Soy β -Conglycinin Peptide Attenuates Obesity and Lipid Abnormalities in Obese Model OLETF Rats

Satoshi Wanezaki¹, Shintaro Saito², Nao Inoue³, Nobuhiko Tachibana¹, Bungo Shirouchi⁴, Masao Sato⁴, Teruyoshi Yanagita⁵, and Koji Nagao^{2*}

¹ Basic Research Institute, R&D, Fuji Oil Co., Ltd., Kinunodai 4-3, Tsukubamirai-shi, Ibaraki 300-2497, JAPAN

² Department of Biological Resource Science, Saga University, Honjo-1, Saga-shi, Saga 840-8502, JAPAN

³ Faculty of Agriculture, Yamagata University, Wakaba-machi 1-23, Tsuruoka-shi, Yamagata 997-8555, JAPAN

⁴ Department of Bioscience and Biotechnology, Graduate School, Kyushu University, Motoooka-744, Nishi-ku, Fukuoka 819-0395, JAPAN

⁵ Faculty of Health and Social Welfare Sciences, Nishikyushu University, Ozaki 4490-9, Kanzaki, Saga 842-8585, JAPAN

Abstract: We previously reported that soy β -conglycinin (β CG) improves obesity-induced metabolic abnormalities, but not obesity, in obese model Otsuka Long-Evans Tokushima fatty (OLETF) rats. In the present study, we aimed to investigate the effects of β CG-derived peptide consumption on obesity and lipid abnormality in OLETF rats. To this end, wild-type Long-Evans Tokushima Otsuka and OLETF rats were provided a normal diet containing 20% casein for four weeks as a control. In addition, we prepared β CG peptide by enzymatic hydrolysis, and OLETF rats were fed a diet in which half of the casein was replaced by β CG peptide (β CG peptide group). Consequently, rats in the β CG peptide group showed decreased abdominal white adipose tissue weight and lipid content (serum and liver triglycerides, and serum and liver cholesterol) compared to control OLETF rats. Further analysis demonstrated that β CG peptide consumption decreased lipogenic enzyme activity and increased lipolytic enzyme activity in the liver of OLETF rats. In addition, suppressive effects on both synthesis and absorption of cholesterol were observed in β CG peptide-fed OLETF rats. These findings suggest that peptidization of β CG enhanced the anti-obese and hypolipidemic effects of β CG.

Key words: β CG peptide, soy protein, obesity, OLETF, lipogenesis, lipolysis

1 Introduction

The metabolic syndrome is known to increase cardiovascular morbidity and mortality, and central obesity is the key component of its development¹⁻³. Although the pathogenesis of metabolic syndrome is complicated, lipid abnormality is proposed as a feature of metabolic syndrome¹⁻³. Many studies suggested that plant proteins could be important modulators of the risks associated with this syndrome^{4,5}. For example, several studies have shown that dietary soy protein reduces cholesterol and triglyceride (TG) levels in animals and humans⁴⁻⁸. Moreover soy β -conglycinin (β CG), a soybean storage protein, has been reported to exert physiological effects such as promoting lipid lowering effects and preventing obesity in several animal models⁹⁻¹³. However, physiological functions of β CG in non-high-fat or non-high-cholesterol diet-fed rodents have not been fully evaluated, and almost all of previous studies have been conducted with diets in which all dietary protein was replaced by β CG even though its es-

sential amino acids are somewhat insufficient in animals growth⁹⁻¹³.

Otsuka Long-Evans Tokushima fatty (OLETF) rats develop multiple metabolic and hormonal abnormalities that shares many features with human obesity¹⁴⁻¹⁶. In addition, these animals exhibit hyperphagia due to a cholecystokinin receptor deficiency, and become obese even by consuming a normal diet (e.g., AIN-76; 7% fat)¹⁷⁻¹⁹. Accordingly, OLETF rats have been suggested as a good model for obesity-induced metabolic dysfunction. We previously reported that the replacement of 10% casein with β CG in non-high fat AIN-76 diet improves hepatomegaly and hepatic lipid accumulation, but not obesity, in obese model OLETF rats¹⁹.

