Chlorella Protein Hydrolysate Attenuates Glucose Metabolic Disorder and Fatty Liver in High-fat Diet-induced Obese Mice

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Abstract: Chlorella (Parachlorella beijerinckii) powder is reported to show a preventive effect against metabolic syndromes such as arteriosclerosis, hyperlipidemia, and hypertension. Approximately 60% of the chlorella content is protein. In order to understand the role of chlorella protein, we prepared a chlorella protein hydrolysate (CPH) by protease treatment. Male C57BL/6 mice were divided into three groups: a normal diet group, high-fat diet (HFD) group, and high-fat diet supplemented with CPH (HFD+CPH) group. The CPH administration improved glucose intolerance, insulin sensitivity, and adipose tissue hypertrophy in the high-fat diet-fed mice. In addition, the HFD+CPH group had significantly decreased liver total cholesterol and triglyceride levels compared with those in the HFD group. Furthermore, the HFD+CPH group had a decreased level of monocyte chemotactic protein-1 (MCP-1) in serum and a lower MCP-1 mRNA expression level in adipose tissue compared with the HFD group. The present study suggests that chlorella protein hydrolysate can prevent a high-fat diet-induced glucose disorder and fatty liver by inhibiting adipocyte hypertrophy and reducing the MCP-1 protein and gene expression.

Key words: chlorella protein hydrolysate, obesity, fatty liver, glucose, MCP-1

1 INTRODUCTION

Metabolic syndrome, which comprises a cluster of metabolic abnormalities, such as hyperlipidemia, diabetes mellitus, and hypertension, is a widespread and increasingly common disease in industrialized countries, and it contributes to the increase in cardiovascular morbidity and mortality3, 5. Non-alcoholic fatty liver disease (NAFLD) is a condition characterized by lipid accumulation in the liver that is not caused by alcohol consumption7. NAFLD develops non-alcoholic steatohepatitis by steatosis alone and can progress to cirrhosis and liver failure. NAFLD is often associated with features of metabolic syndrome and is emerging as the most common liver disease1–6. Insulin resistance plays a major role in the pathophysiology of metabolic syndrome, including NAFLD7. Morbid obesity and fatty liver lead to the development of insulin resistance due to a high blood glucose level, hypertension, and elevated triglycerides8. Excess free fatty acids (FFA) caused by insulin resistance are accumulated in the liver, leading to steatosis. The hypertrophic growth of adipocytes, accompanying obesity, promotes the secretion of monocyte chemotactic factor-1 (MCP-1), which is a key pro-inflammatory factor and can induce insulin resistance8. Therefore, metabolic syndrome, including NAFLD, causes a complex functional disorder of glucose and lipid metabolism.

Chlorella is a genus of green unicellular algae that has been used as a healthy food for a long time. A related species, Parachlorella beijerinckii, has been reported to prevent hypertension9, hyperlipidemia10, insulin resistance11, and arteriosclerosis12, 13 in animals and humans. Our previous study has shown that antihyperinsulinemic effects of P. beijerinckii are due to the modulation of hypertrophy of adipose tissue and adipocytokine secretion in obese mice14. Chlorella contains components such as protein, carotenoids, dietary fiber, and polysaccharides, which have immunopharmacological effects. However, effects of individual components of chlorella on metabolic syndrome remain unknown.

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Recent studies have shown that food protein hydrolysates improve blood pressure by suppressing angiotensin-converting enzyme, which activates angiotensin 1, leading to increased blood pressure\(^{15}\). Egg white hydrolysates have been reported to have antioxidant activity, increase plasma radical-scavenging capacity, and inhibit lipid peroxidation\(^{16}\). Protein accounts for about 60% of the chlorella content. We hypothesized that chlorella hydrolysates produced through the digestive process could partially contribute to the improvement of lifestyle-related diseases. In the present study, we prepared a chlorella protein hydrolysate by protease treatment and investigated the effect of the chlorella protein hydrolysate on abdominal adiposity, glucose metabolism, and lipid parameters in high-fat diet-induced obese mice.

