Effects of Cyclo-His-Pro-enriched yeast hydrolysate on blood glucose levels and lipid metabolism in obese diabetic ob/ob mice

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BACKGROUND/OBJECTIVE: We examined the hypoglycemic and anti-hyperlipidemic effect of yeast hydrolysate (YH) enriched with Cyclo-His-Pro (CHP) in the C57BL/6J ob/ob mouse model.

MATERIALS/METHODS: Mice were separated into 4 groups (8 mice/group) on the basis of blood glucose and body weight: WT control, lean mice given vehicle; ob/ob control, ob/ob mice given vehicle; YH-1, ob/ob mice given 0.5 g/kg of YH; YH-2, ob/ob mice given 1 g/kg of YH. YH in saline or vehicle was administered orally in the same volume every day for 3 weeks.

RESULTS: Mice treated with YH (0.5 and 1 g/kg) for 3 weeks displayed a significant reduction in overall body weight gain and perirenal and epididymal adipose tissue weight compared to the ob/ob control group. Additionally, high-density lipoprotein (HDL) cholesterol, glucose, and atherogenic indexes were significantly decreased in the blood of YH-1 and YH-2 groups compared to the ob/ob control. In ob/ob mice, YH administration significantly improved glucose tolerance and blood insulin levels. These data indicate that YH treatment produces potent hypoglycemic and anti-hyperlipidemic effects by controlling body weight, fat mass, blood lipid, insulin levels, and glucose tolerance.

CONCLUSION: YH could potentially be used as a treatment option for diabetes and hyperlipidemia. The CHP-enriched YH may be a promising strategy in the development of hypoglycemic peptide nutraceuticals.

INTRODUCTION

Diabetes mellitus is a serious, chronic metabolic disorder that significantly impacts the healthcare system as well as the health, quality of life, and life expectancy of individual patients [1]. Diabetes is divided into two major categories: type 1 diabetes (known as insulin-dependent diabetes mellitus or IDDM) and type 2 diabetes (known as non-insulin-dependent diabetes mellitus or NIDDM). Diabetes mellitus is a major endocrine disorder, affecting nearly 10% of the world’s population. At the turn of this century, 171 million individuals were estimated to have diabetes, and by 2030 the number is expected to increase to 366 million [2].

Recently, a variety of ingredients present in natural sources have been suggested to act on a variety of targets by various modes and mechanisms to exert medicinal effects [3, 4]. Since natural sources are usually considered to be less toxic and exhibit fewer side effects than synthetic sources [5], they have excellent therapeutic potential in complicated disorders like diabetes and its complications [6].

In our previous study [7], Flavourzyme and ultrafiltration treatments were selected as the optimal processes for producing yeast hydrolysate with a high content of cyclo-His-Pro (CHP). It is possible that CHP-enriched yeast hydrolysate may be useful as an antioxidative and/or antidiabetic material for the preparation of functional foods [7]. CHP is a naturally occurring cyclic dipeptide consisting of histidine and proline and is a metabolite of thyrotrophin-releasing hormone (TRH). It is also synthesized through other biochemical processes and is found in many protein-rich, processed foods and peptide sources [8]. Since blood levels of CHP are increased after glucose ingestion in humans, CHP activity has been suggested to regulate glycemic control in diabetes [9]. Recently, dietary feeding of CHP plus zinc was shown to significantly improve insulin sensitivity and glucose tolerance in diabetic animal and human subjects [9-11]. Furthermore, several studies have demonstrated that CHP decreased food intake, mimicking the action of leptin, which controls appetite [12,13]. Hence, CHP plays an important role in the regulation of insulin and leptin sensitivity [11,14]. The purpose of this study was to investigate the hypoglycemic and anti-lipidemic effect of CHP enriched yeast hydrolysate (YH) in obese C57BL/6J-ob/ob mice.