Since it has been previously reported that peptides or protein hydrolysates exhibit greater bioactivity than intact proteins or amino acid mixtures^{20, 21}, bioactive peptides have been produced *in vitro* through chemical or enzymatic hydrolysis of several food proteins, to modify and

*Correspondence to: Koji Nagao, Department of Biological Resource Science, Saga University, Honjo-1, Saga-shi, Saga 840-8502, JAPAN

E-mail: knagao@cc.saga-u.ac.jp

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improve the physiological functions of dietary proteins^{21,22}. In soy protein, several bioactive peptides, including hypocholesterolemic, antihypertensive, antioxidant, and appetite suppressive peptides, are derived from its major constituents, glycinin and β CG^{23–26}. However, of the limited number of peptides reported to possess hypotriglyceridemic activity have been identified in hydrolyzed globin from animal blood²⁷. In the present study, we sought to evaluate the diversification and reinforcement of bioactivity of β CG by the artificial peptidization and investigate the effects of β CG-derived peptides on obesity and obesity-induced lipid abnormality in OLETF rats.

2 Materials and Methods

2.1 Animals and diets

All experiments were conducted according to the guidelines provided by the ethics committee for experimental animal care of Saga University (No. 22-042-1, 2013-2015). Six-week-old male OLETF and Long-Evans Tokushima Otsuka (LETO) wild-type rats were purchased from Hoshino Laboratory Animals, Inc. (Ibaraki, Japan), and housed individually in metal cages in a temperature-controlled room (24°C) on a 12 h light/dark cycle. After an adaptation period of one week on a powder chow diet (CE-2; Clea Japan, Tokyo, Japan), the rats were fed with one of the two diets, as shown in Table 1. LETO and control OLETF rats (n = 6 of each) were maintained on the same casein-based diet, while a second group of OLETF rats (n = 6) were fed a similar diet, except that the casein and sucrose in the diet were replaced with 11.43% (w/w) β CG peptide. β CG peptide was prepared from intact β CG (Lipoff,

Fuji oil Co., Osaka, Japan) as follows: β CG was dissolved in water at 5% and hydrolyzed by pepsin (1.0% per substrate) for 2 h under acidic conditions (pH 2.0) at 37°C. Next, the β CG solution was degraded for 2 h using trypsin (1.7% per substrate) and chymotrypsin (0.03% per substrate) under neutral conditions (pH 8.0) at 37°C. After enzymatic hydrolysis, the β CG solution was heated at 80°C for 10 min and centrifuged at 10,000 × g for 10 min. The supernatant (containing β CG peptide) was recovered, freeze-dried, and powdered and used in the experiment. The amino acid composition of β CG peptide was similar to intact β CG, and the molecular weight distribution of β CG peptide, estimated by gel filtration, ranged from 200–15,000 (the peak molecular weight distribution was approximately 5,000). At the end of the experimental period (four weeks) and after a 9-h starvation, while under anesthesia, blood was collected from the abdominal aorta into a syringe. Serum was obtained by centrifugation (1,900 × g, 15 min, 4°C) and stored at –80°C until further analysis. Abdominal (perirenal, epididymal, and omental) white adipose tissues (WAT) and liver were immediately harvested, weighed, and stored at –80°C.

2.2 Measurement of serum parameters

Serum triglyceride (TG), cholesterol, phospholipid, glucose, and non-esterified fatty acids (NEFA) levels were measured using commercial enzyme assay kits (Wako Pure Chemicals).

Biomarkers related to cholesterol metabolism, such as lathosterol, campesterol, and β -sitosterol, in serum were assayed by a gas chromatography-flame ionization detector (GC-FID) system (GC-17A; Shimadzu, Tokyo, Japan) equipped with a capillary column (SPBTM-1, 60 m × 0.25 mm i.d., 0.25 μ m thickness, SUPELCO, Bellefonte, PA, USA) using 5 α -cholestane (Sigma Aldrich Japan) as an internal standard. In brief, to 250 μ L of each serum sample was added 25 μ g of 5 α -cholestane. Samples were saponified with ethanolic KOH and then the sterols were extracted. The extracted sterols were converted to trimethylsilyl (TMS) ethers using the TMS derivatization reagent (BSTFA + TMCS, 99:1, SUPELCO) and then injected to the GC-FID system. The temperatures of the injection port and detector were set at 300°C, respectively. The column temperature was set at 275°C. Helium (purity > 99.995%) was used as the carrier gas. The sterol species were identified by comparison with the retention times of the internal standard and the reference standards (lathosterol, campesterol, and β -sitosterol from Sigma Aldrich Japan) after TMS derivatization.

