1-Trp and 1-Leu-OEt Derivatives of the Monascus Pigment Exert High Anti-Obesity Effects on Mice

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High-fat diets (HFDs) supplemented with the 1-Trp and 1-Leu-OEt derivatives of the monascus pigment were fed to mice. Compared to the HFD group, the average body weight gain of the four HFD-pigment groups was decreased by 13.6–50.9%, and the intra-peritoneal adipose tissue weights were reduced by 16.7–30.5%. The derivatives also reduced the respective serum total cholesterol and triglyceride levels of the mice by 9.7–14.4% and 12.5–17.2%. The 1-Trp derivative greatly increased the HDL-cholesterol level by 64.8–66.4% and the HTR value by 73.8–81.7%. The 1-Leu-OEt and 1-Trp derivatives decreased the total cholesterol level in the mice liver by 9.7–24.2% and 36.2–39.9%, respectively. Reductions in the triglyceride level were 21.5–22.4% for the 1-Leu-OEt derivative and 17.9–18.8% for the 1-Trp derivative. The 1-Leu-OEt derivative exhibited higher in vitro inhibitory activities against HMG-CoA reductase and lipoprotein lipase than the 1-Trp derivative. An in vivo test with mice showed the 1-Trp derivative to have higher anti-obesity effects than the 1-Leu-OEt derivative.

Key words: Monascus species; fungal fermentation; amino acid derivative; in vivo lipid; anti-obesity

An imbalance between the energy intake and expenditure due to fat storage can cause obesity; this is a key risk factor for various diseases, including diabetes, hypertension, cardiovascular complaints, and cancer of the breast, uterus, and small intestine. Physical exercise can be effective for the control and therapy of obesity, but not for everybody. The treatment of obesity, in many cases, is dependent on drug and food therapy. However, anti-obesity drugs can reduce the food intake, alter metabolism, and increase thermogenesis. It is well known that dietary fats are not directly absorbed into the intestines. The fats must be hydrolyzed to glycerol and free fatty acids by lipases, and then the fatty acids can either be used to produce energy or be stored in adipose tissue. If human lipases can be inhibited, the fat absorption that results in obesity can therefore be controlled. The commercial anti-obesity drug, tetrahydrodrolipstatin, is a good example of a lipase inhibitor.

Monascus pigments have been used for many years as natural colorants and as health supplements in East Asia. There are reports regarding the synthesis and biological activities of various amino acid derivatives of monascus pigments. We have previously reported that some amino acid derivatives of monascus pigments showed high in vivo lipase-inhibitory activities, and the leucine ethyl ester derivatives showing the highest activities against lipases. The anti-obesity effects of these two monascus derivatives were investigated in this study by in vivo tests using mice.

Materials and Methods

Chemicals. 1-Tryptophan (1-Trp), the leucine ethyl ester (1-Leu-OEt), and media components for Monascus cultivation were purchased from Sigma-Aldrich Co. Casamino acid, yeast extract, bacto-peptone, and agar powder were bought from Difco Co., and chloroform, methanol, and the other solvents were products of JT Baker Co.

Microorganisms and media. Monascus sp. J101 was used to produce the amino acid derivatives of the monascus pigments. The strain was preserved and spores formed by using a Hiroi agar medium consisting of 10% sucrose, 0.5% casamino acid, 0.3% yeast extract, 0.2% NaNO₃, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.001% FeSO₄·7H₂O, and 2% agar powder in distilled water (w/v). Seeds were cultivated in a Mizutani medium consisting of 5% glucose, 2% bacto-peptone, 0.8% KH₂PO₄, 0.2% CH₃COOH, 0.1% NaCl, and 0.05% MgSO₄·7H₂O in distilled water (w/v). The fermentation medium for producing the monascus pigments consisted of 5% glucose, 0.3% NH₄NO₃, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, and 0.001% FeSO₄·7H₂O in distilled water (w/v). The pH value of all media was adjusted to 6.6 prior to sterilization.

