Preventive Effects of 80% Ethanol Extracts of the Edible Mushroom *Hypsizigus marmoreus* on Mouse Type IV Allergy

Kyoji Yoshino,*,[a] Youko Kondou,[b] Kinuko Ishiyama,[b] Tetsuro Ikekawa,[c] Tsunetomo Matsuzawa,[c] and Mitsuaki Sano[b]

[a]*Department of Chemistry and Biochemistry, Numazu College of Technology, 3600 Ooka, Numazu, Shizuoka 410–8501, Japan,* [b]*Department of Food Science and Nutrition, Nagoya Women's University, 3–40 Sioji-cho, Mizuho-ku, Nagoya 467–8610, Japan, and* [c]*Agricultural Technology Institute of Nagano Farmers Federation, 787–1 Suzaka, Suzaka, Nagano 382–0084, Japan*

(Received September 4, 2007; Accepted November 8, 2007; Published online November 16, 2007)

We investigated the active components responsible for the anti-allergic effect of *Hypsizigus marmoreus* on an oxazolone-induced type IV allergy in male ICR mice. Following both an oral administration at a dose of 250 mg/kg body weight and percutaneous administration at 0.10 mg/ear, significant anti-allergic activities were found in the 80% ethanol-soluble fraction (Fr. 1), and the low molecular weight fraction (Fr. 2, below a molecular weight of *ca*. 12000; Fr. 4, below *ca*. 3000) prepared from Fr. 1. These fractions included ergosterol, mannitol, and trehalose as antioxidants and/or anti-inflammatory components. The anti-allergic activities of these compounds and methionine were equal to or weaker than those of the fractions. Therefore, the molecular weights of the major active components in *Hypsizigus marmoreus* are thought to be less than *ca*. 3000, though ergosterol, mannitol, trehalose, and methionine would not be the main active components.

Key words —— mushroom, *Hypsizigus marmoreus*, anti-allergic effect, mouse, type IV allergy

INTRODUCTION

The increasing prevalence of allergic diseases in the world is worrying. Type IV allergy, one of the major types, is known as delayed type hypersensitivity and occurs via a cell-mediated immune reaction. We previously reported that a 99.5% ethanol extract and a boiling water extract of *Hypsizigus marmoreus* (Peck) Bigel. (bunashimeji), an edible mushroom, inhibited type IV allergy in mice when administered orally and percutaneously.[1)] The boiling water extract exhibited significant anti-allergic activity, though it was less active than the 99.5% ethanol extract. Here, we investigated some of the active components in an 80% ethanol extract of *Hypsizigus marmoreus* for preventive effects on oxazolone-induced type-IV allergy in mice.

MATERIALS AND METHODS

Preparation of Some Fractions from *Hypsizigus marmoreus* —— Fractionation of *Hypsizigus marmoreus* was carried out by the methods shown in Fig. 1. First, a 80% ethanol extract of the fruiting bodies of *Hypsizigus marmoreus* supplied by the Japan Agricultural Cooperatives Zennou Nagano (Nagano, Japan) was prepared. One hundred grams of *Hypsizigus marmoreus* was crushed and extracted under reflux with 700 ml of 80% ethanol at 40°C for 1 hr. The extract was filtered and the filtrate was lyophilized as fraction 1 (Fr. 1). Then, 10 g of Fr. 1 was suspended in 50 ml of water and dialyzed against 2000 ml of water for 24 hr with a cellulose tube (exclusion molecular weight *ca*. 12000, Wako Pure Chem. Ind., Osaka, Japan). The dialysate and non-dialysate were lyophilized as fractions 2 (Fr. 2) and 3 (Fr. 3), respectively. Fr. 2 was ultrafiltrated with a Centriplus YM-3 (exclusion molecular weight *ca*. 3000, Millipore Co., Bedford, MA, U.S.A.). The filtrate and non-filtrate were lyophilized as fractions 4 (Fr. 4) and 5 (Fr. 5). It should be cautious of the molecular weights of substances presumed by dialysis and extra filtration being near values.

