Isolation of Plasma Cholesterol-Lowering Components from
Ningyotake (Polyporus confluens) Mushroom

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Summary The present study was undertaken to isolate component(s) which contributes to the hypocholesterolemic action of Ningyotake (Polyporus confluens) mushroom. The mushroom powder was extracted with 80% ethanol, and the extract and residue were fractionated into five fractions according to the solubility to solvents. When each fraction was added to a diet containing 1% cholesterol and 0.25% sodium cholate and fed to rats, the plasma cholesterol level was significantly decreased only by ethyl acetate-soluble fraction. Therefore, ethyl acetate-soluble fraction was further fractionated by silica gel column chromatography. Two major compounds, which comprised 45.0% and 28.5% of the ethyl acetate-soluble fraction, were obtained in a pure form by the chromatography, and the compounds were identified as grifolin (2-trans,trans-farnesyl-5-methylresorcinol) and neogrifolin (4-trans,trans-farnesyl-5-methylresorcinol), respectively. The addition of grifolin and neogrifolin to the high cholesterol diet was found to lower plasma cholesterol level significantly.

Key Words Ningyotake, Polyporus confluens, mushroom, plasma cholesterol, hypocholesterolemic effect

Certain species of mushroom have been shown to lower plasma cholesterol level in experimental animals (1–5). As a hypocholesterolemic factor of Shiitake (Lentinus edodes) mushroom, eritadenine (4-(6-aminopurine-9-yl)-4-deoxy-D-erythromanic acid) was found by several groups of investigators (6–9). Mushrooms also contain relatively high amounts of polysaccharides. It is well confirmed that certain kinds of soluble polysaccharides from various sources reduce plasma cholesterol when fed to experimental animals. Thus there exist two types of components in mushrooms which lower plasma cholesterol level, i.e., components of low molecular
weight and high molecular weight. Previously we reported that Ningyotake ( \textit{Polyporus confluens} ) mushroom, an edible one widely found in Japan, significantly decreased plasma cholesterol level in rats fed a high cholesterol diet (10). In this study, we tried to isolate the component(s) which contributes to the hypocholesterolemic action of the mushroom.

MATERIALS AND METHODS

\textit{Mushroom.} Ningyotake mushroom was obtained from collectors in Fuku-shima Prefecture. The mushroom was dried at 60–70°C and powdered with a juicer.

\textit{Animals and diet.} Male rats of the Wistar strain (Japan SLC, Hamamatsu) weighing about 110g were used as experimental animals. They were fed experimental diets \textit{ad libitum} for 14d (Exp. 1) or 10d (Exp. 2) in a temperature (24±1°C) and humidity (50–60%)-controlled room with a 12h cycle of light (0600–1800) and dark. The basal diet contained 25% casein, 20% sucrose, 10% lard, 2% corn oil, 5% salt mixture (11), 1% vitamin mixture (11), 0.2% choline chloride, 1% cholesterol, 0.25% sodium cholate and α-corn starch to make 100%. In the experiment 2, 2% cellulose powder was included in the diet at the expense of starch. The mineral and vitamin mixtures were purchased from Oriental Yeast Co., Tokyo. To the basal diet was added each test fraction or test compound obtained from Ningyotake at the expense of starch. At the end of the feeding period, animals were killed by decapitation between 1100 and 1200 following 11 to 12 h starvation.

\textit{Lipid analysis.} Plasma concentrations of total cholesterol, HDL-cholesterol, triglyceride and phospholipid were measured enzymatically using kits (Wako Pure Chemical Ind., Osaka). The difference between total cholesterol and HDL-cholesterol was assumed to be cholesterol associated with VLDL+LDL. Liver lipids were extracted by the method of Folch et al. (12), and the cholesterol and triglyceride in the extracts were measured by the methods of Zak (13) and Fletcher (14), respectively.

\textit{Fractionation of Ningyotake mushroom.} As shown in Fig. 1, 84g (dry weight basis) of the powdered mushroom was extracted with 80% ethanol at room temperature, and fractionated into five fractions (I–V). Each fraction was introduced into the diet so as to correspond to the addition of 6% Ningyotake powder.

\textit{Isolation of major compounds from fraction I.} Thirty grams of fraction I was dissolved in 100ml of \textit{n}-hexane–ethyl acetate (9:1, v/v), and applied to a column (8×60cm) packed with 1,200g of Wako gel C-200 (Wako Pure Chemical Ind., Osaka) equilibrated with \textit{n}-hexane–ethyl acetate (9:1, v/v). The constituents of the fraction I were eluted successively with 7 liters of \textit{n}-hexane–ethyl acetate (9:1, v/v), 8 liters of \textit{n}-hexane–ethyl acetate (8:2, v/v), 5 liters of \textit{n}-hexane–ethyl acetate (6:4, v/v), and 3 liters of methanol. Each fraction (50ml) was checked by thin-layer chromatography with silica gel 60 F\textsubscript{254} (Merck) using \textit{n}-hexane–ethyl acetate (9:1 or 8:2, v/v) as developing solvents. As a color-producing reagent, vanillin–sulfate–ethanol (1:80:20, w/v/v) was used.
Measurements of NMR, IR and mass spectra. Major compounds purified by silica gel (Wako gel C-200) column chromatography of the fraction I of Ningyotake mushroom were analyzed by proton nuclear magnetic resonance (1H-NMR), infrared absorption (IR) and mass spectrometry.