### 2 EXPERIMENTAL PROCEDURES

#### 2.1 Materials

The chlorella protein hydrolysate (CPH) was produced by protease treatment of proteins isolated from *P. beijerinckii* (Chlorella Industry, Tokyo, Japan). Briefly, after cultivating chlorella, 10 L of a chlorella slurry culture solution (115 g/L) was adjusted with sodium hydroxide to pH 10.3 and allowed to stand for 30 min. The solution was then centrifuged for 10 min at 11,000 \(\times g\). The supernatant was autoclaved for 15 min at 120\(^\circ\)C and cooled. The autoclaved solution was adjusted with hydrochloric acid to pH 3.75 and hydrolyzed at 50\(^\circ\)C for 19 h by adding 12 g of pepsin from porcine stomach (Biocon Japan, Nagoya, Japan) with slow stirring. The hydrolysis solution was incubated at 70\(^\circ\)C for 10 min to deactivate the enzyme and was adjusted with sodium hydroxide to pH 7.0. The solution was then desalted from 15.9 to 7.8 mS/cm conductivity using an electrodialyzer (Acilizer 02, Astom Corporation, Tokyo, Japan). A fraction of less than 2,000 molecular weight was collected by ultrafiltration (Sartorius Stedim Japan K.K., Tokyo, Japan), and the filtrate was freeze-dried. Ultimately, 177.5 g of a CPH powder was obtained. The yield of the protein hydrolysate by weight in the chlorella powder was 15.4%. The amino acid composition of CPH was shown in Table 1. Samples for amino acid analysis were prepared by 24-h hydrolysis in 6N HCl at 110\(^\circ\)C. For methionine and cysteine determination, oxidation with performic acid was carried out prior to acid hydrolysis. The amino acid composition was determined using an amino acid analyzer (JLC-500/V2, JEOL, Tokyo, Japan). The tryptophan content was determined by high-performance liquid chromatography after protein hydrolysis with barium hydroxide.

#### 2.2 Animals

C57BL/6 male mice at six weeks of age were obtained from Charles River Japan (Yokohama, Japan). Administration of experimental diets was started after the mice were acclimated for one week to a diet of commercially available CE-2 powder feed (CLEA Japan, Tokyo, Japan). The mice were housed in standard cages (one mouse per cage) in an environment with a temperature of 23 \(\pm\)1.5\(^\circ\)C, humidity of 55 \(\pm\)10\%, and a 12-h light/dark cycle. The animals were allowed *ad libitum* access to respective diets and water. The animals were handled in accordance with the guidelines of the National Institutes of Health (NIH, USA) and the ethical committee for animal experiments of Chlorella Industry Co., Ltd.

#### 2.3 Animal experimental procedure

The mice were randomly divided into three groups (eight mice per group). The normal diet (ND) group was given a normal diet based on AIN-93G. The high-fat diet (HFD) group was given a high-fat diet consisting of the following: 20\% of calories from protein (250 g/kg casein), 50\% of calories from fat (140 g/kg lard, 140 g/kg beef tallow, and 20 g/kg corn oil), and 27\% of calories from carbohydrates (200 g/kg sucrose and 149 g/kg alpha-cornstarch). The high-fat diet included 10 g of a vitamin mix (AIN-93), 35 g of a mineral mix (AIN-93G), 4 g of L-cystine, and 2.5 g of choline bitartrate per kilogram of diet. The third (HFD + CPH) group was fed the high-fat diet with CPH (1 g/kg) at the expense of casein. We chose 1 g of CPH per kilogram because a previous study has shown biological activity at the same level\(^{17}\). The animals were maintained on these diets for 13 weeks. After eight weeks of feeding, blood glucose levels were measured following 4 h of fasting. A glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed after 10 and 11 weeks of feeding, re-

### Table 1  The amino acid composition of CPH.

<table>
<thead>
<tr>
<th>Amino acid (mol%)</th>
<th>Chlorella hydrolysate</th>
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</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>9.50</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.22</td>
</tr>
<tr>
<td>Serine</td>
<td>4.03</td>
</tr>
<tr>
<td>Glutamate</td>
<td>14.78</td>
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<tr>
<td>Proline</td>
<td>5.17</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.41</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.23</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.83</td>
</tr>
<tr>
<td>Valine</td>
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</tr>
<tr>
<td>Methionine</td>
<td>2.06</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.64</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.05</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.66</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.17</td>
</tr>
<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Lysine</td>
<td>7.37</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.54</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>1.21</td>
</tr>
</tbody>
</table>
Beneficial effect of chlorella protein hydrolysate on diet-induced obese mice

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spectively. After the 13-week feeding period, the mice were kept without food overnight and then necropsied under anesthesia with sodium pentobarbital (Nembutal®; Dainippon Sumitomo Pharma, Osaka, Japan) injected intraperitoneally at a dose of 50 mg/kg of body weight. Blood samples were taken from the inferior vena cava. The liver, epididymal, perirenal, and mesenteric adipose tissues were excised, weighed, and stored at −80°C until analysis.

