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Water Extracts of *Paecilomyces tenuipes* Inhibit Cathepsin S-induced Adipocyte Differentiation in 3T3-L1 Cells

Kil-Sun Myoung*, Jung-Hee Lee, Kwang-Sei Lim, and Chul-Sung Huh

R&D Center, Korea Yakult Co., Ltd., Yongin, Gyeonggi, Korea

Abstract Cathepsin S is a cysteine protease that affects extracellular matrix remodeling. Recently, several studies have reported that cathepsin S is involved in obesity. Both mouse and human adipose cells produce this enzyme in the early phase of adipocyte differentiation, where it degrades fibronectin. Cathepsin S gene expression is elevated in the adipose tissue of obese mice as compared to that of lean mice. *Paecilomyces tenuipes* water extracts (PTW) are shown to have an inhibitory effect on cathepsin S activity. In this study, Z-Val-Val-Arg-MCA was used as a cathepsin S-specific substrate in order to examine inhibitory effect of PTW. Supplementing 3T3-L1 cell media with PTW clearly reduced lipid droplet accumulation and cathepsin S-induced adipogenesis. Furthermore, PTW decreased weight gain, subcutaneous adipose tissue growth, the level of serum triglyceride, and total cholesterol in mice fed a high-fat diet. These data suggest that PTW work against adipose cathepsin S and presumably contribute to anti-obese activities.

Keywords: *Paecilomyces tenuipes*, cathepsin S, adipogenesis, obesity

Introduction

Paecilomyces tenuipes (*P. tenuipes or P. japonica*) is a popular entomopathogenic fungus used in folk medicine and health foods in China, Korea, and Japan. The fruiting bodies of this fungus are highly valued as a medical herb. Earlier research has revealed its various biological and pharmacological activities, including immuno-stimulating, anti-oxidant activities, and anti-tumor activities (1-5). Certain bioactive constituents from *P. tenuipes* are reported to have cytotoxic and antibacterial effects (6,7). Furthermore, there is evidence that *P. tenuipes* cultivated on egg yolk can improve lipid and antioxidant metabolism (8); however, the molecular mechanisms involved in lipid metabolism are still unknown.

Cathepsin S is a potent cysteine protease that has the ability to degrade several extracellular matrix elements related to fat mass growth (9). During preadipocyte differentiation, cathepsin S degrades fibronectin, a key component of the extracelluar matrix. Here, preadipocytes are changed from their fibroblastic morphologies into rounded adipocytes; adipocyte differentiation then progresses to completion with fat accumulation in round-shaped cells. Human adipose tissue expresses and secretes cathepsin S protein, where it is up-regulated in obesity; obese subjects have significantly higher circulating cathepsin S concentrations than non-obese subjects (10). In contrast, following gastric surgery when significant losses of fat mass have been induced, the level of cathepsin S protein decreases both in adipocytes and in circulation (11). In this study, the inhibitory effect of *Paecilomyces tenuipes* water extracts (PTW) on cathepsin S was confirmed. By using preadipocyte culture media, it was shown that cathepsin S enzymatic activity, and its induction of adipocyte differentiation, were inhibited by PTW. Furthermore, PTW affected the decrease of weight gain by reducing adipose tissue in animals fed a high-fat diet.

*Corresponding author: Tel: +82-31-899-7845; Fax: +82-31-899-7710
E-mail: duqrl80@re.yakult.co.kr
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Materials and Methods

Assay for cathepsin S activity The modified method of Shindo *et al.* (12) was employed to determine cathepsin S enzymatic activity. The purchased enzyme (Merckbioscience, Darmstadt, Germany) was diluted to 126.87 mU/mL with potassium phosphate buffer [100 mM potassium phosphate (pH 6.5, Sigma-Aldrich, St. Louis, MO, USA), 5 mM dithiothreitol (DTT, Sigma-Aldrich), and 4 mM ethylene-diaminetetraacetic acid (EDTA, Sigma-Aldrich)]. The test mixture contained 20 μL of PTW, 50 μL of cathepsin S solution, and 200 μL of either cathepsin S specific substrate Z-Val-Val-Arg-MCA (10 mM, Peptide Institute, Minoh-shi, Osaka, Japan). The released 4-methyl-coumaryl-7-amide (MCA) cleaved by cathepsin S was quantified using a fluorescence plate reader. All data were the average of triplicate determinations.