Determination of Mouse Type IV Allergic Response —— Oxazolone-induced edema of the ear was used to evaluate the inhibitory effects of the fractions prepared from *Hypsizigus marmoreus* on type IV allergy in mice. Oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one), a sensitizer for type IV allergies, was purchased from Wako Pure Chem. Ind. Male ICR mice, 4 weeks old, were purchased from Japan SLC, Inc., Shizuoka, Japan. The experiment was carried out as follows.[2, 3)] The hair of the abdominal region was carefully cut away, and 0.1 ml of a 0.5% oxazolone

*To whom correspondence should be addressed: Department of Chemistry and Biochemistry, Numazu College of Technology, 3600 Ooka, Numazu, Shizuoka 410–8501, Japan. Tel. & Fax: +81-55-926-5861; E-mail: k-yoshino@numazu-ct.ac.jp

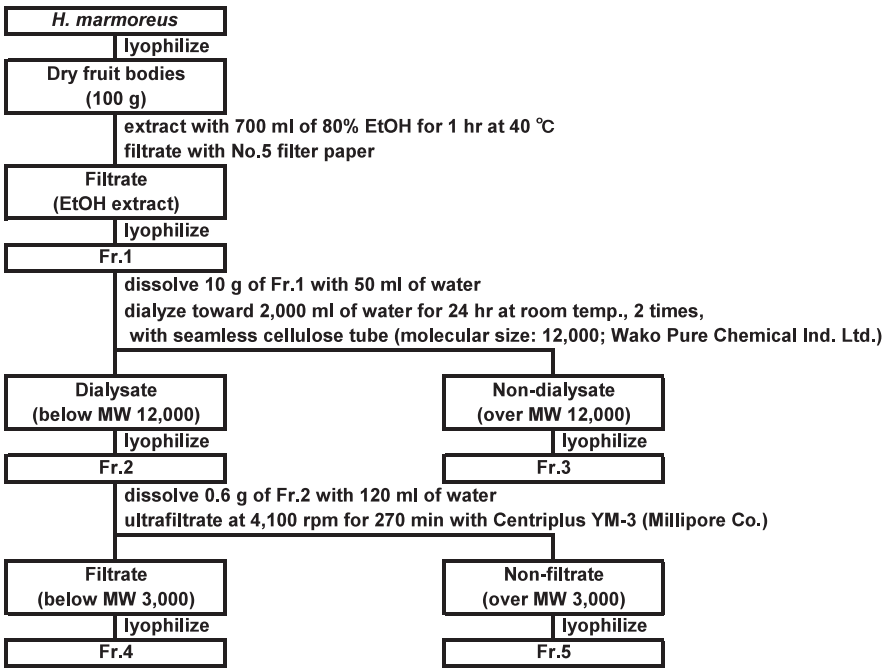

Fig. 1. Preparation of Fractions from *Hypsizigus marmoreus*

solution in ethanol was applied to the skin (sensitization). Five days after the sensitization, 20 μl of a 0.5% oxazolone solution in acetone was applied to both sides of each animal's right ear (challenge). Twenty-four hours after the challenge, the mice were killed under ether anesthesia, and circular samples (5.0 mm in diameter) of both ears were removed using a punching apparatus. The weights of the right (WR) and left (WL) ears were measured. In the case of oral administration, some fractions of *Hypsizigus marmoreus*, ergosterol (Wako Pure Chem. Ind.), D(−)-mannitol (Wako Pure Chem. Ind.), D(+)-trehalose (Wako Pure Chem. Ind.), and L(−)-methionine (Ajinomoto-Takara Co., Tokyo, Japan) were administered to mice 1 hr before the challenge at doses of 200 and/or 250 mg/kg body weight once a day for 3 days. In the case of percutaneous administration, the samples were added to the challenge solution at doses of 0.10 and/or 0.20 mg/ear and applied to the ear. Hydrocortisone, a well-known steroid-type anti-inflammatory agent, was assayed in the same manner. Control mice were not administered any agents. The number of mice in each group was 5. The anti-allergic activity was evaluated by comparing ear swelling ratios between the treated and control groups. The ear swelling ratio was calculated as follows:

$$\text{Ear swelling ratio (\%)} = \frac{\{(\text{WR sample} - \text{WL sample})/\text{WL sample}\}}{\{(\text{WR control} - \text{WL control})/\text{WL control}\}} \times 100.$$

Throughout the experiment, the animals were handled in accordance with "*The Guide for the Animal Experiments in Numazu National College of Technology*."