Cultivation and synthesis of the pigment derivatives. To prepare the spore suspension, Monascus sp. J101 was grown on Hiroi agar slants for 168 h in an incubator at 30°C. The spores were scraped off with a spatula, and a spore suspension of 1 × 10⁸ CFU per ml was prepared. Seeds were cultured by inoculating 5 ml of the spore solution into a 500-ml flask containing 75 ml of the Mizutani medium, and then cultivating for 48 h at 30°C. The seed culture solution for fermentation was inoculated at 7% (v/v) into a 5 liters jar (Korea Fermentor Co., Korea) containing 3 liters of the fermentation medium, and then cultivated for 168 h at 30°C. To synthesize the monascus derivatives, 1-Trp or 1-Leu-OEt was added at 0.7% to the fermentation culture broth, and then incubated for 48 h at 30°C.

Extraction of the monascus pigments. Each culture broth was extracted in ethanol for 24 h on a reciprocally shaken water bath at 30°C. The supernatant was separated by centrifuging for 15 min at 6,000 rpm and then concentrated by evaporation. Unnecessary
pigments were removed by solvent extraction with hexane. The purity of the monascus derivatives was determined by HPLC (HP-1100) in an ODS C18 column (250 × 4.6 mm, 5 μm and Hypersil; Kleinostheim, Germany) with a run time of 40 min, flow rate of 0.8 ml per min, and elution gradient of distilled water/methanol from 100:0 to 30:70.

**Diet breeding of the mice.** Four-week-old male C57BL/6 mice were obtained from Daehan Biolink Co. (Korea) and maintained under conditions of a 12-h light/dark cycle at 23 ± 0.5 °C with a relative humidity of 50 ± 5%. All the mice were group-housed (two or three mice per cage) in the specific pathogen-free (SPF) facility of the Laboratory Animal Research Center of Yonsei University in accordance with the Animal Care and Use Committee of Yonsei University and international guidelines on the ethical use of animals. Forty-eight mice were randomly assigned to six groups consisting of eight mice per group and then maintained for 6 weeks with free access to food and water. Two control groups were fed with either a normal diet (ND; laboratory pellet feed, certified rodent diet #5002, Orient Co., Korea) or a high-fat diet (HFD; Hansam Biotech Co., Korea). Four HFD-pigment groups (LE0.1, LE0.2, Trp0.1 and Trp0.2) were respectively fed with HFD supplemented with either the L-Leu-OEt derivative or the l-Trp derivative at a rate of 0.1 or 0.2 mg per g-mouse per day. The body weight and food intake rate of each mouse were measured once every two days for six weeks. The weight gain was calculated by averaging the difference between the initial and final body weights of each mouse. HFD consisted of 20% casein, 0.3% d-l-methionine, 3% corn oil, 18% shortening, 37% sucrose, 5% cellulose, 1% cholesterol, 0.2% choline bitartrate, a 4.2% mineral mixture, a 1.2% vitamin mixture, 10% corn starch, and 0.004% t-butyldihydroquinone (w/v %).

**Preparation of the mice serum samples and liver homogenates.** After 6 weeks of feeding, all the mice were fasted for 24 h before being anesthetized with ethyl ether. Blood samples were collected through cardiac puncture, and the serum was quickly separated by 20 min of centrifugation at 900 × g. The internal organs were then collected. The liver was homogenized in phosphate-buffered saline (pH 7.4) with a Teflon-glass homogenizer. Each serum sample and liver homogenate were stored at −30 °C until needed for analysis.

**Analysis of lipids and adipose tissue weight.** The total cholesterol contents of each serum sample and liver homogenate were determined by the method of Allain et al. The triglyceride contents were determined by the method of Spayd et al. and the HDL cholesterol contents were measured by using a commercial kit (Asan Pharmaceutical Co., Korea). HTR values (%) were calculated by dividing HDL cholesterol by the total cholesterol content. The weight of the intra-peritoneal adipose tissues was measured after the peritoneal cavity of the mouse had been opened.