Cell culture and adipocyte differentiation 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC). For adipogenic induction, the cells (2 days after confluence) were cultured in differentiation induction medium (Dulbecco's modified essential medium (DMEM, Gibco BRL, Carlsbad, CA, USA) containing 10% fatal bovine serum (FBS, Gibco BRL)) together with a mixture of 0.5 mM isobutylmethylxanthine (IBMX, Sigma-Aldrich), 1 μM dexamenthasone (Sigma-Aldrich), and 10 μg/mL of bovine insulin (Sigma-Aldrich). The 50 μM cathepsin S solution and PTW (100 or 200 μg/mL) were added to the differentiation induction media. Two days following induction, the cells were cultured in post-differentiation medium (DMEM containing 10% FBS and 10 μg/mL of insulin). The PTW were not added to the post-differentiation

Table 1. Composition of experimental diets

Ingredients	Experimental diets[1)]					
	SD		HFD		HFD-PTW	
	g%	kcal%	g%	kcal%	g%	kcal%
Protein	22.1	25.5	24.0	20.0	23.4	20.0
Carbohydrate	50.5	58.2	41.0	35.0	39.9	35.0
Fat	6.3	16.3	24.0	45.0	23.4	45.0
PTW[2)]	-	-	-	-	1.0	-
Total kcal/g	3.47		4.73		4.72	

[1)]SD, standard diet; HFD, high fat-diet; HFD-PTW, high fat-diet with PTW supplementation.
[2)]*P. tenuipes* water extracts.

medium. The cell incubating medium was changed every 2 days. Lipid accumulation was assessed by staining paraformaldehyde-fixed cells with Oil Red-O solution. Cathepsin S enzymatic activity was assessed in preadipocytes 3 hr after exchange to the differentiation induction media. Two-hundred μg/mL of PTW was added to the exchanged media. The cultured cells were washed with phosphate buffered saline (PBS, Gibco BRL) and collected in microcentrifuge tubes containing 300 μL of PBS. Cathepsin S activity was determined using a cathepsin S assay kit (BioVision, Mountain View, CA, USA).

Animals and diets At the beginning of the experiment, male C57BL/6 mice weighing between 8 and 13 g were purchased from the Japan SLC (Kotoh-cho, Japan). The animals were all individually housed in stainless steel cages in an air conditioned room with a controlled temperature (24) and automatic lighting system (alternating periods of 12-hr light/dark). After 1-week of adaptation, the animals were randomly divided into 2 groups: a standard diet (SD) group and high fat-diet (45 kcal% fat, Research Diets, New Brunswick, NJ, USA) group. The high fat-diet group was then randomly divided into 2 sub-groups: a high fat-diet control (HFD) group and a high fat-diet supplemented with PTW (1%, HFD-PTW) group. Table 1 shows the compositions of the experimental diets. The mice were given free access to food and distilled water. Food consumption and weight gain were measured every 3 days. At the end of the experimental period (8 weeks), the mice were anesthetized with urethane following a 5 hr fast. Blood samples were taken from the heart using regular syringes. The plasma and serum were obtained by centrifuging the blood at 60×g for 15 min at 4°C. All samples were stored at −80°C until analyzed.

Measurement of body fat mass and blood sampling Body fat was dissected from epidermal fat pads located in the upper region of the testis. The tissues were removed, weighed, and stored at −80°C until use. The blood was withdrawn to examine plasma glucose and insulin levels. The plasma glucose was determined using an Amplex Red glucose kit (Invitrogen, Carlsbad, CA, USA). The plasma insulin was determined using a mouse insulin kit (Sibayagi, Ishihara, Japan). The serum total cholesterol level was determined using a V-cholesterol kit (Asan Pharmaceutical, Seoul, Korea). The high density lipoprotein (HDL)-cholesterol concentration was determined using an HDL-cholesterol kit (Asan Pharmaceutical). The low density lipoprotein (LDL)-cholesterol was calculated as follows: serum LDL cholesterol concentration (mg/dL)=total cholesterol−HDL cholesterol−(triglyceride/5) (13). The serum triglyceride concentration was measured enzymatically using a kit from Asan pharmaceutical. The serum leptin level was determined using a mouse leptin enzyme-linked immunosorbent assay (ELISA) kit (Biovendor, Modrice, Czech Republic).