Analysis of Ergosterol by High Performance Liquid Chromatography Method —— The ergosterol contents of Fr. 1, 2, and 4 of *Hypsizigus marmoreus* were determined by using high performance liquid chromatography (HPLC, model LC-10AD, Shimadzu Co., Kyoto, Japan). The following analytical conditions were used: column, Wakosil 5C18 (5 μm, 150 × ϕ4.6 mm, Wako Pure Chem. Ind.); column temperature, room temperature; elution solvent, 90% ethanol; flow rate, 1.0 ml/min; detection, UV detector (280 nm, model SPD-10A, Shimadzu Co.). Identification of the compound was due to the comparison of the retention times of the samples and standard.

Analysis of Mannitol and Trehalose by HPLC Method —— The amounts of mannitol and trehalose in Fr. 1, 2, and 4 of *Hypsizigus marmoreus* were determined by HPLC (model LC-10AD, Shimadzu Co.). The analytical conditions were: column, Wakobeads T-100-S (150 × ϕ6.0 mm, Wako pure Chem. Ind.); column temperature, 80°C; elution solvent, 80% acetonitrile; flow rate, 1.0 ml/min; detection: RID (model RID-6A, Shimadzu Co.). The compounds were identified by comparing the retention times of the samples and standards.

Statistical Analysis —— The statistical analysis in this study was conducted with the nonparametric Mann-Whitney U test for differences between the groups, and $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Yield and Anti-allergic Activities of Some Fractions of *Hypsizigus marmoreus*

All fractions prepared from *Hypsizigus marmoreus* were brown powders. The yields of Fr. 1–5, which were expressed as a percentage of the dry weight of *Hypsizigus marmoreus*, were 25.0, 21.7, 3.3, 11.5, and 10.2%, respectively. Therefore, 86.8% of the components in Fr. 1 had a molecular weight smaller than *ca*. 12000 and 46.0%, smaller than *ca*. 3000.

Anti-allergic activities of orally or percutaneously administered Fr. 1–5 of *Hypsizigus marmoreus* are shown in Fig. 2. The doses used were 250 mg/kg body weight for the oral route and 0.10 mg/ear for the percutaneous route. In both cases, Fr. 1 had a significant anti-allergic effect on type IV allergy. The rate of inhibition was 56.3% for the oral administration and 66.3% for the percutaneous administration. The anti-allergic activities of Fr. 1 were almost the same as those of the 99.5% ethanol extract of *Hypsizigus marmoreus*.[1] Though the anti-allergic activity of Fr. 1 was weaker than that of hydrocortisone following the oral administration, it was the same as that of hydrocortisone after the percutaneous administration. Fr. 1 was further fractionated into Fr. 2–5 on the basis of the molecular weights of its components. As shown in Fig. 2, significant levels of anti-allergic activity were found only in the lower molecular weight fractions, Fr. 2 and 4, on oral and percutaneous administration. These fractions inhibited type IV allergy 52.1 and 45.0% via the oral route and 36.4 and 41.9% via the percutaneous route. These results suggest the molecular weights of the major active components in Fr. 1 of *Hypsizigus marmoreus* to be less than *ca*. 3000.

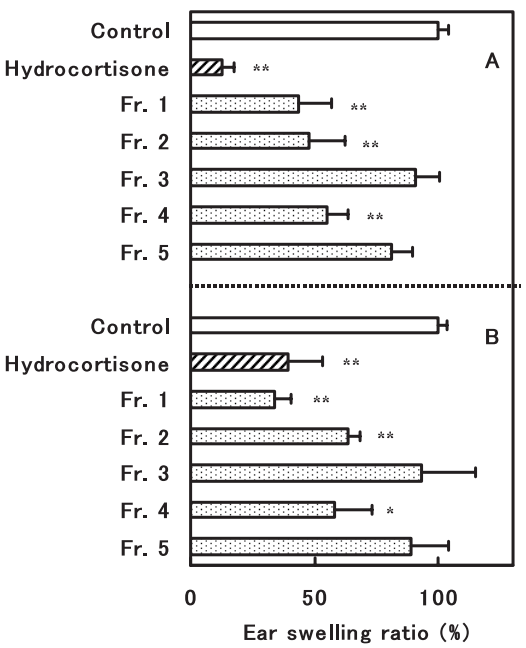

Fig. 2. Effects of the Oral and Percutaneous Administration of Fr. 1–5 Prepared from *Hypsizigus marmoreus* on Type IV Allergy in 5-week-old Male ICR Mice

