Biotechnology and Bioprocess Engineering **2005**, 10: 242-247

Antiobesity Effect of Recombinant Human Caseinomacropeptide in Sprague-Dawley Rat

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Abstract Caseinomacropeptide (CMP) is a glycopeptide of 64 amino acid residues derived from the *C*-terminal of mammalian milk κ-casein. Recently, human CMP (hCMP) was produced from the recombinant yeast *Saccharomyces cerevisiae*. In this study, the antiobesity activity of the recombinant hCMP (rhCMP) was investigated *in vivo* using Sprague-Dawley rats. The rhCMP did not affect the rats fed with a normal fat diet (fat content, 5.0%), but decreased the body weight gain of the rats fed with a high fat diet (fat content, 20%) by up to 19%. Autopsies revealed that the weights of the liver, kidney and adipose tissues decreased when the rats were given the rhCMP, which also reduced the lipid concentrations in the plasma and liver, but enhanced the fecal excretion of lipids. These results suggest that rhCMP prevent the accumulation of lipid by stimulating its fecal excretion, so could be used as a food supplement to alleviate the obesity problem caused by a high fat diet.

Keywords: caseinomacropeptide, antiobesity, rat, food intake, cholecystokinin

INTRODUCTION

Obesity is one of the most common and important health problems worldwide and causes many diseases such as diabetes, hypertension and even cancers [1]. Although multifactorial, overeating and subsequent energy imbalance (intake > consumption) are known to be the most common cause for the obesity.

Caseinomacropeptide (CMP) is a glycopeptide of 64 amino acid residues (106~169) derived from the *C*-terminal of mammalian milk κ-casein. It is produced from proteolysis of casein in the mammalian stomach when milk is consumed [2]. Studies with bovine CMP (bCMP) have shown that the bCMP induces the release of cholecystokinin (CCK), one of the major intestinal regulatory peptides controlling food intake, and reduces the gain of body weight [3-6]. In addition, it was suggested that this activity of CMP was associated with the presence of the carbohydrates moieties in CMP [7-10]. Therapeutic glycoproteins are one of the most important groups of pharmaceutical products [11,12].

Among various CMPs, human CMP (hCMP) is the most desirable for human consumption, due to its of the human origin and low allergenicity to human. In addition, hCMP is known to be more potent than other CMPs in promoting the growth of bifidobacteria and inducing antithrombosis [13,14]. Nevertheless, hCMP was not commercially available, since the massive harvesting of human mother's milk for the production of hCMP used to be practically impossible. Recently, hCMP was produced and purified from a recombinant *Saccharomyces cerevisiae* [15]. Characterization revealed that the glycosylation level of the recombinant hCMP (rhCMP) was very low compared to that of natural bCMP.

The present study was designed to examine the antiobesity activity of the rhCMP using Sprague-Dawley rats. The experiments were carried out with 30 male rats for 4 weeks, while fed with different diets. The weights of the body, organs and adipose tissues were measured and compared to reveal the effect of rhCMP feeding. The concentrations of total cholesterol and triglyceride in the plasma, as well as the lipid content in the liver and fecal excretion, were also measured and compared.

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MATERIALS AND METHODS

Preparation of rhCMP

The rhCMP was produced from a recombinant *S. cerevisiae* harboring the chemically-synthesized hCMP gene [15]. For the construction of cloning vector, the hCMP gene was inserted into a plasmid pYEGα (*GAL* promoter, *URA*3 marker) under the control of *GAL* promoter [16,17].

Table 1. Composition of experimental diets

(Unit: g/100 g diet)

Constituents	Normal diet (ND)	ND + rhCMP 1.0	High fat diet (HFD)	HFD + rhCMP 0.5	HFD + rhCMP 1.0
rhCMP[1)]	-	1.0	-	0.5	1.0
Corn starch	30.0	30.0	15.0	15.0	15.0
Corn oil	5.0	5.0	20.0	20.0	20.0
Casein	20.0	20.0	20.0	20.0	20.0
Methionine	0.3	0.3	0.3	0.3	0.3
Sucrose	35.0	35.0	35.0	35.0	35.0
Cellulose	5.0	5.0	5.0	5.0	5.0
Vitamin mixture[2)]	1.0	1.0	1.0	1.0	1.0
Mineral mixture[3)]	3.5	3.5	3.5	3.5	3.5
Choline bitartrate	0.2	0.2	0.2	0.2	0.2
Butylhydroxytoluene	0.0014	0.0014	0.0014	0.0014	0.0014

[1)] Crude recombinant human caseinomacropepetide (60% purity).