**Inhibition assay for lipoprotein lipase and HMG-CoA reductase.** An enzyme solution was prepared by adding lipoprotein lipase purified from bovine milk to 900 μl of a 10 mM Tris buffer (pH 8.5). After the solution had been mixed with 100 μl of a monascus derivative solution, it was incubated for 15 min at room temperature. To the mixture, 200 μl of a triglyceride solution (0.1 mM NaCl, 0.15 mM Tris buffer, 0.1 mg/ml of heparin, 60 mg/ml of bovine serum albumin, and 5% (v/v) rat serum) was added, and the mixture incubated for 15 min at 25 °C for the enzymatic reaction. The activity of lipoprotein lipase was determined by measuring the amount of released free fatty acids with an enzymatic colorimetric reagent.

A solution of HMG-CoA reductase was prepared by adding the enzyme purified from the HepG2 cell line to 700 μl of a phosphate buffer (300 mM KCl, 240 mM potassium phosphate, 6 mM EDTA, and 15 mM dithiothreitol, at pH 8.5). After the solution had been mixed with 100 μl of a monascus derivative solution, it was incubated for 15 min at room temperature. To this mixture, 100 μl of an NADPH solution and 100 μl of an HMG-CoA solution were added, and the mixture incubated for 10 min at 25 °C for the enzymatic reaction. The activity of HMG-CoA reductase was determined by measuring the decreased amount of NADPH at 350 nm with a spectrophotometer.

**Statistical data analysis.** Each result is expressed as the mean ± SD. Data were analyzed by using the SAS program. Differences between the HDF group and derivative-supplemented diet groups were determined by using Student’s t-test, and significance levels were determined by using an ANOVA test. The level of p < 0.05 is considered as statistically significant.

**Results**

Effect of monascus derivatives on the body and IPAT weights of mice

l-Trp and L-Leu-OEt derivatives of the monascus pigment, which have been reported to be strong inhibitors of lipase, were added to the feed for six groups of mice. Two control groups were respectively fed with a normal diet (ND) and a high-fat diet (HFD). The other four HFD-pigment groups (LE0.1, LE0.2, Trp0.1, and Trp0.2) were respectively fed with HFD supplemented with the monascus derivatives of L-Leu-OEt and l-Trp, (0.1 and 0.2 mg per g-mouse per day).

After the mice had been fed for six weeks with each diet, the food intake rates of the mice dietary groups were measured. As shown in Table 1, the food intake rate for the HFD mice group was 6.8% less than that for the ND group. The rates for the four HFD-pigment mice groups were 2.21–2.27 g per day, corresponding to 3.4–6.0% less than for the HFD group.

The body weight increased gradually with feeding time for all the diet groups. The weight gain of the HFD mice group was 3.91 g, being 110% compared to the ND group. However, the weight gain of the HFD-pigment groups was 1.99–3.38 g, corresponding to 13.60–50.9% less than the HFD group.

The intra-peritoneal adipose tissue (IPAT) weight of the mice after feeding with the various diets was measured (Fig. 1). The IPAT weight of the HFD group increased by only 50% compared to the ND group. However, the weight of the NFD-pigment groups decreased by 16.7–30.5% compared to the HFD group, corresponding to slightly higher values than those for the ND group.

