Fish protein hydrolysate exhibits anti-obesity activity and reduces hypothalamic neuropeptide Y and agouti-related protein mRNA expressions in rats

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ABSTRACT

Fish protein is a source of animal protein that is consumed worldwide. Although it has been reported that the intake of Alaska pollack protein (APP) reduces body fat accumulation and increases muscle weight in rats, the mechanisms underlying these effects are poorly understood. As a possibility, peptides released from APP in the gastrointestinal tract are important to the functions of APP. In the present study, we examined the effects of APP hydrolysate digested artificially with pepsin and pancreatin on white adipose tissue and skeletal muscle. We found that APP hydrolysate group shows significantly lower weight of white adipose tissue and higher weight of soleus muscle than the control group. We also found that APP hydrolysate group reduces food intake and mRNA expressions of neuropeptide Y and agouti-related protein in the hypothalamus compared with the control group. These results may imply that APP hydrolysate exhibits anti-obesity activity by the reduction of appetite and the enhancement of basal energy expenditure by skeletal muscle hypertrophy in rats. The downregulation of orexigenic gene by APP hydrolysate in the hypothalamus may contribute to the reduction of appetite. These results suggest that the effect of APP on anti-obesity and muscle hypertrophy may be induced by peptides released from APP in the gastrointestinal tract.

Alaska pollack (*Theragra chalcogramma*) protein (APP), consumed worldwide is included in foods such as imitation crab, fish cakes, fish sausage, and so on. Recently, we reported that APP intake for 4 weeks enhanced basal energy expenditure by muscle hypertrophy and reduced serum triacylglycerol and body fat accumulation in rats (14). It also has been reported that APP decreases visceral fat accumulation, hypercholesterolemia, hypertriglyceridemia, and hyperglycemia in animals and human (5, 7, 8, 10,

18, 20). Since obesity and hypertriglyceridemia are related to coronary heart disease (2), the anti-obesity, muscle hypertrophy, and hypotriglyceridaemia with APP may be effective in the prevention. However, little is known about the active substances and the metabolism of fish protein. As a possibility, peptides released from APP in gastrointestinal tract may induce the anti-obesity, muscle hypertrophy, and hypotriglyceridaemia of APP.

In the present study, we examined if fish protein hydrolysate digested artificially with pepsin and pancreatin affects white adipose tissue and skeletal muscle weights in rats, compared to the control. As a result, we found that APP hydrolysate exhibited anti-obesity and hypertrophy effects, suggesting that peptides or amino acids released from APP in the gastrointestinal tract have an important role to these

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effects.

Obesity is an epidemic health problem all over the world (4, 19). The maintained balance between energy consumption and energy intake is one of most important factor for normal body weight. A number of endogenous orexigenic and anorexigenic factors in the central nervous system play a key role in energy intake regulation and energy homeostasis. It has reported that food-derived components affected regulation of orexigenic and anorexigenic factors in the hypothalamus. In the present study, we found that fish protein hydrolysate suppressed the energy intake as the novel function. We then examined the effects of fish protein hydrolysate on expressions of energy intake-regulated genes in the hypothalamus by real-time RT-PCR.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (SLC, Shizuoka, Japan) at five weeks of age were raised in stainless wire mesh cages in a room controlled by a 12-hour light-dark cycle (dark phase: 17 : 00–5 : 00) and at a constant temperature ($23 \pm 1^\circ\text{C}$). The animals were housed separately for five days to acclimate them to the environment. Animals were fed regular tap water and an American Institute of Nutrition (AIN)-93 control diet, which is formulated from 200 g/kg of casein (Oriental Yeast Co., Ltd, Tokyo, Japan), 3 g/kg of L-cystine (Wako Pure Chemical Industries., Ltd, Osaka, Japan), 532 g/kg of α -corn starch (Sanwa Starch Co., Ltd, Tokyo, Japan), 100 g/kg of sucrose (Dai-Nippon Meiji Sugar Co., Ltd, Tokyo, Japan), 70 g/kg of soybean oil (RIKEN Nosan-Kako Co., Ltd, Fukuoka, Japan), 50 g/kg of cellulose (Oriental Yeast Co., Ltd.), 35 g/kg of AIN-93G mineral mixture (Oriental Yeast Co., Ltd.), and 10 g/kg of AIN-93 vitamin mixture containing choline bitartrate (Oriental Yeast Co., Ltd.) *ad libitum*. This study was conducted in accordance with the ethical guidelines of the Utsunomiya University Animal Experimentation Committee (Approval No. A14-0010) and was in complete compliance with the National Institutes of Health: Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and to limit experimentation to what was necessary to produce reliable scientific information.