2.3 Measurement of lipid levels in the liver

Hepatic lipid was extracted from the liver by the method described by Folch *et al.*²⁸. Hepatic concentrations of TG and phospholipid were measured according to the methods

Table 1 Composition of experimental diets.

Ingredients	LETO	Control	β CG peptide
		OLETF	
		(%)	
Casein	20.0	20.0	10.0
β CG peptide	–	–	11.43 [†]
Cornstarch	15.0	15.0	15.0
Cellulose	5.0	5.0	5.0
Mineral mixture*	3.5	3.5	3.5
Vitamin mixture*	1.0	1.0	1.0
DL-methionine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Corn oil	7.0	7.0	7.0
Sucrose	48.0	48.0	46.57

β CG: β -Conglycinin

[†] Nitrogen content is equal to 10% casein

* AIN-76

Table 2 Effects of β CG peptide on growth parameters.

	LETO	Control	β CG peptide
		OLETF	
Initial B.W. (g)	116 $\pm$ 2	124 $\pm$ 2*	124 $\pm$ 1
Final B.W. (g)	271 $\pm$ 2	307 $\pm$ 3*	307 $\pm$ 3
B.W. gain (g)	154 $\pm$ 3	183 $\pm$ 4*	184 $\pm$ 3
Food intake (g)	154 $\pm$ 3	183 $\pm$ 4*	184 $\pm$ 3
Food efficiency (g gain/g intake)	0.334 $\pm$ 0.006	0.324 $\pm$ 0.005	0.324 $\pm$ 0.005
Liver weight (g/ 100 g B.W.)	3.45 $\pm$ 0.07	3.44 $\pm$ 0.08	3.35 $\pm$ 0.06

* shows significant difference at $p < 0.05$ vs LETO.

described by Fletcher *et al.*²⁹⁾ and Rouser *et al.*³⁰⁾, respectively. Hepatic cholesterol concentration was analyzed using the Cholesterol E-Test Wako (Wako Pure Chemical Industries, Ltd.).

2.4 Assay for hepatic enzyme activity

A piece of liver from each rat was homogenized in 6 volumes of a 0.25 M sucrose solution containing 1 mM ethylenediaminetetraacetic acid in a 10 mM Tris-HCl buffer (pH 7.4). After the nuclear fractions were precipitated, the supernatants were centrifuged at 10,000 $\times$ g for 10 min at 4°C to obtain mitochondrial fractions. The resulting supernatants were recentrifuged at 125,000 $\times$ g for 60 min to precipitate microsomes, and the remaining supernatant constituted the cytosol fraction. The specific enzymatic activity of two enzymes involved in *de novo* triglyceride synthesis – fatty acid synthase (FAS), malic enzyme and glucose 6-phosphate dehydrogenase (G6PDH) – in the cytosomal fraction, as well as that of the fatty acid translocation enzyme carnitine palmitoyltransferase (CPT) in the mitochondrial fraction, were determined as described elsewhere^{31–34)}. The protein concentration of each fraction was determined according to the Lowry method³⁵⁾, with bovine serum albumin used as the standard.

2.5 Statistical analysis

The data are presented as the means $\pm$ standard error (SE). In order to check a state as a model for obesity, mean differences between LETO and control OLETF rats were determined by the student's t-test (KaleidaGraph ver.4; Synergy Software, Reading, PA, USA). To confirm the efficacy of β CG peptide for OLETF rats, mean differences between control OLETF and β CG peptide groups were determined by the student's t-test. $P < 0.05$ was considered statistically significant.

White adipose tissue (g/100g B.W.)

Fig. 1 Effect of β CG peptide on white adipose tissue weights.