2.4 Glucose and insulin tolerance tests

The GTT was performed on mice at 10 weeks, after 16-h fasting. Glucose (2.0 g/kg of body weight) was administered orally with a feeding needle. Blood samples were collected before and after the glucose loading, at the time points indicated in the figures. The blood samples were taken via tail vein puncture. Blood glucose levels were measured in whole blood with an Accu-Check Aviva blood glucose meter (Roche, Indianapolis, IN, USA). The area under the curve (AUC) was calculated based on the measurement results. The ITT was performed on mice at 11 weeks, after 4 h of fasting. Human insulin (Novolin® R100; Novo Nordisk Pharma, Tokyo, Japan) was injected intraperitoneally at 2.0 IU/kg of body weight. Blood sampling and blood glucose measurement were performed by the same method as in the GTT. The time points are indicated in the figures. The measurement results of ITT are shown as percentages, with the initial blood glucose level normalized to 100%.

2.5 Serum analysis

To obtain mouse serum, the blood samples obtained at the time of dissection were stored for 30 min at room temperature and centrifuged at 2,400 × g for 10 min. The serum obtained from the supernatant was stored at −80°C for use in the following experiment. Blood glucose levels in the serum were measured using the Accu-Check Aviva blood glucose meter. The amounts of insulin and MCP-1 in the serum were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan) and MCP-1 ELISA kit (Bender MedSystems, Burlingame, CA, USA), respectively. Triglycerides (TG) and FFA in the serum were measured using the Labo Assay™ triglyceride kit and Labo Assay™ NEFA kit (Wako Pure Chemical Industries, Osaka, Japan), respectively. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the blood glucose and insulin levels in mice after the end of feeding. The following formula was applied: HOMA-IR = fasting glucose [mg/dL] × fasting insulin [ng/mL]/22.5.

2.6 Liver lipid analysis

Liver lipids were extracted by the method of Floch et al. Triglycerides (TG) and total cholesterol were measured using the Labo Assay triglyceride kit and Labo Assay cholesterol kit (Wako), respectively.

2.7 mRNA isolation and real-time polymerase chain reaction (PCR)

Total RNA was isolated from the epididymal adipose tissue using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Complementary DNA was prepared from the total RNA by reverse transcription (PrimeScript II® 1st Strand cDNA synthesis kit, Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. Relative mRNA levels were measured using a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) via TaqMan analysis with specific primers and probes. The oligonucleotide sequences of the gene-specific primers and probes for the TaqMan analysis of mouse MCP-1 (Mm00441242_m1) and beta-actin (Mm00607939_s1) were obtained from Applied Biosystems. The relative mRNA levels are expressed as the ratio of each copy number to that of beta-actin.

2.8 Morphological analysis of adipose tissue

Epididymal adipose tissue was stored in neutral formalin. We requested the paraffin sectioning and hematoxylin/eosin staining from the Biopathology Research Institute (Oita, Japan). The sections were photographed at a 200× magnification at three different points. The cell sizes per specimen were calculated from a photograph using ImageJ (NIH).

2.9 Statistical analysis

Data are presented as means and standard deviations. The data were tested by one-way analysis of variance, followed by Fisher’s protected least significant difference test to identify significant differences. Differences with p < 0.05 were considered statistically significant.

3 RESULTS

3.1 Effect of CPH on body mass and organ weights

The total body and liver weights of the mice from the HFD group after administration of the diet for 13 weeks were significantly higher than those in the ND group (Table 2). On the other hand, the HFD + CPH group tended to have a lower liver weight. The weights of epididymal, perirenal, and mesenteric adipose tissues were significantly increased in the HFD group compared with those in the ND group. However, there was no apparent difference in the weights of the adipose tissues between the HFD and HFD + CPH groups.

3.2 Effect of CPH on glucose tolerance and insulin sensitivity

After eight weeks of feeding, the HFD group had significantly higher blood glucose levels than those in the ND group. By contrast, the HFD + CPH group showed signifi-
cantly reduced levels of blood glucose compared with those in the HFD group (Fig. 1A).

