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

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Abstract: We previously reported that soy β-conglycinin (βCG) 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 βCG-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 βCG peptide by enzymatic hydrolysis, and OLETF rats were fed a diet in which half of the casein was replaced by βCG peptide (βCG peptide group). Consequently, rats in the βCG 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 βCG 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 βCG peptide-fed OLETF rats. These findings suggest that peptidization of βCG enhanced the anti-obese and hypolipidemic effects of βCG.

Key words: βCG 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 development⁴⁻⁵. Although the pathogenesis of metabolic syndrome is complicated, lipid abnormality is proposed as a feature of metabolic syndrome⁶⁻⁷. Many studies suggested that plant proteins could be important modulators of the risks associated with this syndrome⁸⁻¹⁰. For example, several studies have shown that dietary soy protein reduces cholesterol and triglyceride (TG) levels in animals and humans¹¹⁻¹³. Moreover, soy β-conglycinin (βCG), a soybean storage protein, has been reported to exert physiological effects such as promoting lipid lowering effects and preventing obesity in several animal models¹⁴⁻¹⁶. However, physiological functions of βCG 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 βCG even though its essential amino acids are somewhat insufficient in animals growth¹⁷⁻¹⁹. Otsuka Long-Evans Tokushima fatty (OLETF) rats develop multiple metabolic and hormonal abnormalities that shares many features with human obesity²⁰⁻²¹. 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)²²⁻²³. 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 βCG in non-high-fat AIN-76 diet improves hepatomegaly and hepatic lipid accumulation, but not obesity, in obese model OLETF rats²⁴. Since it has been previously reported that peptides or protein hydrolysates exhibit greater bioactivity than intact proteins or amino acid mixtures²⁵⁻²⁶, bioactive peptides have been produced in vitro through chemical or enzymatic hydrolysis of several food proteins, to modify and

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improve the physiological functions of dietary proteins\textsuperscript{21, 22}. In soy protein, several bioactive peptides, including hypocholesterolemic, antihypertensive, antioxidant, and appetite suppressive peptides, are derived from its major constituents, glycinn and \( \beta \)CG\textsuperscript{23-26}. However, of the limited number of peptides reported to possess hypotriglyceridemic activity have been identified in hydrolyzed globin from animal blood\textsuperscript{27}. In the present study, we sought to evaluate the diversification and reinforcement of bioactivity of \( \beta \)CG by the artificial peptidization and investigate the effects of \( \beta \)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 peak 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) \( \beta \)CG peptide. \( \beta \)CG peptide was prepared from intact \( \beta \)CG (Lipoff, Fuji oil Co., Osaka, Japan) as follows: \( \beta \)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 \( \beta \)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 \( \beta \)CG solution was heated at 80°C for 10 min and centrifuged at 10,000 × g for 10 min. The supernatant (containing \( \beta \)CG peptide) was recovered, freeze-dried, and powdered and used in the experiment. The amino acid composition of \( \beta \)CG peptide was similar to intact \( \beta \)CG, and the molecular weight distribution of \( \beta \)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.

Table 1 Composition of experimental diets.

| Ingredients          | LETO (%) | Control (%) | \( \beta \)CG peptide (%) |
|----------------------|----------|-------------|---------------------------|
| Casein               | 20.0     | 20.0        | 10.0                      |
| \( \beta \)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              | 40.0     | 46.57       |                           |

\( \beta \)CG: \( \beta \)-Conglycinin
\* Nitrogen content is equal to 10\% casein
\* AIN-76

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 \( \beta \)-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 (SPB-1, 60 m × 0.25 mm i.d., 0.25 µm thickness, SUPELCO, Bellefonte, PA, USA) using 5α-cholestan (Sigma Aldrich Japan) as an internal standard. In brief, to 250 µL of each serum sample was added 25 µg of 5α-cholestan. 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 \( \beta \)-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.\textsuperscript{28}. Hepatic concentrations of TG and phospholipid were measured according to the methods.
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Table 2 Effects of βCG peptide on growth parameters.

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

* shows significant difference at \( p < 0.05 \) vs LETO.

