Lipogenesis inhibition by water-soluble components from *Monascus*-fermented rice on 3T3-L1 cells

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Abstract
Obesity is a risk factor for several diseases, and overcoming this problem has become a big challenge in the world. Red mold rice (RMR)/*Monascus*-fermented rice has been traditionally used in East Asian region, and reportedly it contains valuable secondary metabolites that could decrease the risk of several diseases including obesity. Recently, it has been drawn attention that water-insoluble azaphilone pigments produced by *Monascus* fungi can also suppress lipid accumulation. However, there are no information about water-soluble components from RMR to control adipogenesis process in adipocytes. This study therefore focused on lipid accumulation inhibitory activity during differentiation of 3T3-L1 preadipocytes by the water-extracts from RMR. *Monascus pilosus* NBRC4507 was selected as the best strain to induce lipid accumulation inhibitory activity. It produced high inhibitory activity in the water-extracts prepared from RMR cultured at 30°C for 14 days. Thin-layer chromatography (TLC) analyses showed no lovastatin citrinin presence in the water-extracts. Intracellular triglyceride content and glycerol-3-phosphate dehydrogenase activity in 3T3-L1 adipocytes were decreased concentration-dependently by treatment of the water-extracts. In contrast, free glycerol content in the cell culture medium increased in a concentration-dependent manner. These findings suggest that the water-extracts from RMR using *M. pilosus* NBRC4507 effectively inhibit lipid accumulation through lipogenesis inhibition and lipolysis promotion during differentiation of 3T3-L1 cells. Finally, it can be concluded that the water-soluble components of RMR can be used in food industry for health promotion including prevention of obesity.

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Introduction
Obesity and overweight are major risk factors for chronic diseases and reduce life expectancy worldwide. Unhealthy food habits and lack of exercise are closely related to the development of obesity and associated with type 2 diabetes, heart disease, hypertension, cancer, respiratory complications, and osteoarthritis (Kopelman, 2000). An imbalance between energy intake and expenditure induces obesity through adipogenesis process allowing accumulation of excessive energy. Dietary restrictions and medicinal foods are effective in the management of obesity and overweight. Hence a demand for functional or nutraceutical foods has been growing to prevent obesity and overweight.

Red mold rice (RMR) or *Monascus*-fermented rice has been traditionally used as materials for fermentation foods, food coloring, and herbal medicine, for a long period of time in East Asian countries. *Monascus* fungi produce diverse secondary metabolites by solid-state fermentation, and a wide variety of physiological activities of them has been reported by many researchers (Chen *et al.*, 2008).

Monacolin K, identical to lovastatin produced by *Monascus* fungi, is the most famous secondary metabolite which has polyketide structure. Monacolin and its analogues strongly inhibit a rate-limiting enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, in the cholesterol biosynthesis pathway in liver. In addition, *Monascus* fungi can also produce citrinin, a nephrotoxic mycotoxin, and this fact has caused controversy concerning the safety of RMR from a food hygiene perspective. Both monacolin and citrinin are polyketide compounds and have basically water-insoluble properties. Numerous studies have been conducted in regard to adipogenesis inhibitory effects by the water-insoluble polyketide compounds (Chen *et al.*, 2008). However, studies on adipogenesis inhibitory effects by water-soluble compounds of *Monascus*-fermented products are very limited (Chen *et al.*, 2008).

The water-soluble functional ingredient is convenient to use in the food industry and in daily food applications. Therefore, this study focused on the adipogenesis inhibitory activity

of water-soluble components produced by *M. pilosus* NBRC4507 for prevention of obesity. To accomplish this objective, production strain was screened by using lipid accumulation inhibitory assay in 3T3-L1 cells, and the properties of the water-extracts from RMR were further investigated.

Materials and Methods

To prepare RMR, autoclaved non-glutinous rice was inoculated with the spores solution prepared with each *Monascus* strain (5×10^4 spores/mL) and then it was incubated at 30°C for 7, 10, and 14 days. Water-soluble components of RMR was extracted with distilled water were concentrated, freeze-dried, and the resulting powder was stored at -20°C for further experiments.