Protein preparations The adipose cells were washed with phosphate buffered saline (PBS) twice and collected with 300 μL of homogenizing buffer containing 50 mM Tris-HCl (pH 8.0, Sigma-Aldrich), 150 mM NaCl (Sigma-Aldrich), 5 mM EDTA, and 1% Triton X-100. The amount of protein was measured using the Bradford reagent (Bio-Rad, Hercules, CA, USA) method with bovine serum albumin (BSA, Sigma-Aldrich) as the standard.

Western blot analysis Protein extracts of the adipocytes were prepared in homogenizing buffer. The protein extracts were then resolved on 10% Tris-glycine gel. After protein transfer to polyvinyl difluoride (PVDF) membrane (Invitrogen), the membranes were blocked with 5% skim milk (Gibco BRL) and 0.1% Tween 20 (Sigma-Aldrich) in PBS (PBS-T) for 1 hr at room temperature. The membranes were incubated with primary antibody for 1 hr. After washing, the membranes were incubated with secondary antibody coupled to peroxidase (diluted in PBS-T-3% skim milk) for 1 hr. Next, the membranes were washed extensively and incubated with enhanced chemiluminescence detection solution (GE Healthcare, Buckinghamshire, England) and then immediately exposed to X-ray film (GE Healthcare).

Data analysis All results are expressed as the mean± standard deviation (SD). Significant differences among the groups of mice were determined by Turkey's test. Differences with a $p<0.05$ were considered statistically significant.

Results and Discussion

Effects of PTW on cathepsin S activity The PTW inhibited cathepsin S activity in a dose-dependent manner (Fig. 1A). Approximately 250 μg/mL of crude extracts reduced cathepsin S activity by half. Cathepsin S activity was also detected in 3T3-L1 cells. The 3T3-L1 preadipocytes were fully incubated in 6-well plates in which the media were exchanged to differentiation induction media containing 200 μg/mL of PTW. As compared to the untreated controls, the 3T3-L1 cells incubated with PTW for 3 hr showed the decrease in cathepsin S activity (with a 73.0% reduction, Fig. 1B). Cathepsin S has been implicated in angiogenesis, tumor growth, atherosclerosis, and obesity (10,14,15). Increased cathepsin S precursor expression has been observed in obese experimental animals (16). Within adipose tissue, cathepsins, which are lysosomal protease implicated in tissue remodeling, play roles in fibronectin degradation as well as in promoting adipocyte differentiation.

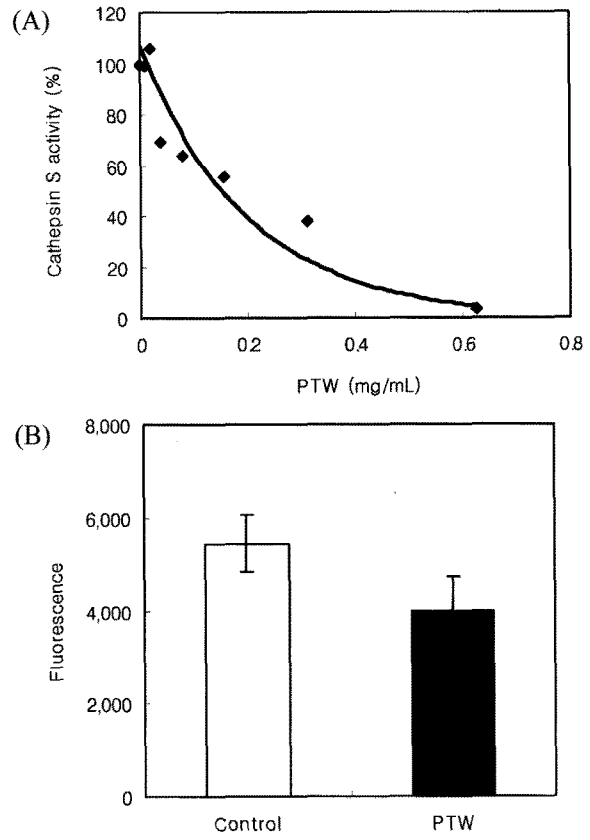

Fig. 1. Effect of PTW on cathepsin S activity. Cathepsin S activity was measured using 10 mM Z-Val-Val-Arg-MCA as the substrate (A). The effect of PTW treatment on cathepsin S activity in 3T3-L1 adipose cells (B).