[2)] Vitamin mixture (per kg): Nicotinic acid, 3 g; Ca pantothenate, 1.6 g; pyridoxine HCl, 0.7 g; thiamin HCl, 0.6 g; riboflavin, 0.6 g; *D*-biotin, 0.02 g; folic acid, 0.2 g; vitamin B_{12}, 0.025 g; vitamin E, 15 g; vitamin A, 0.8 g; vitamin D_3, 0.25 g; vitamin K, 0.075 g; powdered sucrose, 974.655 g.

[3)] Mineral mixture (per kg): $CaCO_3$, 357 g; KH_2PO_4, 196 g; K_3 citrate, 70.78 g; NaCl, 74 g; K_2SO_4, 46.6 g; MgO, 24.4 g; ferric citrate, 6.08 g; $ZnCO_3$, 1.65 g; $MnCO_3$, 0.63 g; $CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$, 0.3 g; KI, 0.01 g; sodium selenate, 0.01 g; ammonium paramolybdate, 0.008 g; chromium potassium sulfate, 0.275 g; sodium metasilicate, 1.45 g; powdered sucrose, 221.227 g.

The strain was constructed so that it have an α-factor secretion signal before the hCMP gene, thus the rhCMP protein was secreted into the culture medium. Fermentation was conducted in a 500-L bioreactor (working volume, 300 L), with YPDG medium (2% yeast extract, 2% glucose, and 3% galactose), at 30°C and pH 5.5~5.7 for 70 h. After fermentation, the culture broth was collected and the cells and particulates removed by continuous centrifugation and microfiltration (pore size, 0.2 μM). The supernatant (250 L) was then applied to a tangential-flow ultrafiltration system, with molecular cut-off membrane (Millipore, Bedford, MA, USA) of 100 and 30 kDa, to remove the large and small proteins, respectively. The retentate (15 L) from the 30 kDa membrane was lyophilized, and a yellowish powder (250 g) of the crude rhCMP obtained. The purity of the crude rhCMP was about 60%, according to HPLC analyses (data not shown), and used for the animal experiments throughout this study, without further purifications.

Animal Experiments

Thirty male Sprague-Dawley rats, weighing approximately 120 g, were used. The rats were housed individually in polycarbonate cages at 22 ± 1°C and 55~60% relative humidity, with a 12:12 light/dark cycle (light, 08:00~20:00). The rats were maintained on a pelletized commercial chow diet for the initial 10 days, then randomly divided into 5 groups (six rats each), which were then fed with experimental diets (Table 1) for 4 weeks. Two different diets, normal and a high fat, with oil content of 5.0% and 20%, respectively, were used. The crude rhCMP was supplemented at a level of either 0.5 or 1.0% in the diets. During the experimental period, the food intake (daily) and body weight (weekly) were recorded. The food intake was determined by measuring the difference in the food weight between that supplied and that remaining after 24 h. The food left after 24 h was discarded, and replaced with fresh food of the same quality during experimental period.

At the end of experiment, all rats from each group were deprived of the diet for 12 h, smothered, and then dissected. Blood samples were collected from the heart with a heparinized syringe. The plasma from the blood was immediately collected by centrifugation at 4,000 × g at 4°C for 20 min, and then stored at -70°C until analysis. The liver, kidney and adipose tissues (perinenal and epididymal fat pad) were removed, and the wet weights measured after rinsing with saline solution. The organ samples were stored at -70°C for further lipid analyses. Feces were collected during the last week of the experiment, weighed and stored at -70°C until analyses.

Lipid Analysis

The concentrations of total cholesterol and triglyceride in plasma were measured enzymatically, using a commercial diagnostic kit (Asan Pharmaceutical Co., Seoul, Korea). Livers were crushed in a homogenizer (Polytron PT-MR3100, Kinematica AG, Littau, Switzerland), and the total lipids extracted with a chloroform:methanol (2:1, v/v) mixture using a modification of the method of Folch *et al.* [18]. Fecal total lipids were extracted with ether using a soxhlet extraction apparatus.