Lipid accumulation inhibitory activity of the RMR water-extracts was examined using 3T3-L1 cells, cultured in a 6-well plate with a basal medium (Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin) at 37° C in 5% CO_2. After confluence, the medium was changed to a differentiation medium (DMEM containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 µM 3-isobutyl-1-methylxanthine, 0.5 mM dexamethasone, and 5 µg/mL insulin) for differentiation induction, and the cells were cultured for 2 days. Then the cells were further cultured with a lipid accumulation medium (DMEM containing 10% FBS, 1% penicillin/streptomycin, and 5 µg/mL insulin) and added the water extracts from RMR, at a final concentration of 0.5, 0.75, and 1 mg/mL, and the medium was changed every 2 days during 8 days of differentiation. Lovastatin, at a final concentration of 5 µM or 10 µM, was used as a positive control. After 8 days of differentiation, oil red stain was done, and the coupled oil red pigment coupled with intracellular lipid droplets was extracted with isopropanol, and the resulting absorbance was measured at 510 nm (Benchmark Plus microplate reader, Bio-Rad).

Lovastatin and citrinin in the water-extracts of RMR were verified by the TLC methods of Jaivel and Marimuthu (2010) and Pepeljnjak andOžegović (2002), respectively. Triglyceride (TG) content and glycerol-3-phosphate dehydrogenase (GPDH) activity in3T3-L1 cells were determined using commercial assay kits according to the manufacturer's instructions. Amount of free glycerol in the cell culture medium was quantified colorimetrically using commercial assay reagents. TG content, GPDH activity, and free glycerol content were normalized to the protein concentration.

Statistical analyses were performed using GraphPad Prism 5 software, and data were presented as means ± SD (three independent experiments, $n = 3$).

Results and Discussion

Monascus pilosus (NBRC4507) was selected as the good strain to produce active water-soluble components. In this study, all of the tested water-extracts were prepared from this strain. The absence of both lovastatin and citrinin in the water-extracts of RMR was confirmed by TLC analyses. Thus, these results suggest that the lipid accumulation inhibitory activity exhibited in this study may be caused by new physiological substances in the water-extracts of RMR rather than by lovastatin or citrinin.

As shown in Figure 1, the lipid accumulation inhibitory activity gradually increased depending on the concentrations of the water-extracts and the cultivation period of RMR. The water-extracts from RMR cultured for both 10 and 14 days increased the inhibitory activity in a concentration-dependent manner, and those cultured for 14 days had greater inhibitory activity than those of 10 days at any given concentrations. Especially, 1.0 mg/mL of the water-extract from RMR cultured for 14 days showed significantly higher inhibitory activity than that of 5 µM lovastatin controls. The result of this experiment suggests that an adequate production of the lipid accumulation inhibitory activity in the water-soluble components from RMR needs longer cultivation time. In fact, the growth of *Monascus* species by solid-state fermentation is very slow, and it takes a long cultivation period for production of the water-insoluble secondary metabolites such as lovastatin and citrinin. Therefore, it strongly suggests that the water-soluble components of 14 days cultured RMR have mild lipogenesis inhibitory activity which differs from the effects of lovastatin and citrinin. The intracellular TG content treated with the water-extracts from RMR cultured for 14 days decreased in a concentration-dependent manner. Decreasing of the intracellular TG content level gave close agreement with the results of oil red stain.