Preparation of APP hydrolysate. Alaska Pollack (*Theragra chalcogramma*) fillets (Nippon Suisan Kaisha, Ltd., Tokyo, Japan) were freeze-dried and ground. The fat component was extracted using hot

ethanol (65°C, 60 min, 2 times) and removed by centrifugation. The remaining ethanol was dried in a vacuum dryer (60°C, 24 h). Nutritional analyses of casein and fish protein sources were performed at Japan Food Research Laboratories (Tokyo), as in our previous report (14).

APP (20 mg/mL) were suspended in water just before digestion. Enzymatic digestion by pepsin [enzyme:substrate (E:S) ratio = 1 : 100] (Sigma-Aldrich, St. Louis, MO, USA) was performed at 37°C for 3 h (pH 2.0). The hydrolysate was subsequently boiled for 10 min to inactivate the enzyme. Following pepsin digestion, enzymatic digestion by pancreatin [E:S = 1 : 20] (Sigma-Aldrich) was performed at 37°C for 3 h (pH 7.4). The hydrolysate was subsequently boiled for 10 min to inactivate the enzyme. The hydrolysate was freeze-dried and kept at -20°C until experiment.

Experimental protocols. After five days of acclimatization, eighteen rats were divided into the three groups. APP hydrolysate dissolved in saline was administered intraperitoneally twice a day (10 : 00–11 : 00 and 16 : 00–17 : 00) at the dose of 0, 100, or 300 mg/kg body weight in each group. Throughout the 3-day duration of the experiment, weights of body and food consumed were recorded every morning for each animal, and then the food was replenished. On Day 3, the rats were killed by decapitation, and the liver, spleen, kidney, white adipose tissues (perirenal and epididymal adipose tissues), interscapular brown adipose tissue, and skeletal muscles (soleus, extensor digitorum longus, and gastrocnemius) were removed and weighed. In the weighing of white adipose tissues and skeletal muscles, the sum of the weights of the right and left sides was measured.

In another experiment, twelve rats were divided into the two groups. APP hydrolysate dissolved in saline was administered intraperitoneally twice a day (10 : 00–11 : 00 and 16 : 00–17 : 00) at the dose of 0 or 300 mg/kg body weight in each group for 2 days. The rats were killed by decapitation, and the hypothalamus and white adipose tissues (perirenal and epididymal adipose tissues) were removed. Each hypothalamus was kept in RNA later RNA Stabilization Reagent (QIAGEN Sciences Inc., Germantown, MD) until RNA extraction at -20°C .

Real-time RT-PCR. Total RNA was extracted from the hypothalamus using the QIAzol Lysis Reagent (QIAGEN Sciences Inc.) and RNeasy Mini Kit (QIAGEN Sciences Inc.). The total RNA was tran-

Table 1 Primer sequences used in the present study

Genes		Sequences	Product size (bp)	NCBI ID
Orexigenic factor				
NPY	sense	TGTGGTGATGGGAAATGAAA	87	NM_012614
	antisense	CAGGCAGACTGGTTTCACAG		
AgRP	sense	CGTGTGGGCCCTTTATTAGA	82	NM_033650
	antisense	TGCTTGTGTATCCTTGCCTA		
Galanin	sense	CTCACAGGCAAGAGGGAGTT	93	NM_033237
	antisense	AGTGCGGACGATATTGCTCT		
Anorectic factor				
POMC	sense	GAAGATGCCGAGATTCTGCT	86	NM_139326
	antisense	ACCAGCTCCACACGTCTATG		
CRH	sense	TGGATCTCACCTTCCACCTT	80	NM_031019
	antisense	GTTGCTGTGAGCTTGCTGAG		
Urocortin	sense	GTTGTCCATCGACCTCACCT	97	NM_019150
	antisense	TATGATGCGGTTCTGCTCTG		
CART	sense	CCCTACTGCTGCTGCTACCT	89	NM_017110
	antisense	CACGGCAGAGTAGATGTCCA		
Housekeeping gene				
β-actin	sense	GATCATTGCTCCTCCTGAGC	89	NM_031144
	antisense	TGATCCACATCTGCTGGAAG		

scribed to cDNA with random 6 mers by Prime-Script™ RT Master Mix (Takara Bio Inc., Shiga, Japan). For quantitative PCR, we amplified the cDNA using the LightCycler® 96 System (Roche Diagnostics Co., Mannheim, Germany) with THUNDERBIRD® qPCR Mix (Toyobo Co., Osaka, Japan) and each primer specific for rat neuropeptide Y (NPY), agouti-related peptide (AgRP), galanin, pro-opiomelanocortin (POMC), corticotrophin-releasing hormone (CRH), urocortin, and cocaine and amphetamine-regulated transcript (CART) according to the manufacturer's instructions. These primer sequences are shown in Table 1. The reactions were cycled 45 times with denaturation at 95°C for 10 s, and with annealing and elongation at 65°C for 60 s. The relative expression level of each mRNA was normalized using the mRNA level of β -actin.