Keywords: Yeast hydrolysate, blood glucose, diabetes mellitus, cyclo-his-pro
MATERIALS AND METHODS

Preparation of yeast hydrolysate

An 8% yeast suspension was hydrolyzed for 48 h using Flavourzyme (endoprotease and exopeptidase from Aspergillus oryzae). The hydrolysis temperature was 50°C for crude enzyme, and the enzyme/yeast substrate ratio (E/S) was 1/100 for enzyme. Hydrolysis of yeast with enzyme was performed in 0.01 M phosphate buffer. The solution pH was adjusted to 7.0 for Flavourzyme before hydrolysis was initiated. Inactivation of enzyme was done by heating at 90°C for 5 min. The yeast hydrolysate obtained from enzymatic hydrolysis was first passed through a 0.2-μm membrane filter (Satocon cassette, Sartorius, Germany). A portion of the solution was removed immediately and the filtrate was then pumped through a 10-kDa molecular weight cut-off membrane (Satocon cassette). The yeast hydrolysate obtained from the filtration, and the resulting substance was dried and used as the yeast hydrolysate (YH).

Animals and diets

The experimental protocol was reviewed and approved by the Korea University Animal Care Committee (KUIACUC 2009 0420-2). Female C57BL/6-ob/ob mice (ob/ob mice, 50 ± 3 g) and C57BL/6J lean wide type mice (WT mice, 21 ± 3 g) were obtained at 8-9 weeks of age from Daehan-BioLink Co. (Seoul, Korea). They were individually housed in plastic cages with grated stainless steel floors. The colony room was maintained at 24 ± 1°C with 60% atmospheric humidity and a 12 h light/12 h dark cycle. The mice had ad libitum access to water and a commercial diet (Samyang Co., Seoul, Korea) containing the following (g/kg of diet): moisture, 80; protein, 230; fat, 35; fiber, 50; and carbohydrate, 600. They were fed with normal chow and water ad libitum during an initial adaptation period of 1 week.

After an adaptation period, the mice were separated into 4 groups (8 mice/group) on the basis of blood glucose and body weight: WT control, lean mice given vehicle; ob/ob control, ob/ob mice given vehicle; YH-1, ob/ob mice given 0.5 g/kg of YH; YH-2, ob/ob mice given 1 g/kg of YH. YH in saline or vehicle was administered orally as the intragastric injection using oral gavage with a ball tip needle in the same volume every day for 3 weeks. The dosage was decided on the basis of previous studies including toxicological studies [7,15-17].

Measurement of body weight, food intake, blood lipid levels, organ weight, and the fat pad

Body weight and food intake were measured every 2 days for 3 weeks. At the end of the experimental period, the mice were anesthetized with CO2, and blood was collected in tubes treated with anticoagulant heparin. Blood was separated by centrifugation at 3,000 × g for 15 min at 4°C and stored at -70°C until required for further analysis. The liver, spleen, kidney, perirenal fat, and epididymal fat were removed and weighed after sacrificing the animals. Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs. In toxicological experiments, comparison of organ weights between treated and untreated groups of animals have conventionally been used to evaluate the toxic effect of the test article [18,19]. Triacylglycerol, total cholesterol, and high-density lipoprotein (HDL) cholesterol levels in blood were measured using the FUJI DR8-CHEM 3500 i (Fuji Photo Film Co., Osaka, Japan). Low-density lipoprotein (LDL) cholesterol levels were estimated using a method validated by Friedewald et al. [20].

Measurement of fasting blood glucose and insulin

Fasting blood glucose was measured after 3 weeks of treatment with YH, during which the animals were fed normal diets. Blood glucose levels were monitored every week using venous blood from the tail vein after a 12 h fast with a glucose analyzer (Superglucocard II, Arkray Inc., Kyoto, Japan) based on the glucose oxidase method [21]. Blood was collected from the heart using a heparinized syringe. The blood was separated by centrifugation at 3,000 × g for 15 min at 4°C. The level of insulin in the blood was measured using a commercially available mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Shibayagi Co., Gunma, Japan).