**Effect of monascus derivatives on the lipid contents of the mice serum and liver**

After six weeks of diet feeding, the serum total cholesterol and triglyceride contents of the HFD group respectively increased by 25.0% and 42.7% when compared to the ND group (Fig. 2). Compared to the HFD group, the cholesterol and triglyceride contents of the four HFD-pigment groups respectively decreased by

**Table 1.** Effects of the l-Leu-OEt and l-Trp Derivatives on the Body Weights and Food Intake Rates of Mice

<table>
<thead>
<tr>
<th>Diet Group</th>
<th>Body weight (g)</th>
<th>Weight gain (g)</th>
<th>Food intake rate (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>ND</td>
<td>23.66 ± 0.61</td>
<td>25.53 ± 1.20</td>
<td>1.86 ± 0.98</td>
</tr>
<tr>
<td>HDF</td>
<td>23.61 ± 0.35</td>
<td>27.52 ± 1.35</td>
<td>3.91 ± 1.23</td>
</tr>
<tr>
<td>LE0.1</td>
<td>23.72 ± 0.78</td>
<td>26.41 ± 1.07</td>
<td>3.38 ± 1.18</td>
</tr>
<tr>
<td>LE0.2</td>
<td>23.24 ± 1.12</td>
<td>25.68 ± 1.24</td>
<td>2.44 ± 1.21</td>
</tr>
<tr>
<td>Trp0.1</td>
<td>23.59 ± 1.20</td>
<td>25.08 ± 1.36</td>
<td>1.99 ± 0.88</td>
</tr>
<tr>
<td>Trp0.2</td>
<td>23.75 ± 0.64</td>
<td>25.60 ± 0.79</td>
<td>2.22 ± 0.61</td>
</tr>
</tbody>
</table>

* Differences between HDF group and derivative-supplemented diet groups are significant at p < 0.05. ND, normal diet; HDF, high fat diet; LE0.1, HDF + 0.1 mg l-Leu-OEt derivative per g-mouse per day; LE0.2, HDF + 0.2 mg l-Leu-OEt derivative per g-mouse per day; Trp0.1, HDF + 0.1 mg l-Trp derivative per g-mouse per day; Trp0.2, HDF + 0.2 mg l-Trp derivative per g-mouse per day.*
9.7–14.4% and by 12.5–17.2%. However, there were no significant differences in the cholesterol and triglyceride levels among the four HFD-pigment groups.

The HDL cholesterol content in the serum was measured after feeding each diet (Fig. 3). The HDL cholesterol content of the HFD mice group was 11.0% lower than the ND group. In the case of the HFD-pigment groups, the HDL-cholesterol contents of the LE0.1 and LE0.2 groups were similar to that of the ND group, whereas the respective contents of the Trp0.1 and Trp0.2 groups were 64.8% and 66.4% higher. The HTR level of the HFD group was 34.8% lower than the ND group. The HTR levels of the LE0.1 and LE0.2 groups were slightly higher than that for the HFD group, whereas the respective levels of the Trp0.1 and Trp0.2 groups were 73.8% and 81.7% higher.

The liver lipid contents of the different diet mice groups were determined (Fig. 4). The total cholesterol and triglyceride contents of the HFD group were 80.0% and 60.0%, respectively, higher than the ND group. However, the cholesterol contents of the LE0.1, LE0.2, Trp0.1, and Trp0.2 HFD-pigment groups were respectively 9.7, 24.2, 36.2, and 39.9% lower than the HFD group. Their triglyceride contents were also lower, by 21.5, 22.4, 17.9, and 18.8%, respectively.

**Effect of the monascus derivatives on the reaction of cholesterol synthesis-related enzymes**

The inhibitory effects of the l-Leu-OEt and l-Trp derivatives on the *in vitro* reactions of the cholesterol synthesis-related enzymes, i.e., HMG-CoA reductase and lipoprotein lipase, were investigated. As shown in Fig. 5, the l-Leu-OEt and l-Trp derivatives inhibited HMG-CoA reductase by 16.5% and 11.3%, respectively. The inhibitory degrees of the l-Leu-OEt derivative and l-Trp derivative for lipoprotein lipase were 27.0% and 9.5%, respectively.

**Discussion**

The l-Leu-OEt and l-Trp derivatives of the monascus pigment, which has been reported to be a strong inhibitor of lipase,22,23) showed considerable anti-obesity effects on mice. Although the derivatives were less inhibitory than the commercial drug, Orlistat, they can be developed into a functional food ingredient because of the advantage of being a GRAS compound.

