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Original Article

Effect of Fish Protein and Peptides on Lipid Absorption in Rats

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Summary

The digestion products of protein can interrupt the intestinal absorption of acidic and neutral sterols. The intake of certain protein hydrolysates is more effective in lowering serum cholesterol than the intake of intact protein. In this study, we hypothesized that, compared to intact fish protein, dietary fish peptides prepared by the treating of fish protein with papain alter lipid absorption in rats. Male Wistar rats were divided into the following three dietary groups, each composed of seven rats: casein (20 %), fish protein (10 %) + casein (10 %), and fish peptides (10 %) + casein (10 %), each with cholesterol (0.5 %) and cholic acid (0.1 %). Compared with dietary casein, dietary fish peptides decreased serum and liver cholesterol, whereas fecal acidic and neutral sterols excretions were higher. The fish protein diet had similar effects as the fish peptides diet, 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 suggested that the intake of fish peptides is more effective in the suppression of lipid absorption than the intake of intact fish protein. This finding may be of benefit to patients with hypercholesterolemia.

Introduction

Epidemiological studies in Greenland Inuit and Japanese fishing villages suggest that dietary fish and marine animals can prevent coronary heart disease[1, 2]. The beneficial effects of fish intake are attributed to n–3 polyunsaturated fatty acids (PUFAs) in fish oil, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[3, 4]. Dietary n–3 PUFAs decrease serum triacylglycerol, although they do not lower serum cholesterol[5]. Therefore, it is not possible to explain the health function of fish-based foods solely in terms of EPA and DHA. General dietary habits include not only fish oil but also whole fish, which provide many additional nutrients, such as protein. Some studies have demonstrated that, compared with dietary casein, dietary fish protein reduces plasma cholesterol concentration in experimental animals[6–9].

It has often been argued that protein digestion products interrupt the intestinal absorption of acidic and neutral sterols. Several reports have shown that peptides prepared by the hydrolysis of plant proteins exhibited hypocholesterolemic activity in serum, which may be due to the increased excretions of fecal acidic and neutral sterols[10]. These reports also indicated that animal peptides prepared by treating pork meat with papain or β-lactoglobulin with trypsin had hypocholesterolemic activity[11, 12], although intact animal proteins in beef, pork, and turkey failed to reduce cholesterol in the plasma or liver[13]. Dietary fish peptides have many beneficial effects properties: they are antihypertensive, antioxidative, and immunomodulating[14–16]. Few studies, however, have focused on the hypocholesterolemic effects of dietary fish peptides in the serum and liver of experimental animals[7]. In this study, we used rats fed cholesterol diets to evaluated the effects of dietary fish peptides prepared using papain and fish protein on lipid absorption, especially acidic and neutral sterols.

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Materials and Methods

1. Preparation of fish protein and peptides

Fillets of Alaska pollock (*Theragra chalcogramma*) 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, USA) for 2 min after water deprivation. The resulting meat was treated with cold acetone, ethyl acetate, and *n*-hexane to remove protein-associated lipids. The meat was then dried under N_2 gas and stored at −30℃.

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

Table 1 presents the nutrient 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℃ for 24 hour. The ash amount was analyzed by direct ignition at 550℃ for 24 hour.

Table 1 Nutrients in dietary proteins (%)

Components	Dietary protein		
	Casein	Fish protein	Fish peptides
Crude protein	89.2	89.6	89.1
Crude fat	1.4	1.1	0.4
Moisture	7.7	7.7	8.3
Ash	1.6	1.6	2.2

The molecular weight (MW) distribution of casein, fish protein, and fish peptides were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with broad-range or polypeptide SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA, USA).