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. The protein concentration of each fraction was determined according to the Lowry method, with bovine serum albumin used as the standard.

2.5 Statistical analysis
The data are presented as the means ± 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 βCG peptide for OLETF rats, mean differences between control OLETF and βCG peptide groups were determined by the student's t-test. \( P < 0.05 \) was considered statistically significant.

3 Results and Discussion
This study evaluated whether the consumption of βCG peptide could affect obesity and lipid abnormality in obese model OLETF rats.

3.1 Effect of βCG 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 βCG 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-
βCG did not decrease serum TG levels of OLETF rats. Previous studies showed that cholesterol levels were observed in the control group. In addition, lower hepatic TG and phospholipid levels were lower in βCG peptide-fed rats than those in control. In glucose and NEFA levels did not differ, TG, cholesterol and PL levels were lower in βCG peptide-fed rats than those in control. In glucose and NEFA levels did not differ, TG, cholesterol and PL levels were lower in βCG peptide-fed rats than those in control. In HDL-cholesterol levels, we observed that physiological functions of βCG are slightly difficult to evaluate on a high-sucrose diet and the anti-obesity effects of βCG peptide diet are stronger than βCG diet.

3.2 Effect of βCG peptide on lipids and glucose levels

Lipid levels (TG, cholesterol and phospholipid) 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 βCG peptide-fed rats than those in control. In addition, lower hepatic TG and cholesterol levels were observed in the βCG peptide group than the control group in OLETF rats. Previous studies showed that βCG did not decrease serum TG levels of OLETF rats. Similarly, we observed that hypolipemic effects were enhanced by the peptidization of βCG.

3.3 Effect of βCG peptide on triglyceride metabolism

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

Activities of FAS, key enzymes in the regulation of PA 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 βCG peptide diet compared to the control OLETF rats (Fig. 2). These data suggest that βCG peptide administration lowers TG levels through the suppression of hepatic lipogenesis in obese OLETF rats, which is consistent with previous studies.

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 βCG peptide feeding in the mitochondrial fraction of the liver (Fig. 2). These results suggest that βCG 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 βCG feeding did not alter hepatic lipolytic enzyme activities in OLETF rats, it can be inferred that βCG gained additional bioactivity by the peptidization.

3.4 Effect of βCG peptide on cholesterol metabolism

To gain insight into the effect of dietary βCG 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 campessterol and β-sitosterol, are sterol isomers that cannot be synthesized

| Table 3  | Effects of βCG peptide on serum and liver parameters. |
|----------|------------------------------------------------------|
|          | LETO                       | Control                  | βCG peptide          |
| 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 βCG in high-starch AIN-93G diet lowers mesenteric adipose tissue weight in OLETF rats. Our previous study, however, the replacement of 10% casein with βCG in high-sucrose AIN-76 diet could not affect WAT weights in OLETF rats. Collectively, these results suggest that physiological functions of βCG are slightly difficult to evaluate on a high-sucrose diet and the anti-obesity effects of βCG peptide diet are stronger than βCG diet.
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 βCG peptide diet compared to the control OLETF rats (Fig. 3). These data suggest that βCG 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 βCG peptide diet compared to the control OLETF rats (Fig. 3). These data suggest that suppression of cholesterol absorption by βCG peptide contribute to a decrease in cholesterol levels in obese OLETF rats.

4 Conclusions

The present study shows that the dietary intake of βCG peptide leads to an anti-obesity effect and alleviates lipid abnormality in OLETF rats. In particular, both hypolipogenic effect and enhanced lipolytic effects of βCG 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 βCG 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 absorbed\(^{41}\) and has higher lipolysis-stimulating\(^{39}\) and antioxidant activities\(^{40}\) than those of intact soy protein, we hypothesized that peptideization of βCG 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

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**Fig. 2** Effect of βCG peptide on hepatic enzyme activities.

Rats were fed the Control diet or βCG peptide diet for four weeks. Values are expressed as the mean ± 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 βCG peptide diets in OLETF rats (\(p < 0.05\)).
actions of βCG 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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