In the GTT, the HFD group had significantly higher blood glucose levels than those in the ND group at the time points of 0, 90, and 120 min after the glucose loading (Fig. 1B). Compared with the HFD group, these levels in the HFD + CPH group were significantly reduced at 30, 90, and 120 min. On the other hand, there was no difference between the HFD + CPH and ND groups at any time point. Similarly, the GTT AUC of the HFD group was significantly larger compared to that of the other two groups (Fig. 1C).

In the ITT, the percentage of blood glucose in the HFD group was significantly higher than that in the ND group at 15, 30, and 60 min after the insulin injection (Fig. 1D). Compared with the HFD group, the HFD + CPH group demonstrated a significant reduction of the blood glucose percentage at the time points of 15, 30, and 60 min.

### 3.3 Effect of CPH on serum parameters and liver lipids

The HFD and HFD + CPH groups had significantly increased serum glucose levels compared to that in the ND group (Table 3). In contrast, the serum insulin levels and HOMA-IR in the HFD + CPH group were significantly lower than those in the HFD group. The serum concentrations of

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of CPH on body mass and organ weights.</th>
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<tbody>
<tr>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>Food intake (kcal/day)</td>
<td>16.0 ± 1.3</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>17.8 ± 0.8</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>29.8 ± 1.8a</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.15 ± 0.10a</td>
</tr>
<tr>
<td>Adipose tissue weight (g/100g body weight)</td>
<td></td>
</tr>
<tr>
<td>Epididymal</td>
<td>2.78 ± 0.41a</td>
</tr>
<tr>
<td>Perirenal</td>
<td>1.16 ± 0.35a</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>0.92 ± 0.39a</td>
</tr>
</tbody>
</table>

Fig. 1  Effect of CPH on blood glucose and insulin tolerance tests.
(A) Fasting blood glucose level of at 8 weeks of feeding. (B) Glucose tolerance test at 10 weeks of feeding. (C) Area under the curve for glucose tolerance test at 10 weeks of feeding. (D) Insulin tolerance test at 11 weeks of feeding. Values are shown as means ± SDs (n = 8). Means in the same superscripts (abc) show no significant differences, while those with different superscripts (abc) differ significantly at p < 0.05.
TG and FFA were comparable among the three groups. Interestingly, the HFD + CPH group had significantly reduced liver TG levels compared to those in the HFD group. The HFD group tended to have higher liver total cholesterol levels than those in the ND group ($p = 0.062$). By contrast, liver total cholesterol in the HFD + CPH group was significantly reduced compared to that in the HFD group.

3.4 Effect of CPH on epididymal adipose tissue

Next, we assessed whether the CPH supplementation could prevent the HFD-induced adipose tissue hypertrophy. Figure 2 shows enlarged photographs of the abdominal adipose tissue of each dietary group. The adipocyte cells in the HFD group were significantly larger than those in the ND group. However, the HFD + CPH group had the adipocyte cell size significantly reduced compared to that in the HFD group.

3.5 Effect of CPH on MCP-1 serum levels and adipose MCP-1 mRNA expression

We also evaluated the MCP-1 protein and gene expression in serum and adipose tissue. The serum MCP-1 levels were significantly higher in the HFD group than those in the other two dietary groups (Fig. 3A). An increase in MCP-1 mRNA expression in adipose tissue was observed in the HFD group, but the expression level was also significantly decreased in the HFD + CPH group (Fig. 3B).

| Table 3 Effect of CPH on serum parameters and liver lipids. |
|-----------------|-----------------|-----------------|
|                 | ND              | HFD             | HFD+CPH         |
| Glucose (mg/dl) | 86 ± 16a        | 139 ± 28b       | 119 ± 22b       |
| Insulin (ng/ml) | 0.42 ± 0.13a    | 0.82 ± 0.15b    | 0.60 ± 0.18c    |
| HOMA-IR         | 1.62 ± 0.55a    | 5.08 ± 1.23b    | 3.25 ± 1.45c    |
| TG (mg/dl)      | 67.5 ± 8.3      | 71.9 ± 14.7     | 73.2 ± 12.4     |
| FFA (mEq/l)     | 0.69 ± 0.18     | 0.75 ± 0.15     | 0.70 ± 0.13     |
| TG (mg/g)       | 39.2 ± 11.0a    | 51.5 ± 11.5b    | 31.9 ± 10.0c    |
| T-cho           | 4.41 ± 0.86ab   | 5.50 ± 0.94c    | 3.68 ± 0.50b    |

Fig. 2  Effect of CPH on adipocyte size in abdominal white adipose tissue. Hematoxylin and eosin staining of epididymal adipose tissue section from (A) ND, (B) HFD, (C) HFD + CPH groups and adipose cell size (D) are shown. Values are shown as means ± SDs ($n = 8$). Means in the same superscripts (abc) show no significant differences, while those with different superscripts (abc) differ significantly at $p < 0.05$. Scale bar = 200 μm.