2. Animals care and experimental diets

The experimental protocol was reviewed and approved by the Animal Ethics Committee of Kansai Medical University. It 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 (Kyoto, Japan) were kept in an air-conditioned room (temperature, 21–22℃; humidity, 55–65%; lights on, 08:00–20:00) and had free access to tap water and feed. Rats were fed AIN-93G diets according to the recommendations of the American Institute of Nutrition[17]. After a 5-day acclimation to the AIN-93G diet, rats were divided into the following three dietary groups, each composed of seven rats: casein (CAS) diet; fish protein plus casein (FP) diet; and fish peptides plus casein (FPH) diet, each with 0.5% (w/w) cholesterol and 0.1% (w/w) cholic acid. Table 2 presents the composition of the experimental diets prepared according to AIN-93G and Table 3 presents the amino acid composition of the experimental diets that was determined by a commercial service (Japan Food Research Laboratories, Tokyo, Japan).

Food consumption and body weight were recorded every two days. Feces were collected from each group every 24 hours for 7 days prior to sacrifice. After feeding for 4 weeks, rats were weighed and sacrificed under pentobarbital 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

Table 2 Composition of experimental diets (g/kg diet)

Components	Dietary groups		
	CAS	FP	FPH
Casein	200	100	100
Fish protein	–	100	–
Fish peptides	–	–	100
Dextrinized corn starch	132	132	132
Corn starch	391.5	391.5	391.5
Sucrose	100	100	100
Cellulose	50	50	50
AIN-93G 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
Cholesterol	5	5	5
Cholic acid	1	1	1

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

Table 3 Amino acid composition in the experimental diets (g/kg diet)

Aminoacid	Dietary groups		
	CAS	FP	FPH
Alanine	5.4	8.9	8.7
Arginine	6.6	10.3	10.3
Aspartic acid	12.6	17.6	17.5
Cystine	0.9	1.5	1.5
Glutamic acid	38.0	37.3	37.5
Glycine	3.2	5.4	5.4
Histidine	5.4	5.1	5.0
Isoleucine	9.8	9.9	9.8
Leucine	16.8	17.5	17.3
Lysine	14.2	18.0	17.7
Methionine	5.2	6.1	6.0
Phenylalanine	9.0	8.4	8.4
Proline	20.0	13.5	13.4
Serine	9.2	9.2	9.4
Threonine	7.4	8.7	8.7
Tryptophan	2.2	2.3	2.2
Tyrosine	10.0	9.1	9.1
Valine	12.0	11.4	11.1
Lysine/Arginine	2.6	2.0	2.0

acid synthesis and cholesterol metabolism[18]. Blood was collected without anticoagulant, and serum was obtained by centrifugation at 1,500 *g* for 15 min and stored at −80°C until analysis. Liver and abdominal white adipose tissues of the epididymis were extirpated rapidly and then weighed, rinsed with 0.9% NaCl, and frozen in liquid nitrogen followed by storage at −80°C until analysis.

3. Lipid analysis

Serum cholesterol, high–density lipoprotein-cholesterol (HDL-C), and low–density lipoprotitaein-cholesterol (LDL-C) analyses were performed by a commercial service (Japan Medical Laboratory, Osaka, Japan).

Liver cholesterol content was analyzed by gas-liquid chromatography (GC-14B, Shimadzu, Kyoto, Japan) using 5α-cholestane as an internal standard.

Fecal neutral sterol content was analyzed with SE-30 using a GC-14B instrument and 5α-cholestane as an internal standard. Fecal acidic sterol content was measured according to the method of Bruusgaard *et al.*[19]. Fecal nitrogen content was determined by the Kjeldahl method.

4. *In vitro* digestion of protein

Casein, fish protein, and fish peptides were digested by the method of Iwami *et al.*[20] 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., St. Louis, MO, USA). After 180 min of incubation, the pepsin was inactivated by neutralization with NaOH and porcine pancreatin (Sigma Chemical Co., St. Louis, MO, USA) 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.

5. 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.*[21].

6. Micellar solubility of cholesterol

Micellar solubilities of cholesterol 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.*[22] with some modifications. Cholesterol content was measured using an enzymatic assay kit (Cholesterol-E-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

7. Statistical Analysis

Data were represented as means ± standard deviation (SD). Statistical analyses between multiple groups were determined by analysis of variance (ANOVA). Statistical comparisons were made using the Tukey–Kramer test. Differences with $p < 0.05$ were considered significant.