Oral glucose tolerance test (OGTT)

Blood glucose levels were monitored every week after a 12 h fast with venous blood from the tail vein using a glucose analyzer. After overnight fasting, on the day of animal sacrifice, 0-min blood was taken from tip of the tail vein from all the mice. The mice, which had been administrated YH (1 g/kg body weight) for 30 min, received an oral load of 30% glucose solution (2 g glucose/kg of body weight). Blood samples were collected from the tail vein 10, 20, 30, 60 and 100 min after the oral glucose load and treated as previously demonstrated for blood glucose analysis [22]. The blood glucose levels were expressed as increments from the baseline. Incremental areas under the response curves (AUC) were calculated using the trapezoidal rule.

Pancreas immunohistochemistry (IHC)

The microtome (Leica Co., Heidelberg, Germany). Then, they were deparaffinized in xylene twice, for 5 min each time, and then rehydrated with graded ethanol. For IHC, rehydrated sections were treated with 3% H2O2 in methanol for 30 min to block endogenous peroxidase, and then washed with 0.01 mol/L phosphate buffer for 10 min and immunostained with primary antibody, monoclonal mouse anti-insulin (1:1000), or anti-glucagon (1:200) antibody (Lab Vision Co., CA, USA). The antigen-antibody complex was visualized by an avidin-biotin peroxidase complex solution using an ABC kit (Vector Lab, CA, USA). The staining intensities of the sections were assessed in a quantitative fashion according to the microdensitometric method, which is based on optical densities and area according to the Image J program (National Institutes of Health).

Statistical analysis

All statistical analyses were performed using the Statistical
Package for Social Sciences (SPSS) version 12.0 (SPSS Inc., IL, USA). The differences among groups were evaluated by one-way analysis of variance (ANOVA) and Duncan’s multiple range tests. All data were reported as the mean and standard error of the mean (SEM). A level of \( P < 0.05 \) was used as the criterion for statistical significance.

**RESULTS**

**Change in body weight and food consumption**

We prepared YH with high-content CHP using various proteases (Neutrase, Alcalase, Ficin, Flavourzyme and Protamex). These proteases produced YH containing CHP concentrations greater than 200 \( \mu \)g/g. Specially, Flavourzyme-treated hydrolysate contained the highest CHP content (674.0 \( \mu \)g/g) of the all tested proteases in our previous report [7]. The YH had a molecular weight of less than 10 kDa and was composed of water (5.4%), crude fat (0.8%), crude protein (64.9%, 14% glutamic acid), carbohydrates (26.9%), ash (0.9%), and CHP (680 \( \mu \)g/g). YH also contained a high content of Zn (154 \( \mu \)g/g, data was not shown) [7].

To determine whether YH administration over 3 weeks can influence obesity, body weights were measured regularly. Body weight was found to be significantly higher in ob/ob control group (\( P < 0.05 \)) than WT control group. YH administration dose-dependently reduced liver weights by 12% and 21% in the YH-1 and YH-2 groups, respectively, compared to the ob/ob control group. Adipose tissue acts as a major storage site for excess energy, in addition to its role as an endocrine organ; thus, it is important for the regulation of energy homeostasis [24, 25]. In order to examine the effect of YH administration on body fat accumulation, adipose tissue weight in ob/ob mice was measured. Compared to WT control, perirenal and epididymal adipose tissue weight was 3- to 7-fold greater in the ob/ob control group (\( P < 0.05 \)). Perirenal adipose tissue weights in the YH-1 and YH-2 groups were 5.80 ± 0.07 g and 5.12 ± 0.17 g, respectively, and both treated groups weighed significantly less than the ob/ob control group (6.76 ± 0.08 g) (\( P < 0.05 \)). Additionally, animals treated with YH-2 displayed a significant reduction in epididymal adipose tissue weight compared to ob/ob control (\( P < 0.05 \)). In the ob/ob control group, epididymal adipose tissue weight was 6.68 ± 0.10 g/100 g body weight, while YH-1 and YH-2 groups were 6.39 ± 0.15 g and 5.94 ± 0.13 g/100 g body weight, respectively.