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4 DISCUSSION

Our results support the hypothesis that a protein hydrolysate derived from chlorella protects mice from diet-induced obesity and impairment of glucose and lipid metabolism. The dietary intake of CPH clearly reduced the blood glucose levels over the course of eight weeks and improved glucose intolerance. In addition, significant reductions in the liver total cholesterol and TG levels, serum insulin level, and HOMA-IR score were demonstrated in the HFD + CPH group compared with the values obtained for the HFD group. The CPH supplementation of the HFD diet also suppressed the adipose tissue hypertrophy.

The interesting findings in the present study were the reductions of the serum MCP-1 level and adipose tissue MCP-1 mRNA expression after 13 weeks of CPH intake. Chronic inflammation is closely related to obesity and metabolic syndrome, including type 2 diabetes and atherosclerosis. The expansion of adipocytes due to obesity activates pro-inflammatory pathways and induces production of inflammatory cytokines such as MCP-1. Recent studies have highlighted the critical role of MCP-1 in the induction of inflammation and macrophage infiltration into adipose tissue. High levels of MCP-1 production are also associated with obesity. A role for MCP-1-induced insulin resistance in obesity was demonstrated in previous studies. Transgenic mice with adipose tissue-specific expression of MCP-1 showed macrophage infiltration into adipose tissue, an increased hepatic TG content, and insulin resistance. By contrast, MCP-1 knockout mice fed a high-fat diet had drastically reduced levels of macrophage accumulation in adipose tissue and hepatic steatosis when compared with those in high-fat diet-fed wild-type mice. Moreover, inhibition of MCP-1 function by the acute expression of a dominant negative mutant of MCP-1 ameliorated insulin resistance in diabetes db/db mice and in high-fat diet-fed wild-type mice. These reports clearly demonstrated the association among increased expression of MCP-1, macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in mice. Likewise, our present results suggest that downregulation of MCP-1 in adipocytes by CPH contributed to the amelioration of these metabolic disorders. A previous study also indicated that whole chlorella powder had the ability to inhibit MCP-1 expression in adipose tissue of mice fed a high-fat diet.

The CPH diet significantly reduced the HFD-induced high blood glucose level as determined by the GTT, except at 60 min, which showed a decreasing tendency. On the other hand, the CPH diet reduced the blood glucose level at all time points after insulin injection in the ITT. These tests share certain similarities in that the CPH diet group at 13 weeks had a significantly reduced insulin level; however, the blood glucose level did not change significantly. The HOMA-IR is widely used to estimate the insulin resistance in human and animal models. The present study demonstrated a CPH-mediated improvement of the HOMA-IR score, suggesting that CPH improves the insulin resistance, which is the essential first pathologic step in the development of NAFLD and dyslipidemia. CPH also prevented the HFD-mediated liver TG accumulation. It is therefore reasonable to assume that the improvement of insulin resistance by CPH might contribute to the attenuation of fatty liver.

In the present study, the serum TG level was slightly higher in the CPH group, although the liver TG level in the same group was significantly lower when compared with that in the HFD group. In contrast, serum FFA in the CPH group showed a trend to decrease and liver total cholesterol was clearly reduced.

Taken together, the present study suggests that CPH improves the insulin resistance induced by HFD and attenuates the fatty liver. As a possible mechanism, CPH might ameliorate the insulin resistance by suppressing the MCP-1 secretion from adipose tissue and liver lipid accumulation. Reduction of liver TG by the administration of a whey hydrolysate and soy protein hydrolysates was also reported. We found a significant reduction of liver TG levels in the CPH group compared to those in the HFD group. However, the molecular mechanism is not clear yet, and thus further studies are required.

5 CONCLUSIONS

The present study suggests that chlorella protein hydrolysate contributes to the improvement of insulin resistance and fatty liver in high-fat diet-induced obese mice. Thus, daily intake of chlorella protein hydrolysate may provide beneficial health effects.
References


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