Table 2. Changes in body weights, food intakes and food efficiency ratios (FER) of rats fed experimental diets for 4 weeks

Group	Initial body weight (g) [NS]	Final body weight (g)	Body weight gain (g/day)	Food intake (g/day) [NS]	FER
Normal diet (ND)	249.8 ± 8.09	361.4 ± 13.73^{c}	3.83 ± 0.25^{c}	21.70 ± 1.34	0.19 ± 0.01^{a}
ND + rhCMP 1.0	250.0 ± 9.00	364.2 ± 6.17^{c}	4.04 ± 0.18^{cb}	22.25 ± 0.89	0.19 ± 0.01^{a}
High fat diet (HFD)	249.2 ± 10.82	400.0 ± 3.67^{a}	4.77 ± 0.18^{a}	19.50 ± 0.29	0.24 ± 0.01^{b}
HFD + rhCMP 0.5	249.4 ± 10.49	391.5 ± 13.68^{ab}	4.37 ± 0.27^{ab}	20.35 ± 0.48	0.23 ± 0.01^{b}
HFD + rhCMP 1.0	249.5 ± 10.13	376.5 ± 3.35^{bc}	3.86 ± 0.21^{c}	20.09 ± 0.54	0.20 ± 0.01^{a}

FER = g of body weight gain /g of food intake.
Data are expressed as mean ± standard error of 6 rats per group.
[NS] Values in the corresponding column are not different to each other with statistical significance.
[a-c] The values with different superscript in the same column are significantly different between the groups ($p < 0.05$).

Table 3. The weight ratios (g/100 g body weight) of the liver, kidney and adipose tissues in rats fed experimental diets for 4 weeks

Group	Liver	Kidney	Perinenal fat pad	Epididymal fat pad
Normal diet (ND)	3.98 ± 0.23	0.87 ± 0.03	1.06 ± 0.07	1.30 ± 0.02
ND + rhCMP 1.0	3.97 ± 0.40	0.90 ± 0.05	0.93 ± 0.10	1.28 ± 0.06
High fat diet (HFD)	4.15 ± 0.11	0.96 ± 0.06	1.26 ± 0.10	1.45 ± 0.06
HFD + rhCMP 0.5	3.90 ± 0.23	0.90 ± 0.05	1.06 ± 0.18	1.31 ± 0.20
HFD + rhCMP 1.0	3.78 ± 0.24	0.87 ± 0.03	0.95 ± 0.15	1.19 ± 0.28

Data are expressed as mean ± standard error of 6 rats per group.

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Data were expressed as the mean ± SEM, with statistically significant difference between the groups means evaluated by two-way ANOVA and Duncan's multiple range tests.

RESULTS

Body Weight Gain and Food Intake

Table 2 shows the changes in the food intake and body weight during the experimental 4 week period. When rhCMP was not included in the diet, the final body weights of the groups fed the high fat diet (HFD) were remarkably higher than those of the groups fed the normal diet (ND). In the ND group, the effect of rhCMP supplementation was not observed; final body weight, food intake rate and food efficiency ratio (FER = g of body weight gain/g of food intake) were about the same, regardless of rhCMP supplementation. Conversely, in the HFD groups, the feeding of rhCMP showed a dose-dependent reduction in the final body weights and weight gains. The supplementation of rhCMP lowered the body weight gains by 8.4 and 19.1% at doses of 0.5 and 1%, respectively. With the 1.0% rhCMP supplementation, the body weight gain in the HFD group was close to that in the ND group. It is noticeable that the food intake rate between the HFD groups was not affected by rhCMP. This indicates that rhCMP does not reduce appetite, but decreases the body weight gain upon food intake, as shown by the lower FERs in the HFD groups fed with rhCMP.

Weights of Organs and Adipose Tissues

Table 3 shows the relative weights of the liver, kidney and adipose tissues (perinenal and epididymal fat pad) at the end of the experiment. The weights of organs were normalized to the body weights of the rats from which the organs were taken. When rhCMP was not included in the diet, the HFD group exhibited higher weights than the ND group. The effect of rhCMP supplementation was negligible in the ND groups; whereas, in the HFD groups, the ratios of the organ to body weight decreased in a dose-dependent manner with rhCMP supplementation.