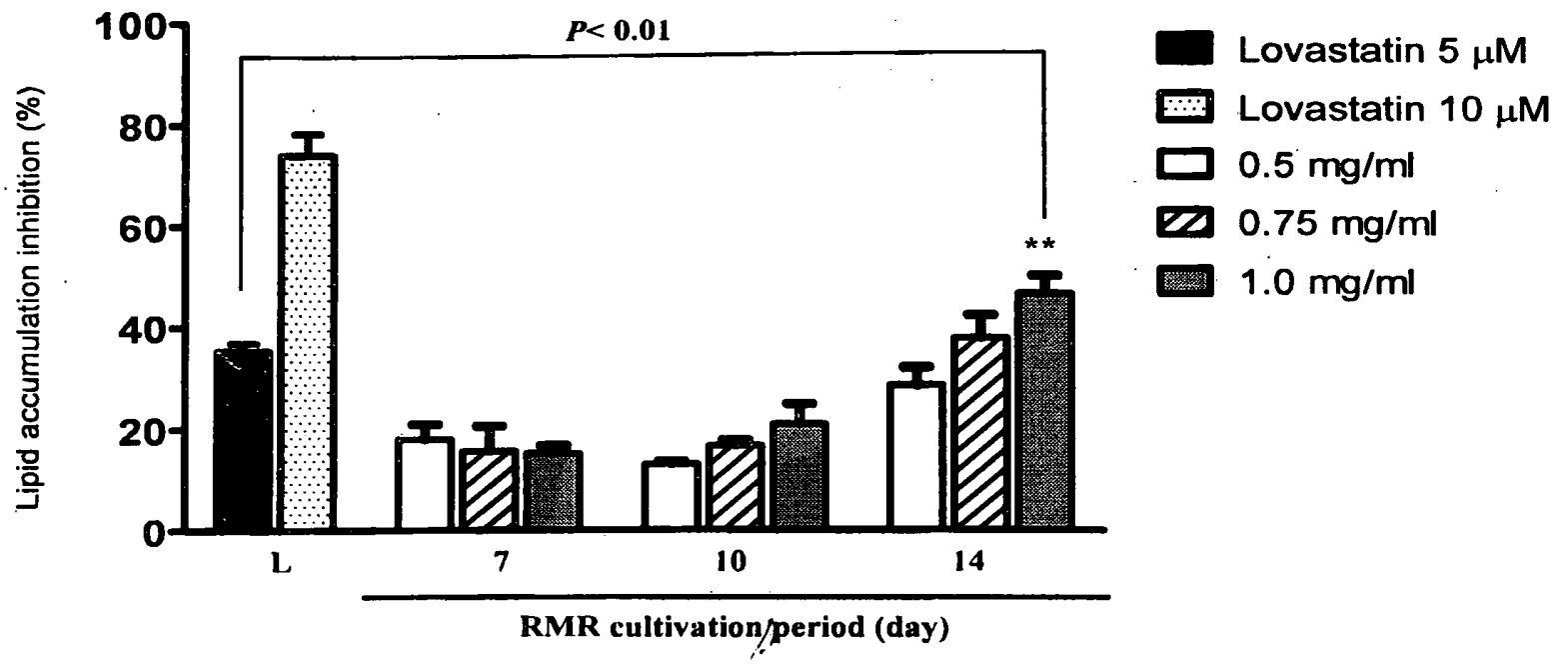

Figure1: Effects of water-extracts from RMR on lipid accumulation in the differentiated 3T3-L1 adipocytes. Lipid accumulation inhibitory activity of the water-extracts from RMR cultured for 7, 10, and 14 days at a final concentration of 0.5, 0.75, and 1 mg/mL, and lovastatin (L) at 5 μM and 10 μM. The degree of inhibition was expressed as a percentage of the control as the mean ± SD (n = 3).

GPDH activity in the differentiated 3T3-L1 cells was concentration-dependently reduced by the treatment with the water-extracts from RMR cultured for 14 days. The results indicated that the water-extracts from RMR effectively reduce TG biosynthesis from glucose by reduction of GPDH activity during adipocyte differentiation. On the other hand, the glycerol release level in the culture medium increased in a concentration-dependent manner of the water-extracts of RMR. These results thus showed a significant negative correlation between lipogenesis expressed as the TG content and lipolysis expressed as the glycerol release level. Therefore, these results strongly suggest that the water-soluble components from RMR inhibit lipid accumulation during differentiation of adipocytes, by both lipogenesis inhibition and lipolysis promotion.The intracellular TG level and lipid accumulation in the adipocytes is determined by the balance between lipogenesis and lipolysis (Ailhaud, 1982). GPDH, a marker of the late phase of differentiation, is one of the key enzymes playing a primary role in lipid metabolism involved in TG biosynthesis via glycolysis. Although the water-extracts from RMR reduced the GPDH activity, the lovastatin control showed only a small reduction of the enzyme activity. This effect of lovastatin is supported by previous study (Chen *et al.*, 2008). Therefore, the water-extracts of RMR may inhibit lipogenesis by a different mechanism from that of lovastatin.

In conclusion, the water-soluble components of RMR which is practically non-existent of lovastatin and citrininan significantly inhibited the adipogenesis process. After further studies, these findings can be used in food industry for health promotion including management of obesity.

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