Results and Discussion

Several researchers have suggested that, compared with dietary casein, dietary fish protein better reduces plasma cholesterol concentrations in laboratory animals[6-9]. It has also been found that peptides formed from pork meat by papain hydrolysis exhibit hypocholesterolemic activity in animals[11], but that pepsin– and trypsin–hydrolyzed pork meat exhibit no plasma hypocholesterolemic effect[23]. We hypothesized that, compared with intact fish protein, dietary fish peptides prepared using papain alter cholesterol metabolism in rats. This study examined the effect of fish peptides on serum and liver cholesterol contents and compared it with the effects of casein and fish protein in rats fed cholesterol diets. This study used fish protein that was detected mostly as two bands (approximately, 200 and 45 kDa), which may be derived from myofibrillar protein. The fish peptides were detected at a MW < 5 kDa (data not shown).

The growth parameters of body weight gain, energy intake, food efficiency, and organ weights of relative liver and epididymal white adipose tissue weights among the groups (date not shown) did not differ.

Table 4 presents the serum and liver lipid contents of rats fed the casein, fish protein, and fish peptides diets. The FP group tended to have a lower serum cholesterol content

Table 4 Serum and liver lipid contents of rats fed experimental diets for 4 weeks

	Dietary groups		
	CAS	FP	FPH
Serum (mg/dL)			
Cholesterol	78.9 ± 7.9[a]	71.1 ± 7.2[ab]	64.9 ± 7.8[b]
HDL-C	49.6 ± 7.0	53.2 ± 6.8	52.4 ± 4.0
LDL-C	7.7 ± 0.5[a]	6.6 ± 0.8[b]	6.4 ± 0.5[b]
Liver (mg/g protein)			
Cholesterol	443 ± 50[a]	345 ± 44[b]	299 ± 48[b]

Data represent means ± SD (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.
HDL-C, high–density lipoprotein–cholesterol; LDL-C, low–density lipoprotein–cholesterol.

and significantly lower serum LDL-C and liver cholesterol contents compared with the CAS group. The FPH group had significant lower serum cholesterol, LDL-C, and liver cholesterol contents than the CAS group, even lower than the FP group. The serum HDL-C content did not differ among the groups. These results suggest that dietary fish peptides are more effective in decreasing serum cholesterol and LDL-C contents than dietary fish protein.

Two main causes have been put forth in regard to the decreased serum and liver cholesterol contents related to dietary protein. One hypothesis is that it relates to the amino acid composition of the protein, in particular, the ratio of lysine/arginine[24], and the content of specific amino acids, namely, methionine[25], cysteine[26], and glycine[27]. However, the amino acid composition of the FP and FPH diets proved to be similar to that of the CAS diet, and the difference can not be explained in terms of differences in lysine/arginine, methionine, cysteine, and glycine content alone (Table 2). The other hypothesis involves an intradigestive tract effect, namely, that the digestibility of dietary proteins and the physicochemical properties of digestion products in the digestive tract are related to cholesterol metabolism[28]. Nagata *et al*. found that the degree of serum cholesterol lowering depends on the extent of fecal excretion of steroids[29]. To clarify the mechanism of the decrease in serum and liver cholesterol contents related to the fish protein and peptides diet, fecal sterols and nitrogen excretions were analyzed. Table 5 presents the fecal acidic sterol, neutral sterol, total sterols, and nitrogen excretions of rats fed the casein, fish protein, and fish peptides diets. The FP group tended to have higher fecal acidic sterol, and significantly higher fecal neutral sterol and total sterols excretions compared with the CAS group. The FPH group had significantly higher fecal acidic sterol, neutral sterol, and total sterols excretions than the CAS group, and significantly higher neutral sterol and total sterols excretions compared with the FP group. Dietary fish protein and peptides might thus effectively inhibit the absorption of sterols. Compared to rats fed casein, rats fed fish protein and peptides had decreased serum and liver cholesterol contents as a result of the suppression of sterols absorption. The results suggested that, compared with dietary fish protein, dietary fish peptides were more effective in increasing fecal sterols excretions. In addition, Higaki *et al*. suggested that feeding soy protein stimulated the fecal excretion of bile acid. They demonstrated that the increase in fecal bile acid was accompanied by an increase in fecal nitrogen excretion[30]. In this study, the FP and FPH groups had higher fecal nitrogen excretion than the CAS group (Table 5). The enhancement of fecal sterols excretions by fish protein and peptides was at least partly due to the indigestible protein and peptides remaining after digestion because fecal nitrogen excretion was increased.