Values are means ± S.E.M. $N = 5$. (A) Oral administration at a dose of 250 mg/kg body weight. (B) Percutaneous administration at a dose of 0.10 mg/ear. Significant differences from control value; $^{*}p < 0.05$, $^{**}p < 0.01$.

Components in Fr. 1, 2, and 4 of *Hypsizigus marmoreus*

A type IV allergic response is often accompanied by severe inflammation, an important process in the development of type IV allergies. Active oxygen species, which are produced in activated macrophages and during the enzymatic oxidation of arachidonic acid for the formation of chemical mediators such as leukotrienes and prostaglandins, play an important role in the inflammatory process. It was reported that some antioxidants, tea catechins[4] and superoxide dismutase,[5] could inhibit inflammation. Tea catechins also exhibited anti-allergic effects on type IV allergy in mice.[6] In our other reports, feeding of a diet containing 5–10% dried powder of extracts of *Hypsizigus marmoreus* increased the antioxidative activity in mouse plasma.[7,8] Among the components of mushrooms, ergosterol and its peroxides,[9] mannitol,[10] trehalose,[11] and methionine[12] are known to exhibit antioxidative and/or anti-inflammatory activities. It was also reported that *Hypsizigus marmoreus* contains these compounds. The amount of ergosterol in dry whole fruiting bodies of *Hypsizigus marmoreus* was 0.43%.[13] The amount of mannitol and trehalose was 1.3 and 2.1%,[14] respectively, and that of free methionine was 0.046%.[15] The molecular weights of ergosterol, mannitol, trehalose, and methionine were less than 3000, being 396.7, 149.2, 342.3, and 182.2, respectively.

Ergosterol, mannitol, and trehalose made up 0.51 ± 0.06, 11.2 ± 0.5, and 26.9 ± 1.8% of Fr. 1,

respectively. We compared the difference between the yields and the contents of some components in Fr. 1, which was 80% ethanol extract, and the 99.5% ethanol extract, which was reported in our previous paper.[1] The yield of the 99.5% ethanol extract was 20.2%. The contents of ergosterol, mannitol, and trehalose were 0.79 ± 0.01, 10.8 ± 0.6, and 24.8 ± 0.5% in the 99.5% ethanol extract. The contents of total sugar (measured as glucose by anthrone-sulfuric acid method) were 38.1 ± 4.9 in Fr. 1 and 43.5 ± 1.4 in the 99.5% ethanol extract. As described above, the anti-allergic activities of Fr. 1 and the 99.5% ethanol extract were also well alike. From these results, we thought the components in Fr. 1 and the 99.5% ethanol extract were much the same. We didn't analyze the methionine content of any fraction of *Hypsizigus marmoreus* in this study, because it was thought to be extremely low as compared to the amounts of ergosterol, mannitol, and trehalose. Mannitol and trehalose made up 18.8 ± 1.4 and 24.0 ± 0.8% of Fr. 2, and 22.8 ± 1.4 and 26.8 ± 1.2% of Fr. 4, respectively. Ergosterol was not detected in either fraction. These results suggest that the fractionizing process decreased the ergosterol content of the fractions of *Hypsizigus marmoreus*, increased the mannitol content, and had no effect on the trehalose content.