Supplementing the monascus derivatives to a high-fat diet lowered both the food intake rate of the mice and their weight gain (Table 1). The l-Trp derivative appeared to be more effective for weight control than the l-Leu-OEt derivative. However, the opposite would have been expected, because the l-Leu-OEt derivative was better than the l-Trp derivative in our previous *in vitro* tests of the lipase-inhibitory activity.22 This was probably due to the l-Leu-OEt derivative being easily hydrolyzed in mice, leading to the reduction of lipase-inhibitory activity. On the other hand, it is known that white adipose tissues store a large amount of triglycerides during an energy-excess period. The reduction in intra-peritoneal adipose tissue (IPAT) weight of the mice (Fig. 1) induced by feeding the monascus derivatives is considered to have been partially responsible for the decreased body weight, probably by inhibiting fat absorption leading to a decrease in fat storage.
The serum total cholesterol and triglyceride levels of the mice were lowered by feeding either the L-Trp derivative or the L-Leu-OEt derivative (Fig. 2). There was no significant difference between the effect of the two derivatives. However, the L-Trp derivative considerably increased the HDL cholesterol level, whereas the L-Leu-OEt derivative did not (Fig. 3). The HTR value was substantially elevated by the L-Trp derivative. On the other hand, the liver weight of the mice was not significantly affected by feeding either the L-Trp derivative or the L-Leu-OEt derivative (data not shown), whereas the liver cholesterol and triglyceride levels were significantly reduced (Fig. 4). While the L-Trp derivative was more effective than the L-Leu-OEt derivative for reducing the total cholesterol level in the liver, the L-Leu-OEt derivative was a little better than the L-Trp derivative for reducing the triglyceride level. This apparently reflects a difference in the metabolism of excess energy in mice depending on the derivative type. The effect of the L-Trp derivative on reducing the cholesterol level was greater in the liver than in the serum, possibly due to higher inhibition against cholesterol synthesis in the liver than against cholesterol absorption in the serum.

The fact that both derivatives decreased the total cholesterol level, but increased the HDL-cholesterol level is apparently related to their inhibitory actions on both HMG-CoA reductase and lipoprotein lipase. Derivative inhibition of HMG-CoA reductase (Fig. 5), which is a rate-limiting step in cholesterol biosynthesis, can lead to a reduction in the plasma total cholesterol and LDL cholesterol levels. On the other hand, lipoprotein lipase is a key enzyme in lipoprotein metabolism that catalyzes the hydrolysis of core triglycerides in both triglyceride-rich lipoproteins and very-low-density lipoproteins (VLDL), resulting in the generation of free fatty acids. Free cholesterol, phospholipids, and apolipoproteins are produced by the hydrolysis of triglyceride-rich lipoproteins. These molecules provide key substrates for the maturation of HDL particles. The positive correlation between the lipoprotein lipase activity and corresponding HDL cholesterol concentration shown in our study has been observed in many different normal and dyslipidemic human populations.

According to our previous report, the L-Leu-OEt derivative was more effective than the L-Trp derivative at inhibiting in vitro lipase reactions. However, in vivo tests with mice revealed the opposite pattern. This effect was probably caused by a difference in the chemical stability of the L-Leu-OEt and L-Trp derivatives in the mouse body. In general, the ester moiety of the L-Leu-OEt derivative is unstable under highly acidic conditions.
conditions. We observed that the L-Leu-OEt derivative was considerably degraded in a 2 N HCl solution (data not shown), indicating that the derivative can be degraded during passage through the stomach.

In conclusion, the L-Leu-OEt and L-Trp derivatives of the monascus pigment were effective for reducing the body weight gain of mice and for lowering the cholesterol and triglyceride contents in the mice serum and liver. In particular, the HDL level and HTR value were greatly increased with use of the L-Trp derivative.

Acknowledgments

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References