Statistical analysis. Data for animal experiments were analyzed by one-way analysis of variance, and differences between means were tested at p<0.01 using Duncan's multiple range test (15) when F value was significant at p<0.05.

RESULTS

1. Effects of each fraction of Ningyotake mushroom on plasma cholesterol (Exp. 1)

Table 1 shows growth, food intake, liver weight and liver lipid content in rats fed the basal diet or diets supplemented with each fraction of Ningyotake mushroom. Feeding of fraction IV slightly but significantly depressed the growth of animals as compared with control animals. Liver weight per 100 g body weight was significantly lower in rats fed the fraction IV. The liver cholesterol content was significantly decreased by fractions I and IV, and the liver triglyceride content was decreased by all the fractions.
Table 1. Body weight gain, food intake, liver weight and liver lipid content in rats fed high cholesterol diets supplemented with various fractions of Ningyotake mushroom (Exp. 1).

| Diet                  | Body wt. gain (g/14 d) | Food intake (g/14 d) | Liver wt. (% of body wt.) | Liver lipids (mg/g) |
|-----------------------|------------------------|----------------------|---------------------------|---------------------|
|                       |                        |                      |                           | CHOL                |
| 25% Casein (25C)      | 78 ± 2<sup>a,b</sup>   | 166 ± 3<sup>ab</sup> | 4.92 ± 0.08<sup>a</sup>  | 66.6 ± 2.1<sup>a</sup> 58.3 ± 2.0<sup>a</sup> |
| 25C + 0.80% fr. I     | 79 ± 2<sup>b</sup>     | 159 ± 4<sup>b</sup>  | 4.96 ± 0.10<sup>a</sup>  | 56.4 ± 1.3<sup>b</sup> 41.7 ± 2.2<sup>b</sup> |
| 25C + 1.21% fr. II    | 80 ± 3<sup>b</sup>     | 166 ± 4<sup>ab</sup> | 4.80 ± 0.09<sup>a</sup>  | 64.3 ± 2.2<sup>a</sup> 43.9 ± 1.6<sup>a</sup> |
| 25C + 0.61% fr. III   | 83 ± 1<sup>ab</sup>    | 170 ± 2<sup>ab</sup> | 4.88 ± 0.10<sup>b</sup>  | 65.0 ± 2.3<sup>a</sup> 46.6 ± 2.4<sup>b</sup> |
| 25C + 0.46% fr. IV    | 69 ± 2<sup>c</sup>     | 159 ± 3<sup>ab</sup> | 4.29 ± 0.09<sup>b</sup>  | 55.9 ± 1.8<sup>b</sup> 39.2 ± 3.6<sup>b</sup> |
| 25C + 2.89% fr. V     | 87 ± 2<sup>a</sup>     | 176 ± 3<sup>a</sup>  | 4.78 ± 0.10<sup>b</sup>  | 65.9 ± 1.8<sup>a</sup> 44.2 ± 2.2<sup>b</sup> |

<sup>1</sup>Values are mean ± SE for 10 (25C) or 6 rats; values in a column not sharing the same superscript letter are significantly different at p < 0.05. CHOL, cholesterol; TG, triglyceride.

Table 2. Plasma lipid concentrations in rats fed high cholesterol diets supplemented with various fractions of Ningyotake mushroom (Exp. 1).

| Diet                  | Plasma lipid concentration (mg/dl) |
|-----------------------|-----------------------------------|
|                       | Total CHOL | HDL-CHOL | (VLDL + LDL)-CHOL | TG | PL |
| 25% Casein (25C)      | 383 ± 42<sup>a,c</sup>           | 19 ± 2<sup>b</sup> | 363 ± 42<sup>a</sup> | 138 ± 12<sup>a</sup> | 210 ± 10<sup>a</sup> |
| 25C + 0.80% fr. I     | 180 ± 21<sup>b</sup>             | 27 ± 2<sup>b</sup> | 153 ± 22<sup>b</sup> | 148 ± 10<sup>a</sup> | 176 ± 11<sup>b</sup> |
| 25C + 1.21% fr. II    | 377 ± 46<sup>a</sup>             | 20 ± 3<sup>abc</sup> | 358 ± 46<sup>a</sup> | 130 ± 13<sup>ab</sup> | 207 ± 9<sup>ab</sup> |
| 25C + 0.61% fr. III   | 302 ± 31<sup>ab</sup>            | 23 ± 2<sup>ab</sup> | 278 ± 31<sup>ab</sup> | 129 ± 12<sup>ab</sup> | 189 ± 8<sup>abc</sup> |
| 25C + 0.46% fr. IV    | 275 ± 20<sup>ab</sup>            | 15 ± 2<sup>c</sup> | 261 ± 21<sup>ab</sup> | 98 ± 16<sup>b</sup> | 161 ± 6<sup>c</sup> |
| 25C + 2.89% fr. V     | 324 ± 22<sup>ab</sup>            | 24 ± 3<sup>ab</sup> | 300 ± 23<sup>ab</sup> | 118 ± 7<sup>ab</sup> | 190 ± 7<sup>abc</sup> |

<sup>1</sup>See footnote 1 in Table 1. PL, phospholipid.