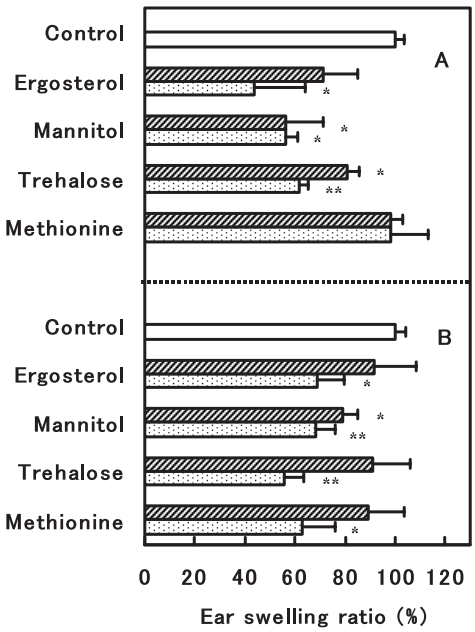

Fig. 3. Effects of the Oral and Percutaneous Administration of Ergosterol, D-Mannitol, D-Trehalose, and L-Methionine on Type IV Allergy in 5-week-old Male ICR Mice

Values are means ± S.E.M. $N = 5$. (A) Oral administration at a dose of ▨ 200 mg/kg body weight and ⬚ 250 mg/kg body weight. (B) Percutaneous administration at a dose of ▨ 0.10 mg/ear and ⬚ 0.20 mg/ear. Significant differences from control value; $^{*}p < 0.05$, $^{**}p < 0.01$.

Anti-allergic Activities of Ergosterol, Mannitol, Trehalose, and Methionine

We examined the anti-allergic effects of these compounds on type IV allergy in mice. The anti-allergic activities of ergosterol, mannitol, methionine, and trehalose administered orally at doses of 200 and 250 mg/kg body weight and percutaneously at 0.10 and 0.20 mg/ear are shown in Fig. 3. Via the oral route, mannitol and trehalose had significant effects at both doses, though ergosterol was effective only at 250 mg/kg. Methionine showed no significant anti-allergic activity at any dose. Via the percutaneous route, mannitol had significant anti-allergic effects at both doses, though ergosterol, methionine, and trehalose exhibited significant activity only at 0.20 mg/ear. These results suggest that the oral and percutaneous administration of these compounds was effective against type IV allergy in mice. However, the anti-allergic activities of all these compounds were equal to or weaker than those of Fr. 1, 2, and 4 of *Hypsizigus marmoreus* at a dose of 250 mg/kg body weight for oral administration and 0.10 mg/ear for percutaneous administration. We examined the effect of the percutaneous administration of a mixture of these components at doses equal to those determined in 0.10 mg of Fr. 1/ear. No significant anti-allergic effect was obtained: the ear swelling ratio was 108 ± 2.02%. The anti-allergic activities of ergosterol, mannitol, trehalose, and methionine would insensibly contribute to those of Fr. 1, 2, and 4 of *Hypsizigus marmoreus*. Except *Hypsizigus marmoreus*, some components of low molecular weight in various mushrooms were reported as anti-inflammatory and/or antioxidative compounds. The analogues of grifolic acid in *Albatrellus dispansus* (Lloyd) Canf. et Gilbm. (koumoritake) and the diterpenes such as sarcodonin A and neosarcodonin A in *Sarcodon scabrosus* (kerouji) were anti-inflammatory compounds.[16, 17] Cycloleucomelone-leucodi-hexaacetate in *Boletopsis leucomelas* (kurokawa), and grifolin and the analogues of grifolic acid in *Albatrellus dispansus* (Lloyd) Canf. et Gilbm. and *Albatrellus yasudai* (numeriaitake) were the inhibitors of 5-lipoxygenase, an enzyme producing leucotrienes.[16, 18] Neogrifolin derivatives in *Albatrellus ovinus* (ningyoutakemodoki) were antioxidative compounds.[19] All of the molecular weights of these compounds are less than 1000. Though the total

amount of ergosterol, mannitol, and trehalose was approximately 39, 43, and 50% in Fr. 1, 2, and 4 of *Hypsizigus marmoreus*, a small amount of other active components could exist in Fr. 1. On the other hand, the fractionizing process did not increase the anti-allergic activities of the fractions of *Hypsizigus marmoreus* as shown in Fig. 2. A combination of the effects of some components in Fr. 1 can be anticipated. Further study is needed to identify the other anti-allergic components in the 80% ethanol extract of *Hypsizigus marmoreus*.

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