Table 5 Sterol and nitrogen contents in daily excreted feces for 7 days prior to sacrifice in rats fed experimental diets for 4 weeks (mg/day)

	Dietary groups		
	CAS	FP	FPH
Acidic sterol	5.7 ± 0.8^{b}	6.8 ± 0.8^{ab}	7.9 ± 1.4^{a}
Neutral sterol	37.6 ± 9.4^{b}	61.2 ± 3.7^{a}	76.2 ± 8.2^{a}
Total sterol	43.0 ± 9.5^{c}	68.0 ± 4.3^{b}	84.5 ± 9.4^{a}
Nitrogen	27.9 ± 2.4^{b}	34.3 ± 2.8^{a}	38.0 ± 2.1^{a}

Data represent means ± SD (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.

Previous studies have suggested that soy protein[31] and egg ovomucin[32] suppressed the micellar solubility of cholesterol and enhance bile acid binding capacity *in vitro* resulting in increased fecal steroid excretion. Proteins that are insoluble digestion products to mammalian digestive enzymes are known as resistant proteins that act to decrease blood cholesterol levels[33]. The present study examined digested casein, fish protein and peptides using an *in vitro* digestion model[20] to divide each sample into the insoluble digestion products before measuring the production rate, the micellar solubility of cholesterol and the bile acid binding capacity.

Fig. 1 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. Fig. 2 illustrates the taurocholate and deoxycholate binding capacities of the

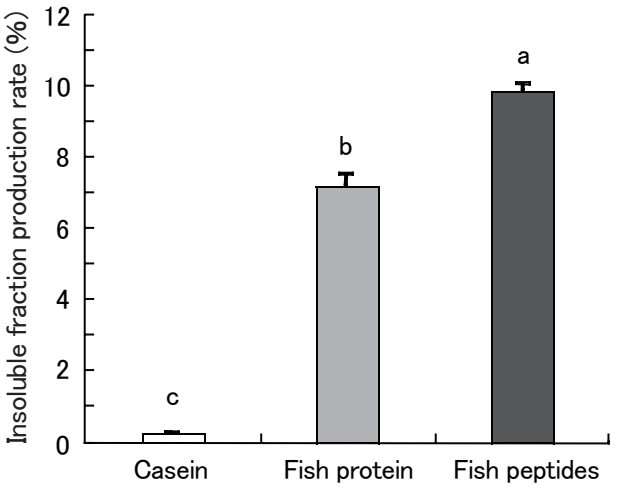

Fig. 1 Production rate of the insoluble digestion products of casein, fish protein, and fish peptides hydrolysates.

Data represent means ± SD (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.