In this study, perirenal and epididymal fat adipose tissue and body weights were dramatically increased in ob/ob controls compared to WT control (Tables 1 & 2). However, after YH treatment (0.5 and 1 g/kg), body weight gain and adipose tissue weight significantly decreased. These results indicate that CHP in YH suppressed fat accumulation in adipose tissue, resulting in reduced body weight gain.

**Lipid levels in blood**

An imbalance between triacylglycerol hydrolysis and synthesis is critical during the development of obesity [26]. Blood lipid levels were measured after 3 weeks of YH administration in mice. Compared to the WT control, blood triacylglycerol and total cholesterol levels were significantly increased by 2.0-fold.
in ob/ob controls ($P < 0.05$, Table 3). Blood triacylglycerol and total cholesterol levels were not significantly reduced in the YH-1 and YH-2 groups compared to the ob/ob control group. However, HDL-cholesterol levels were markedly higher in animals treated with YH-1 and YH-2 than ob/ob controls by 104% and 155%, respectively. Blood LDL-cholesterol and atherogenic index were significantly higher in the ob/ob control than WT control ($P < 0.05$), but YH administration reduced significantly these levels ($P < 0.05$). Particularly, after 3 weeks of YH-2 treatment, blood concentrations of LDL-cholesterol, and the atherogenic index were significantly reduced by 50% and 77%, respectively, compared to the ob/ob control ($P < 0.05$). These results indicate that YH administration reduced the levels of obesity-associated lipids released into the blood.

**Fasting blood glucose levels**

Table 4 shows fasting blood glucose levels of YH-treated groups and controls. In the ob/ob control group, the blood glucose level was increased by approximately 400 mg/dL. As reported previously by Lin et al. [27], ob/ob mice had significantly elevated glucose levels compared to WT control. The ob/ob mice treated with two different doses (0.5 and 1 g/kg) of YH displayed significantly reduced fasting blood glucose levels compared to ob/ob control ($P < 0.05$). After 3 weeks of treatment, the fasting blood glucose levels of the YH-2 group (246.12 ± 13.09 mg/dL) were significantly lower than the ob/ob control group (364.98 ± 15.12 mg/dL) ($P < 0.05$). As a result, in vivo treatment with YH caused a significant reduction in blood glucose levels.

**OGTT and blood insulin level**

To investigate whether the inhibition of hyperglycemia in YH-treated animals was affected by insulin, an OGTT assessment was performed (Fig. 1). WT control, ob/ob control, and YH-treated groups showed significant increases in blood glucose levels (209.93 ± 1.69, 413.35 ± 4.20, 397.0 ± 1.22, and 396.95 ± 2.20 mg/dL, respectively) in glucose gavages after 20 min (Fig. 1A, $P < 0.05$). The ob/ob control group showed slowly recovery to its baseline glucose level, even after 100 min. However, the YH groups (YH-1 and -2) showed significantly lowered blood glucose levels compared to the ob/ob control after 100 min ($P < 0.05$). The AUC for glucose decreased by 10.2% and 11.9% in YH-1 and YH-2 groups, respectively, compared to ob/ob controls ($P < 0.05$). Insulin controls many different metabolic processes in energy homeostasis, including glucose levels [28]. Blood insulin concentrations in YH groups decreased by 33% and 38%, respectively, compared to the ob/ob control (Fig. 1B, $P < 0.05$). Taken together, these data indicate that ob/ob mice treated with YH have improved glucose tolerance and hyperinsulinemia compared to ob/ob controls.