Plasma Lipid Concentrations

Fig. 1 shows the effect of rhCMP on the concentrations of total cholesterol and triglyceride in the plasma. In the ND groups, the total cholesterol level was about the same, regardless of rhCMP supplementation, but decreased by 6.2 and 20.1% in the HFD groups fed 0.5 and 1% rhCMP, respectively. The plasma triglyceride levels were also affected by rhCMP supplementation. The levels were reduced in both the ND and HFD groups, and it was noticed that the ND groups show higher triglyceride concentrations than the HFD groups. High carbohydrate and low fat diets have been reported to raise the plasma

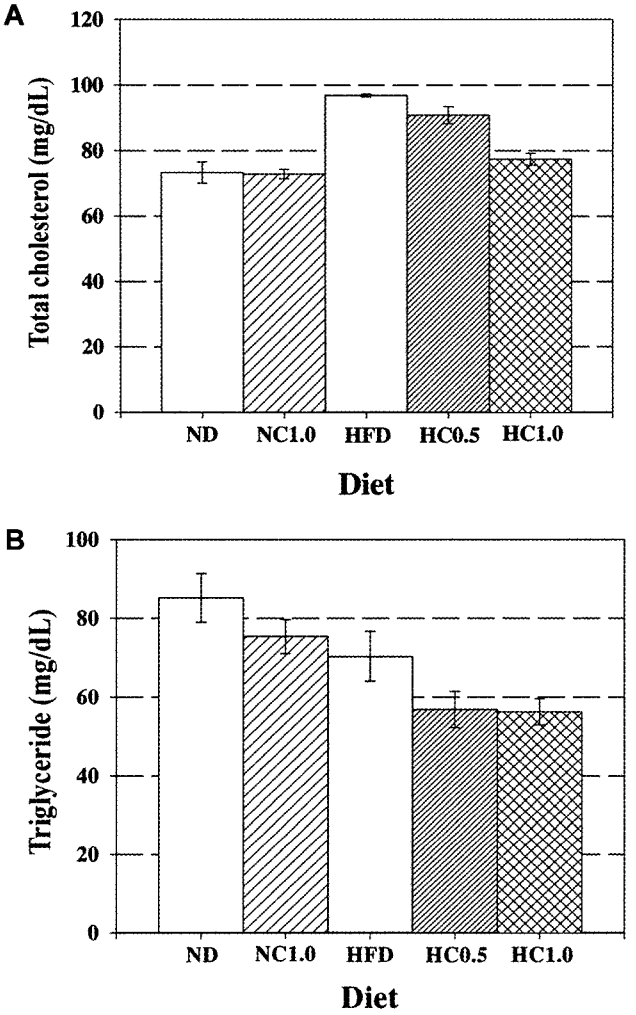

Fig. 1. Effect of recombinant human CMP (rhCMP) on the total cholesterol (A) and triglyceride (B) concentrations in the plasma of the rats fed experimental diets for 4 weeks. ND, normal diet; NC1.0, ND + rhCMP 1.0%; HFD, High fat diet; HC0.5, HFD + rhCMP 0.5%; HC1.0, HFD + rhCMP 1.0%. Data are expressed as mean ± standard error of 6 rats per group.