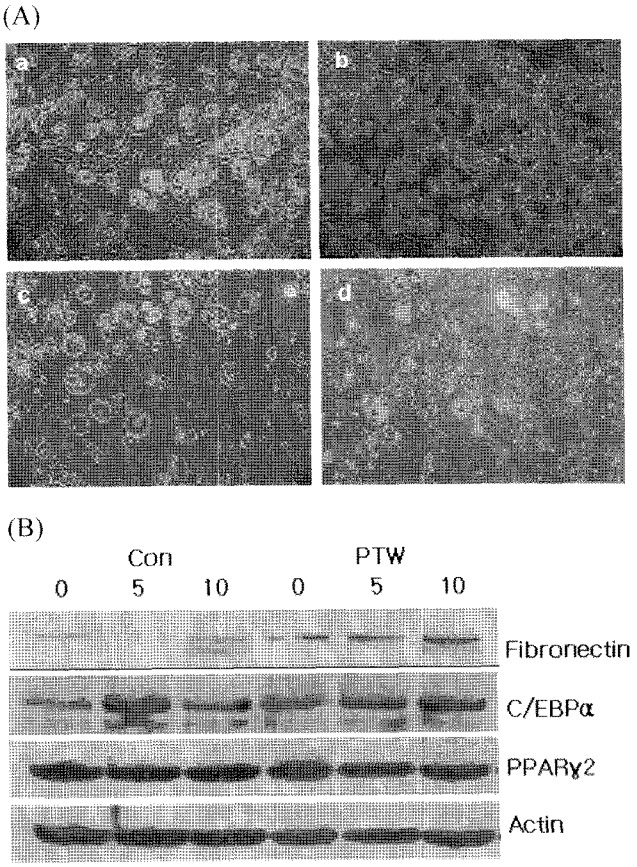

Fig. 2. Effects of PTW on adipose cells differentiation. (A) Differentiation of adipose cells. 3T3-L1 adipocytes were differentiated (a) with 50 μM/mL of cathepsin S (b). The PTW dose-dependently (c, 100 μg/mL; d, 200 μg/mL) inhibited adipocytes differentiation induced by 50 μM/mL of cathepsin S. (B) Effect of PTW on protein expression during 3T3-L1 adipose cells differentiation. Con, control; PTW, PTW treated adipose cells.

Inhibitory effect of PTW on preadipocyte differentiation The PTW were added to differentiation induction media (100 or 200 μg/mL, day 0) in order to evaluate its effect on adipocyte differentiation. After 2 days of incubation, the induction media were exchanged with post-differentiation media without PTW supplementation. At day 10, lipid accumulation was assessed by Oil Red-O staining. Cathepsin S treatment induced adipose cell differentiation, but this effect was reduced dose-dependently by PTW treatment (Fig. 2A). The protein expressions indicating adipocytes differentiation were also measured, where 200 μg/mL of PTW reduced the protein level of PPARγ2 (peroxisome proliferators-activated receptor γ2) and C/EBPα (CCAT/ enhancer binding protein α) in 10-day differentiated cells (Fig. 2B). In contrast, the PTW supplementation increased the level of fibronectin protein, which was degraded by cathepsin S in the differentiation process (11). The PTW supplementation in preadipose cells inhibited cathepsin S activity and adipose cell differentiation.