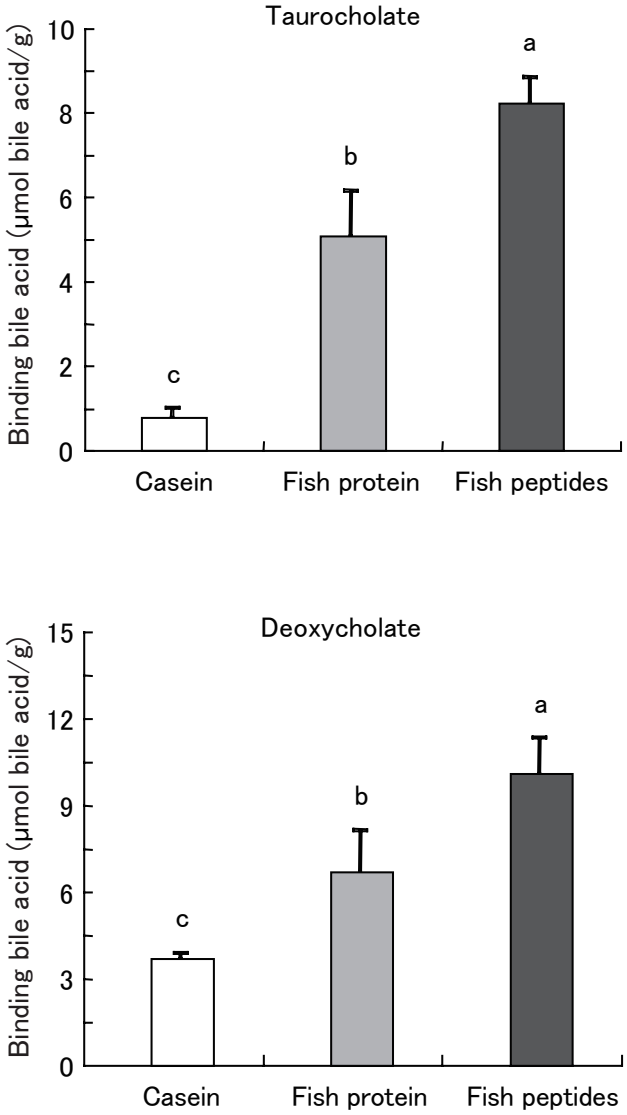

Fig. 2 Taurocholate and deoxycholate binding capacities of the insoluble digestion products of casein, fish protein, and fish peptides hydrolysates.

Data represent means ± SD (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.

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. 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. Fig. 3 illustrates the micellar solubilities of cholesterol 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 cholesterol compared with the insoluble digestion products of casein. These results suggested that the in-

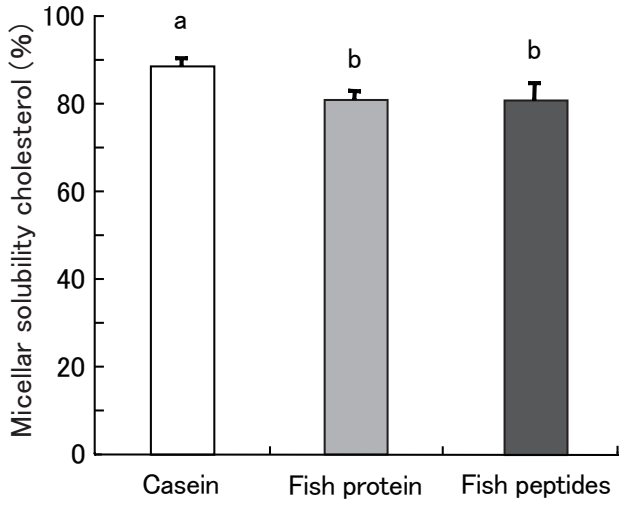

Fig. 3 Effects of the insoluble digestion products of casein, fish protein, and fish peptides hydrolysates on the micellar solubility of cholesterol.

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

creased 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 cholesterol.

In conclusion, the present study revealed that, compared to dietary casein, dietary fish protein and peptides decreased serum and liver cholesterol contents through the enhancement of fecal acidic and neutral excretions. Furthermore, dietary fish peptides prepared using papain had a greater more hypocholesterolemic effect than dietary intact fish protein. The hypocholesterolemic effect was mediated by the indigestible protein and peptides remaining after the digestion of fish peptides. This study found that fish peptides have a hypocholesterolemic effect, which may be beneficial in the prevention of arteriosclerosis.

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