Table 2 shows plasma lipid concentrations. The plasma total cholesterol level was significantly decreased only by fraction I. The feeding of fraction I increased HDL-cholesterol level and decreased (VLDL + LDL)-cholesterol level. The plasma triglyceride level was decreased by fraction IV, and the plasma phospholipid level was decreased by fractions I and IV.

2. Effects of major compounds isolated from fraction I on plasma cholesterol (Exp. 2)

Fraction I of Ningyotake mushroom was further fractionated by silica gel column chromatography since the plasma cholesterol-lowering effect of this fraction was the largest of all the fractions as shown in the experiment 1. Two major compounds (1 and 2) were purified by the chromatography as judged from thin-layer chromatography and 1H-NMR; many of minor compounds remained in

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impure form. As shown in Table 3, the major compounds comprised 73.5% of the fraction I. Hence, we decided to examine whether or not the major compounds have a plasma cholesterol-lowering effect. The compounds 1 and 2 were added to the basal diet at the levels of 0.56% and 0.30%, respectively, which corresponded to the addition of 10% Ningyotake powder.

Table 4 shows growth, food intake, liver weight, and liver and plasma lipid concentrations in rats fed the basal diet or diets supplemented with compounds 1 and 2. The compounds did not affect the growth and food consumption of animals. The relative liver weight was slightly increased and the liver cholesterol content was significantly decreased by the both compounds. The plasma total cholesterol level was significantly decreased by the both compounds, although the effect of 0.56% compound 1 was larger than that of 0.30% compound 2. The plasma HDL-cholesterol level was significantly increased by the both compounds, while the (VLDL+LDL)-cholesterol level was significantly decreased. The plasma tri-glyceride level was enhanced by the both compounds.

From the spectra of NMR, IR and mass, compounds 1 and 2 were identified as grifolin (2-trans, trans-farnesyl-5-methylresorcinol) (16,17) and neogrifolin (4-...
DISCUSSION

It is widely accepted that many of soluble dietary fibers have a plasma cholesterol-lowering effect. The fraction IV of Ningyotake mushroom is considered to contain soluble polysaccharides. In fact, this fraction was highly viscous when dissolved in water. However, feeding of the fraction IV did not lead to a significant reduction of plasma cholesterol, although it decreased plasma triglyceride and phospholipid levels. Likewise, the fraction V, which is considered to contain insoluble polysaccharides, had no significant effect on the plasma cholesterol level. Therefore, it appeared that the contribution of dietary fibers to the hypocholesterolemic effect of Ningyotake mushroom is relatively small.

Instead, the present study showed that lipophilic compounds with low molecular weight in the fraction I is predominantly responsible for the effect of Ningyotake mushroom. This is in contrast to the case of Shiitake mushroom in which a water-soluble compound, eritadenine, is responsible for the hypocholesterolemic effect of the mushroom. To our knowledge, there is no other report in which a hypocholesterolemic effect of edible mushroom was shown to be attributable to the presence of lipophilic compound. The present study demonstrated that two major compounds, grifolin and neogrifolin, of the fraction I were found to have a plasma cholesterol-lowering effect. But, the possibility that some of minor compounds also have a plasma cholesterol-lowering effect cannot be excluded.

Grifolin was first isolated as an antibiotic constituent of a mushroom, *Grifola confluens* (18). Thereafter, several reports have shown that grifolin and neogrifolin exist also in mushrooms such as *Albatrellus confluens* (19), *Polyporus dispansus* (Japanese name, Komoritake) (17), and *Polyporus confluens* (17), although *Albatrellus confluens* was presumed to be a synonym of *Polyporus confluens* (17). Further, it is also reported that farnesylphenols other than grifolin and neogrifolin exist in some mushrooms (17,18). Thus, it is expected that some species of mushroom other than Ningyotake also have a plasma cholesterol-lowering effect because of the
presence of farnesylphenols in those mushrooms.

Our previous study showed that the feeding of Ningyotake mushroom at a 5% level of diet could decrease plasma cholesterol level without development of fatty livers, in contrast to the case of Shiitake mushroom, in rats fed a high cholesterol diet. In agreement with this, grifolin and neogrigolin did not evoke fatty livers. The mechanism by which grifolin and neogrigolin decrease plasma cholesterol level is not known at present, although the feeding of Ningyotake mushroom was shown to enhance fecal excretion of steroid, particularly neutral steroids (10). Grifolin and neogrigolin significantly decreased the content of liver cholesterol, but the extent of reduction seems to be smaller than that of reduction of plasma cholesterol, suggesting a possibility that there exists some mechanism(s) other than enhancement of fecal steroid excretion for the hypocholesterolemic effect of grifolin and neogrigolin. Further studies on the structure-activity relationship and on the mechanism of the plasma cholesterol-lowering effect of farnesylphenols are now in progress.

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