Anti-obesity activity of PTW To determine effects of PTW on adipose tissue, an animal experiment was performed. Each mouse in the HFD-PTW group consumed approximately 1 g/kg of body weight (BW) of the PTW daily for 8 weeks. During the 8 weeks experimental period, food intake amounts, and body weight were monitored twice/week. In the HFD-PTW group, body weight gains were significantly reduced in comparison to the HFD group, the negative control (Table 2). At the end of the experiment, the mice administered PTW had a 24.6% reduction in body weight ($p<0.05$) as compared to the HFD group. Food intake was also decreased in the PTW treated group; however, diet efficiency remained lower than in the HFD group. To test whether the reductions in body weight were caused by decreased adiposity, the animals were sacrificed and their epidermal fat pads (acquired from upper regions of the testis) were weighed. When compared with the negative control group, the HFD-PTW group had a dramatic decrease in epidermal adipose tissue (Table 2), with a 63.1% reduction of the epidermal fat pads. These data indicate that PTW may possess anti-obesity activity *in vivo*. PTW presumably worked against adipose cathepsin S, and decreased the weight gain and subcutaneous adipose tissue growth in mice fed a high-fat diet.

Blood glucose and plasma lipids Most studies reporting on mushroom consumption have shown hypoglycemic and hypolipidemic effects, especially with entomopathogenic fungi (17-19). For example, the oral administration of *Cordyceps militaris* decreased fasting serum glucose levels in pancreatectomized rats by increasing the glucose disposal

Table 2. Effects of PTW supplementation on weight gain and fat mass[1)]

	SD	HFD	HFD-PTW
Calorie (kcal/g)	3.47	4.68	4.64
Food intake (g)	214.9±4.94[2)]	153.4±7.9	126.4±6.5***
Weight gain (g)	8.2±0.9	16.3±2.3	8.0±1.2***
Efficiency of diet (%)[3)]	3.7±0.5	10.7±1.0	5.8±1.0***
Epidermal fat pads (g)	0.53±0.06	1.71±0.19	0.63±0.06***

[1)]SD, standard diet; HFD, high fat-diet; HFD-PTW, high fat-diet with PTW supplementation; *Compared with HFD group (*p<0.05, **p<0.01, ***p<0.001, t-test unpaired comparison). [2)]Values are mean±SD (n=10/group). [3)]Efficiency of diet=Weight gain (g)/Food intake (g)×100.

Table 3. Effects of PTW on blood glucose and serum lipids[1)]

	SD	HFD	HFD-PTW
Fasting blood glucose (mg/dL)	237.2±24.9	245.7±50.5	243.1±19.5
Serum insulin (ng/mL)	0.5±0.2	4.3±2.0	0.5±0.1***
Serum triglyceride (mg/dL)	94.9±11.1	95.7±26.7	83.8±2.9
Total cholesterol (mg/dL)	143.9±6.6	241.5±24.7	165.9±18.2***
HDL-cholesterol (mg/dL)	63.5±7.5	96.1±13.8	77.3±7.0**
LDL-cholesterol (mg/dL)	69.0±11.8	126.3±24.8	66.6±19.3***
Leptin (pg/mL)	26.0±5.9	209.5±62.7	24.2±16.7***

[1)]SD, standard diet; HFD, high fat-diet; HFD-PTW, high fat-diet with PTW supplementation. Values are mean±SD (n=10/group); *Compared with HFD group (*p<0.05, **p<0.01, ***p<0.001, t-test unpaired comparison).

rate (20); it also increased hepatic glucokinase activities (21). *P. tenuipes* also has a hypoglycemic effect in non-insulin dependent diabetes mellitus patients (22), as well as lipid metabolism effects in diet-induced obese rats (4,23). In this study, however, neither a high-fat diet nor PTW supplementation affected fasting blood glucose levels (Table 3). Meanwhile, PTW supplementation dramatically recovered the increase in serum insulin levels in the HFD group, in which the serum insulin levels of the HFD-PTW group were similar to those of the SD group. And the HFD-PTW group had significantly lower serum total cholesterol concentrations than the HFD group. The serum triglycerides were also affected by PTW supplementation, with the HFD-PTW group showing relatively lower levels than the SD group. The LDL-cholesterol levels were calculated by the Friedewald formula (13). The HFD group had a 1.8-fold increase in LDL-cholesterol as compared to the SD group, and the level of the HFD-PTW group was similar to the SD group. Lastly, the serum leptin levels were significantly lower in the HFD-PTW group as compared to the HFD group. PTW has a potential anti-obese activity and hypolipidemic effects in diet-induced obese mice. It is now possible to produce *P. tenuipes* on a large-scale due to development of artificial cultivation technique (8). PTW could become a promising therapeutic agent for obesity.

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