Statistical analysis. Data are expressed as mean $\pm$ standard errors (SE). Statistical comparisons between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey-Kramer's test or the unpaired Student's *t*-test. Statistical significance was defined as $P < 0.05$. Statistical calculation was performed with the StatView software.

RESULTS

Effects of APP hydrolysate on food intake and body weight

Total food intake for 3 days in the APP hydrolysate

group decreased dose-dependently compared with the control group (Fig. 1A). In Day 1 and Day 2, the food intake in the APP hydrolysate (300 mg/kg body weight) group was significantly lower than that in the control group (Fig. 1B). In addition, total body weight gain for 3 days in the APP hydrolysate group decreased dose-dependently compared with the control group (Fig. 1C). There were no significant differences between the control and APP hydrolysate groups in the liver, kidney, and spleen weight (data not shown).

Effects of APP hydrolysate on weights of white adipose tissue and skeletal muscle

Weight of perirenal adipose tissue in the APP hydrolysate (for 3 days, 100 and 300 mg/kg body weight) group was significantly lower than that in the control group (Fig. 2A). Weight of epididymal adipose tissue in the APP hydrolysate (300 mg/kg body weight) group tended to be lower than that in the control group (Fig. 2B). Sum of perirenal and epididymal adipose tissue weights in the APP hydrolysate group decreased dose-dependently compared with the control group (Fig. 2C), suggesting that APP hydrolysate exhibited anti-obesity activity. There were no significant differences between the control and APP hydrolysate groups in interscapular brown adipose tissue weight (data not shown).

Weight of the soleus skeletal muscle in the APP hydrolysate group (for 3 days, 100 mg/kg body weight) was significantly higher than that in the control

Fig. 1 Effects of fish protein hydrolysate on food intake and body weight gain in rats. Rats were peritoneally administered saline or APP hydrolysate for 3 days and food intake (A, B) and body weight (C) were measured. Each value represents mean $\pm$ SEM ($n=6$). Asterisks (*) indicate a significant difference in comparison with the control (0 mg/kg of APP hydrolysate) rats ($P < 0.05$). Sharp signs (#) indicate a tendency to be difference in comparison with the control rats ($P < 0.1$).

Fig. 2 Effects of fish protein hydrolysate on white adipose tissue weight in rats. Rats were administered saline or APP hydrolysate for 3 days. On Day 3, perirenal adipose tissue (A) and epididymal adipose tissue (B) were removed and measured. Total adipose tissue weight (C) was calculated. Each value represents mean $\pm$ SEM ($n=6$). Asterisks (*) indicate a significant difference in comparison with the control (0 mg/kg of APP hydrolysate) rats ($P < 0.05$). Sharp signs (#) indicate a tendency to be difference in comparison with the control rats ($P < 0.1$).

Fig. 3 Effects of fish protein hydrolysate on skeletal muscle weight in rats. Rats were administered saline or APP hydrolysate for 3 days. On Day 3, soleus muscle (A), gastrocnemius muscle (B), and extensor digitorum longus muscle (C) were removed and measured. Each value represents mean $\pm$ SEM ($n=6$). Asterisks (*) indicate a significant difference in comparison with the control (0 mg/kg of APP hydrolysate) rats ($P < 0.05$). Sharp signs (#) indicate a tendency to be difference in comparison with the control rats ($P < 0.1$).

Fig. 4 Effects of fish protein hydrolysate on the gene expression of NPY (A) and AgRP (B) in rat hypothalamus. Each value represents mean $\pm$ SEM ($n=6$). Asterisks (*) indicate a significant difference in comparison with the control (0 mg/kg of APP hydrolysate) rats ($P < 0.05$).

group, although weights of the soleus skeletal muscle in another APP hydrolysate group (for 3 days, 300 mg/kg body weight) tended to be higher than that in the control group (Fig. 3A). There were no significant differences between the control and APP hydrolysate groups in the gastrocnemius and extensor digitorum longus skeletal muscle weights (Fig. 3B and 3C).