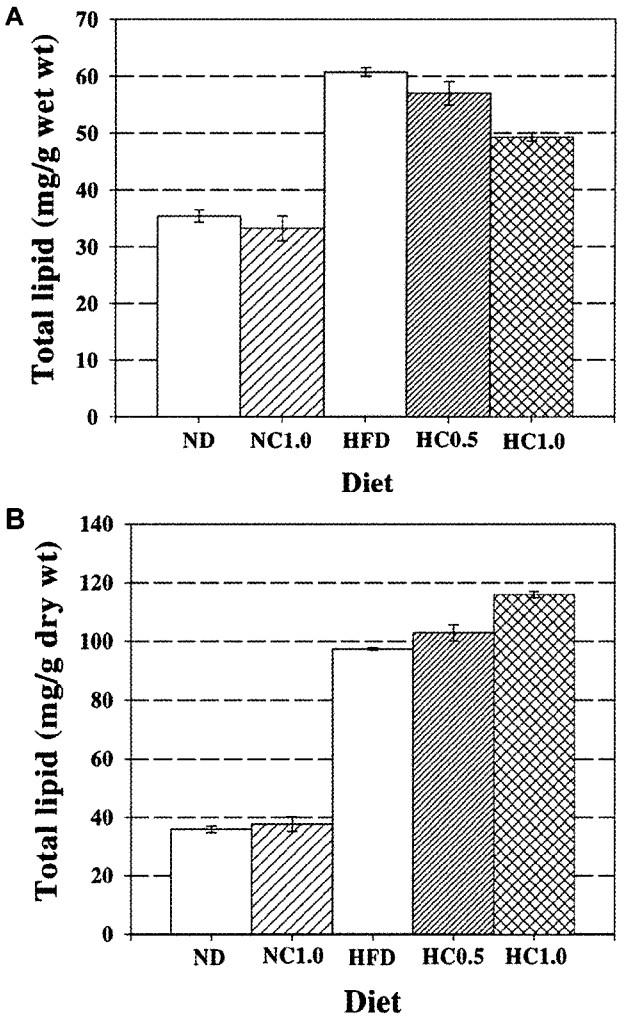

Fig. 2. Effect of recombinant human CMP (rhCMP) on the total lipid concentrations in the liver (A) and feces (B) in rats fed experimental diets for 4 weeks. Symbols are the same as in Fig. 1. Data are expressed as mean ± standard error of 6 rats per group.

triglyceride level [19-21], which was confirmed by the present result.

Liver and Fecal Lipid Excretion

Fig. 2 shows the total lipid concentration of the liver and feces between the different diet groups. The effect of rhCMP was negligible in the ND groups, but significant in the HFD groups. The lipid concentration in the liver was significantly higher in the HFD than the ND groups. The supplementation of rhCMP in the HFD group reduced the liver lipid content in a dose dependent manner, but could not fully offset the increased lipid concentration, even with 1.0% supplementation. The fecal total lipid increased in the HFD compared to the ND groups, which were elevated in a dose-dependent manner.

DISCUSSION

Obesity has become more prevalent in contemporary society. Although the etiology of obesity is complex, dietary factors, particularly the consumption of high fat diet, is considered an important risk factor for its development [22-24].

In order to examine the antiobesity effect of rhCMP, an animal test was conducted, focusing on the changes in food intake, body weight, organs and adipose tissues weights, plasma lipid concentrations, and total lipid concentrations of liver and feces. A crude rhCMP, with 60% purity, was supplemented in the diet at either 0.5% or 1.0%. During the 4 week experimental period, no rat died and no adversary effect on the growth of the rats was observed with the oral administration of rhCMP. We may conclude that the oral administration of rhCMP is not

harmful, at least, to rats for the experimental 4 weeks period.

The rhCMP supplementation in the diets led to lower final weights, weight gains and FER in rats fed with HFD. The results from autopsy showed, that rhCMP reduced the weights of organs and adipose tissues relative to the body weights, as well as the lipid concentrations in the plasma and liver. In addition, although the amount of food intake remained unchanged, the fecal excretion of lipids increased, in a dose-dependent manner, upon rhCMP supplementation. These results clearly suggest that rhCMP has an antiobesity effect on rats consuming a high fat diet. They also suggest that this effect is related to the change of lipid metabolism during the digestive process, in such a way as to prevent the accumulation of lipids inside the body, but allows them to be excreted through feces.

The biological activity of proteins are mostly related with their three dimensional structures. Since rhCMP is a peptide, which is easily degraded in the stomach, the presence of biological activity with orally-administered rhCMP raises some questions as to its mechanism. It has been reported that, when administered by an intravenous injection, the bCMP induces the release of CCK, an appetite suppressing hormone, resulting in reduced food consumption [3-6]. In addition, this activity of CMP was associated with the presence of the carbohydrates moieties in CMP [7-10]. The rhCMP used in the present study had a much lower level of glycosylation than natural CMP [13], and its supplementation to the diet did not affect the appetite, as suggested by the same food consumption, regardless of the presence of the rhCMP. This suggests that the antiobesity effect of the rhCMP observed in the present study is not related with CCK. Further studies are required to elucidate the mechanism for the antiobesity effect of rhCMP. However, as the fecal excretion of lipids increased upon the feeding of rhCMP, it is probable that rhCMP inhibits the action of lipase in the digestive organs.

In summary, we examined the antiobesity effect of rhCMP produced from the yeast *S. cerevisiae*, using an animal test. For the rats fed a high fat diet, the supplementation of rhCMP reduced the body weight gain, weight ratios of the liver, kidney and adipose tissue, and the lipid contents in the plasma and liver. In addition, the fecal excretion of lipid was increased. This indicates that rhCMP has an antiobesity effect on the rats fed a high fat diet by suppressing the absorption and accumulation of fat, and could be developed as a food additive for this purpose.

Acknowledgements This study was supported by the Brain Korea 21 project and the Brain Busan 21 project, one of the authors, Dr. Y.-K. Oh, was supported by 2004 postdoctoral study grant of Pusan National University.

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[Received March 14, 2005; accepted May 9, 2005]