**Immunoreactivities for insulin and glucagon in pancreatic islets**

The quantitative analysis results for insulin and glucagon immunoreactivities in the pancreatic islets of the ob/ob mice are shown in Fig. 2. YH-treated ob/ob mice (YH-1 and YH-2) displayed pancreatic islets with significantly enhanced insulin staining compared to the staining intensity of the ob/ob control ($P < 0.05$). The IHC intensities of glucagon in YH-treated groups (YH-1 and YH-2) were significantly reduced compared to the ob/ob controls ($P < 0.05$).

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### Table 4. Effects of yeast hydrolysate (YH) on blood glucose levels in ob/ob mice

| Time   | WT control | ob/ob control | YH-1 | YH-2 |
|--------|------------|---------------|------|------|
| 0 week | 109.21 ± 4.41  | 339.66 ± 18.69 | 377.39 ± 11.09 | 340.03 ± 15.22 |
| 1st week | 110.14 ± 4.72  | 357.79 ± 20.22 | 286.12 ± 17.35 | 251.56 ± 14.33 |
| 2nd week | 114.53 ± 3.36  | 371.31 ± 21.11 | 286.21 ± 15.33 | 246.31 ± 12.03 |
| 3rd week | 113.19 ± 4.01  | 364.98 ± 15.12 | 284.31 ± 16.11 | 246.12 ± 13.09 |

WT control, lean mice given vehicle; ob/ob control, ob/ob mice given vehicle; YH-1, ob/ob mice given 0.5 g/kg of YH; YH-2, ob/ob mice given 1 g/kg of YH. All experimental data were Mean ± SEM. Values with different superscript letters within the same row are significantly different at $P < 0.05$ by Duncan’s multiple range test.
DISCUSSION

Recently, there has been a growing interest in hypoglycemic agents from natural products [3, 4], because natural sources are usually considered to be less toxic with fewer side effects than synthetic sources. Many traditional remedies for diabetes mellitus use natural sources, and over 200 pure phytochemicals are currently known to have hypoglycemic properties [29]. CHP found in many protein-rich processed foods or peptide sources [8], suggested to relate glycemic control in diabetes, demonstrated that mimicking the action of leptin [12,13], the regulation of insulin and leptin sensitivity [11,14]. In this study, we investigated whether administrating with CHP enriched YH could control and reduce the blood glucose level and lipid metabolism observed in obese C57BL/6J-ob/ob mice. The reduction of body weight gain can be correlated with reduction of fat mass, indicating that reduced fat mass may lead to reduced body weight [30]. In this study, perirenal, epididymal fat adipose tissue, and body weights were dramatically increased in the ob/ob control group when compared to the WT control mice, respectively (Table 1, 2). However, after administration with YH (0.5 and 1 g/kg of body weight), the body weight gain and adipose tissue weights were significantly decreased. These results indicate that CHP in YH suppressed fat accumulation in adipose tissue resulting in reduced body weight gain.

Blood triacylglycerol and total cholesterol levels were significantly higher in the ob/ob control group compared to the WT control group, whereas blood triacylglycerol and total cholesterol levels were decreased pattern in the YH administration groups. According to den Boer et al. [31], triacylglycerol are synthesized in the liver, secreted into the bloodstream as very low-density lipoproteins (VLDL), and transported to adipose tissues. Consequently, the quantification of triacylglycerol levels showed the amount of lipids in the liver. Furthermore, the blood HDL-cholesterol level was higher in YH administration groups than in the ob/ob control group (Table 3). HDL-cholesterol plays an important role in reverse cholesterol transport pathway, carrying cholesterol esters and triacylglycerol from perirenal adipose tissues to liver for removal [32,33]. In this study, we demonstrate that CHP in YH promote the efflux of cholesterol accumulation by increasing blood HDL-cholesterol level. Taken together, these results suggest that CHP in YH suppresses lipid accumulation, as well as levels of triacylglycerol and total cholesterol, and results in reduction of body weight gain and adipose tissue weight.

