated glucose uptake (45). The WFP+C diet inhibited increased serum NEFA contents compared with the RFP+C diet, indicating that WFP might possibly stimulate insulin activity, resistance, and sensitivity. A previous study reported that cod proteins reduced liver TAGs through the enhancement of basal energy expenditure by inducing muscle hypertrophy (46). In addition, glucose-lowering peptides, including Ala-Asn-Gly-Glu-Val-Ala-Gln-Trp-Arg and Gln-Trp-Arg, have been identified in cod protein samples after enzymatic digestion (47). Further investigation is needed to clarify the effects of dietary WFP and RFP on insulin activity and glucose metabolism and the consequences of the release of bioactive peptides in the digestive tract.

CONCLUSION

These results suggest that WFP decrease the liver cholesterol content, whereas the RFP do not in rat animal models with free access to experimental diets. This research shows for the first time the differential effects of cod proteins and tuna proteins on the serum and liver lipid profiles in Wistar rats fed a non-cholesterol- or cholesterol-containing diet under the no fasting condition.

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The authors declare no conflict of interest.

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