Effects of APP hydrolysate on mRNA expression related with appetite in hypothalamus on the second day of administration

APP hydrolysate significantly decreased *NPY* mRNA expression in the hypothalamus when compared with control (Fig. 4A). Also, APP hydrolysate significantly decreased *AgRP* mRNA expression in the hypothalamus when compared with control (Fig. 4B). There was no significant difference between groups

Table 2 The effects of APP hydrolysate on mRNA expressions of appetite related factors in rats

		Control	Fish protein hydrolysate
		(target gene mRNA/ β -actin mRNA)	
Orexigenic factor			
Galanin	($\times 10^{-2}$)	5.14 $\pm$ 0.41	5.42 $\pm$ 0.26
Anorectic factor			
POMC	($\times 10^{-2}$)	2.28 $\pm$ 0.22	2.45 $\pm$ 0.39
CRH	($\times 10^{-3}$)	3.30 $\pm$ 0.29	3.42 $\pm$ 0.30
Urocortin	($\times 10^{-4}$)	3.43 $\pm$ 0.99	2.60 $\pm$ 0.37
CART	($\times 10^{-1}$)	1.83 $\pm$ 0.12	1.85 $\pm$ 0.11

in *galanin*, *POMC*, *CRH*, *urocortin*, and *CART* mRNA expressions in the hypothalamus (Table 2). These results suggested that gene expression in the potent orexigenic *NPY/AgRP* neurons was inhibited by intraperitoneal administration of APP hydrolysate.

DISCUSSION

In the present study, we examined that APP hydrolysate affected obesity, muscle hypertrophy, and appetite in rats on the third day of administration. We found that APP hydrolysate decreased white adipose tissue weight and increased soleus skeletal muscle weight. Further, APP hydrolysate decreased food intake. Although the reduction of energy intake in rats administered APP hydrolysate is not sufficient to account for the anti-obesity activity, the reduction of energy intake would be related to reduce white adipose tissue weight partially. Mechanism of hypertrophy by APP hydrolysate remains to be unknown. Further investigation will elucidate about muscle fibre type, muscle fibre size, and degradation and synthesis of muscle protein.

In our previous study, APP oral intake for 4 weeks decreased visceral white adipose tissue weight and increased skeletal muscle weight in rats (9, 14). As an additional function, APP intake exhibited hypotriglyceridemia and lowered atherogenic index, risk factors for coronary heart disease (9). Although anti-obesity and muscle hypertrophy effects were observed with APP intake, the obvious mechanism was unclear. Therefore, in the present study, we prepared APP hydrolysate digested artificially with pepsin and pancreatin. We found that the APP hydrolysate exhibited significantly anti-obesity and muscle hypertrophy effects. There were no changes by administration of APP hydrolysate in the liver, kidney, and spleen weights, which was consistent with the previous results of APP intake. These data suggested that

active substances may be included in APP hydrolysate (*i.e.*, APP-derived peptides released in the gastrointestinal tract). Further investigation will elucidate to identify amino acid sequences of peptides as active substances.

It has been reported that hydrolysates of food protein exhibit anti-obesity activity. Vaughn *et al.* reported that soy protein hydrolysate reduced body weight gain in rats (23). Mun *et al.* and Lee *et al.* reported that corn gluten hydrolysate reduced food intake and body weight in normal and obese rats (11, 15). Ochiai *et al.* reported that egg-white protein hydrolysate decreased food intake and fat accumulation, and increased muscle weight (17). Kaneko *et al.* and Miyazaki *et al.* reported that rubiscolin-6, peptide derived from Rubisco, which is a major protein in green leaves such as spinach, reduced food intake in mice fed a high-fat diet (6, 13). Nakato *et al.* reported that soy-ghrelin derived from soy protein stimulated the appetite in mice (16). Our data showed that fish protein hydrolysate decreased appetite inversely with soy derived peptide. It might suggest that the order of amino acid in released peptide is an important factor on determination of up- or down-regulation of appetite. These results suggested that regulation of fat accumulation and/or appetite may be impact by food-derived peptides.

Food intake is regulated mainly by the interaction of orexigenic *NPY/AgRP* and anorexigenic *POMC/CART* neurons (1, 12). These neurons locate mainly in the arcuate nucleus of hypothalamus. In the present study, we found that *NPY* and *AgRP* mRNA expressions in rats administered APP hydrolysate were significantly lower compared with the control. There was no change of *POMC* and *CART* mRNA expressions by the APP hydrolysate administration. Additionally, there was no change of *galanin*, *CRH*, *urocortin* mRNA expressions in the APP hydrolysate group. The activation of *NPY/AgRP* neurons induces

potent hyperphagia and subsequently causes obesity (3, 21, 22). These results suggest that APP hydrolysate suppresses the gene expression of *NPY* and *AgRP* in hypothalamus, and decreases food intake and white adipose tissue weight in rats.

In summary, fish protein hydrolysates decrease the energy intake and inhibit the mRNA expression of *NPY* and *AgRP*, which are orexigenic neuropeptides. Thus, fish protein hydrolysates may increase skeletal muscle weight and decrease white adipose tissue weight. These results might imply that muscle hypertrophy and anti-obesity activities by fish protein intake result from fish protein derived peptides.

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