One of the most important effects of insulin is its ability to stimulate glucose transport into muscle and fat. Prospective studies of populations at high risk for type 2 diabetes have suggested that in most patients, the initial inherited lesion is insulin resistance. Insulin-stimulated glucose disposal is markedly reduced in patients with type 2 diabetes [34]. In ob/ob mice, by 6 weeks of age, insulin resistance and hyperinsulinemia are well developed [35]. Our experimental animals were profoundly hyperinsulinenic, since the average value for WT control group is under 6 ng/mL (Fig. 1). After YH administration, blood insulin levels were significantly reduced under fasting condition, suggesting that YH improved insulin resistance in the ob/ob mice.

The insulin resistance exhibited by type 2 diabetic patients is complicated by obesity. Past studies have shown that insulin sensitivity in type 2 diabetes patients improves with weight loss [28], possibly due to an improvement in insulin-stimulated glucose transport into muscle [36]. Our results showed that the CHP in YH possessed both anti-diabetic and anti-obese activity in ob/ob mice. The mechanisms of actions of CHP for the improvement of insulin resistance remain to be elucidated. Data from epidemiological studies [37, 38] and clinical trials [39, 40] showed that hyperglycemia was the principal cause of the complications associated with diabetes. Thus, effective control of blood glucose level is a key step in preventing or reversing diabetic complications and improving the quality of life in both type 1 and type 2 diabetic patients [28]. In this study, we measured fasting blood glucose every week after YH administration; we found that these compounds progressively reduced blood glucose levels in ob/ob mice (Table 4). And then, data from the present study demonstrated that administration of YH significantly reduced fasting blood glucose level and improved glucose tolerance in ob/ob mice (Fig. 1). It is possible that glucose tolerance improvement was achieved by restoring...
insulin sensitivity, and in turn, normalizing insulin-mediated glucose utilization.

Similar to our results, in which prostate extract containing zinc and CHP significantly decreased blood glucose and improved glucose tolerance and insulin sensitivity in genetically type 2 diabetic Goto-Kakizaki (GK) rats and ob/ob mice [9,41]. It is indicating that CHP has a strong ability to stimulate intestinal zinc absorption and cellular zinc uptake, and glucose utilization [41,42]. Zinc deficiency critically affects diabetes because zinc activates insulin receptor β-subunits, thereby exerting an influence in glucose metabolism [41]. Although the exact mechanism of anti-diabetes activity of CHP is not clearly established, it is highly possible that CHP is involved in the regulation of insulin sensitivity by stimulating zinc metabolism because of the following arguments: oral intake of CHP plus zinc significantly improves glucose tolerance in diabetic and CHP stimulates zinc transport mechanisms across the small intestine and cell membrane of muscle cells to increase zinc use [42]. Accordingly, it is assumed that the YH containing CHP might affect zinc metabolism, which plays an important role in the synthesis and secretion of insulin sensitivity that increase glucose uptake.

In this study, insulin staining was enhanced in pancreatic tissue from the YH-treated ob/ob mice, and plasma insulin levels were higher in the YH groups as compared to those in the ob/ob control group. We observed that a concentration of plasma insulin and insulin immunoreactivity in the pancreatic islets were significantly higher in the YH groups than in the ob/ob control group.

CHP-enriched YH ameliorated fasting blood glucose levels and glucose tolerance, and reduced hyperlipidemia and body weight in leptin-deficient C57BL/6j-ob/ob mice. While the molecular mediators responsible for the hypoglycemic and anti-hyperlipidemic activity of CHP-enriched YH were not identified in this study, we found that CHP-enriched YH treatment could be useful for treating individuals with diabetes and hyperlipidemia. YH may be a new strategy in the development of hypoglycemic peptide nutraceuticals. Further studies may lead to new therapeutic approaches for treatment and/or prevention of diabetes.

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