Rats were fed the Control diet or β CG peptide diet for four weeks. Values are expressed as the mean $\pm$ standard error for six rats. See Table 1 for composition of diets. ^a A significant difference was observed between LETO and control diet-fed OLETF rats (at $p < 0.05$). ^b A significant difference was observed between control and β CG peptide diets in OLETF rats ($p < 0.05$).

3 Results and Discussion

This study evaluated whether the consumption of β CG peptide could affect obesity and lipid abnormality in obese model OLETF rats.

3.1 Effect of β CG peptide on growth parameter

Accompanied by higher food intake, weight of WATs increased in control diet-fed OLETF rats compared to LETO rats (Table 2, Fig. 1). Although there was no difference in the weight of liver, the weight of epididymal and perirenal abdominal WATs in the β CG peptide group was significantly lower than that in the control group of OLETF rats (Table 2, Fig. 1). Recently, Kawabeta *et al.* reported that the re-

Table 3 Effects of β CG peptide on serum and liver parameters.

		Control	βCG peptide
	LETO	OLETF	
Serum parameters			
TG (mg/dL)	55.9 ± 5.8	118 ± 10*	78.4 ± 5.2 [†]
CHOL (mg/dL)	127 ± 3	169 ± 6*	129 ± 3 [†]
PL (mg/dL)	159 ± 7	227 ± 5*	177 ± 6 [†]
Glucose (mg/dL)	152 ± 9	193 ± 12*	168 ± 5
NEFA (mEq/L)	0.621 ± 0.028	0.733 ± 0.021*	0.660 ± 0.044
Liver parameters			
TG (mg/g liver)	8.50 ± 0.68	16.3 ± 0.6*	10.2 ± 0.5 [†]
CHOL (mg/g liver)	3.63 ± 0.13	3.75 ± 0.14	3.26 ± 0.08 [†]
PL (mg/g liver)	36.4 ± 0.6	37.2 ± 0.7	36.5 ± 0.5

TG: triglyceride, CHOL: cholesterol, PL: phospholipid

NEFA: non esterified fatty acid

* shows significant difference at $p < 0.05$ vs LETO.[†] shows significant difference at $p < 0.05$ vs Control.

placement of 10% casein with β CG in high-starch AIN-93G diet lowers mesenteric adipose tissue weight in OLETF rats³⁶⁾. Our previous study, however, the replacement of 10% casein with β CG in high-sucrose AIN-76 diet could not affect WAT weights in OLETF rats¹⁹⁾. Collectively, these results suggest that physiological functions of β CG are slightly difficult to evaluate on a high-sucrose diet and the anti-obesity effects of β CG peptide diet are stronger than β CG diet.

3.2 Effect of β CG peptide on lipids and glucose levels

Lipid levels (TG, cholesterol and phospholipids) in the liver and serum, serum glucose and serum NEFA were significantly higher in the control OLETF group than in the LETO group (Table 3). There were also significant differences in the levels of serum and liver components between the groups in OLETF rats (Table 3). Although serum glucose and NEFA levels did not differ, TG, cholesterol and phospholipid levels were lower in β CG peptide-fed rats than those in control. In addition, lower hepatic TG and cholesterol levels were observed in the β CG peptide group than the control group in OLETF rats. Previous studies showed that β CG did not decrease serum TG levels of OLETF rats^{19, 36)}. Similarly, we observed that hypolipidemic effects were enhanced by the peptidization of β CG.

3.3 Effect of β CG peptide on triglyceride metabolism

To further examine the effect of β CG peptide on the liver, hepatic enzymes related to TG metabolism were analyzed.

Activities of FAS, key enzymes in the regulation of FA *de novo* synthesis, and G6PDH and malic enzyme, which provide the NADPH required for fatty acid synthesis, were

significantly higher in the liver of OLETF rats compared with LETO rats. In OLETF rats, malic enzyme (Fig. 2) activity was not different between the groups, but the activity of FAS and G6PDH were significantly lower in rats maintained on the β CG peptide diet compared to the control OLETF rats (Fig. 2). These data suggest that β CG peptide administration lowers TG levels through the suppression of hepatic lipogenesis in obese OLETF rats, which is consistent with previous studies^{19, 36)}.

The activity of CPT, a key enzyme of mitochondrial fatty acid β -oxidation, differed slightly between LETO and OLETF rats. However, in OLETF rats, CPT activity was markedly enhanced by β CG peptide feeding in the mitochondrial fraction of the liver (Fig. 2). These results suggest that β CG peptide administration alleviates hepatic TG accumulation partly through enhancement of lipolysis in the livers of obese OLETF rats. Additionally, given the results from previous studies using both AIN-76 and AIN-93G diet composition indicating that β CG feeding did not alter hepatic lipolytic enzyme activities in OLETF rats^{19, 36)}, it can be inferred that β CG gained additional bioactivity by the peptidization.

3.4 Effect of β CG peptide on cholesterol metabolism

To gain insight into the effect of dietary β CG peptide on cholesterol metabolism, we analyzed biomarkers related to cholesterol metabolism in the serum (Fig. 3). To evaluate whole-body cholesterol metabolism, cholesterol synthesis and absorption need to be measured. Lathosterol is a precursor of cholesterol *de novo* cholesterol synthesis, and its serum level can be used as an index of cholesterol synthesis in the body. Plant sterols, such as campesterol and β -sitosterol, are sterol isomers that cannot be synthesized

Fig. 2 Effect of β CG peptide on hepatic enzyme activities.

Rats were fed the Control diet or β CG peptide diet for four weeks. Values are expressed as the mean $\pm$ standard error for six rats. See Table 1 for composition of diets. ^a A significant difference was observed between LETO and control diet-fed OLETF rats (at $p < 0.05$). ^b A significant difference was observed between control and β CG peptide diets in OLETF rats ($p < 0.05$).

in animal body and their presence in the serum showed positive correlations with the absorption rate of dietary sterols^{37,38}.

Serum lathosterol levels were slightly higher in OLETF rats than LETO rats. In OLETF rats, serum lathosterol levels were significantly lower in rats on the β CG peptide diet compared to the control OLETF rats (Fig. 3). These data suggest that β CG peptide administration lowers cholesterol levels partly through the suppression of cholesterol synthesis in obese OLETF rats.

Serum plant sterol levels, the indices for cholesterol absorption, were significantly higher in OLETF rats than LETO rats. In OLETF rats, levels of serum plant sterols, campesterol and β -sitosterol, were significantly lower in rats on the β CG peptide diet compared to the control OLETF rats (Fig. 3). These data suggest that suppression of cholesterol absorption by β CG peptide contribute to a decrease in cholesterol levels in obese OLETF rats.

4 Conclusions

The present study shows that the dietary intake of β CG peptide leads to an anti-obesity effect and alleviates lipid abnormality in OLETF rats. In particular, both hypolipogenic effect and enhanced lipolytic effects of β CG peptide in the liver may provide a mechanistic basis for the observed effects. Additionally, suppressive effects on both synthesis and absorption of cholesterol can be attributed to the hypocholesterolemic effect of β CG peptide.

Although its enhancement mechanism has not yet been completely elucidated, soy protein hydrolysate exerts stronger lipid lowering effects than intact soy protein^{39,40}. Since previous studies reported that hydrolyzed soy protein is more rapidly and efficiently absorbed⁴¹ and has higher lipolysis-stimulating³⁹ and antioxidant activities⁴⁰ than those of intact soy protein, we hypothesized that peptidization of β CG exposes some bioactive amino acid R groups or bioactive peptide sequences, conferring these properties. Further investigations are required to clarify the bioactive structures and biological mechanism of

Fig. 3 Effect of β CG peptide on levels of biomarkers related to cholesterol metabolism in serum. Rats were fed the Control diet or β CG peptide diet for four weeks. Values are expressed as the mean $\pm$ standard error for six rats. See Table 1 for composition of diets. ^a A significant difference was observed between LETO and control diet-fed OLETF rats (at $p < 0.05$). ^b A significant difference was observed between control and β CG peptide diets in OLETF rats ($p < 0.05$).

actions of β CG peptide to determine whether it can improve or prevent obesity-induced metabolic disorders in humans.

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Conflict of Interests

The authors declare no conflicts of interest associated with this manuscript.

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