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Antimutagenic effects of subfractions of Chaga mushroom (*Inonotus obliquus*) extract

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ABSTRACT

Inonotus obliquus is a mushroom commonly known as Chaga that is widely used in folk medicine in Siberia, North America, and North Europe. Here, we evaluated the antimutagenic and antioxidant capacities of subfractions of *Inonotus obliquus* extract. The ethyl acetate extract was separated by vacuum chromatography into three fractions, and the fraction bearing the highest antimutagenic activity was subsequently separated into four fractions by reversed phase (ODS-C_{18}) column chromatography. The most antimutagenic fraction was then separated into two subfractions (subfractions 1 and 2) by normal phase silica gel column chromatography. Ames test analysis revealed that the subfractions were not mutagenic. At 50 μg/plate, subfractions 1 and 2 strongly inhibited the mutagenesis induced in *Salmonella typhimurium* strain TA100 by the directly acting mutagen MNNG (0.4 μg/plate) by 80.0% and 77.3%, respectively. They also inhibited 0.15 μg/plate 4NQO-induced mutagenesis in TA98 and TA100 by 52.6–62.0%. The mutagenesis in TA98 induced by the indirectly acting mutagens Trp-P-1 (0.15 μg/plate) and B(α)P (10 μg/plate) was reduced by 47.0–68.2% by the subfractions, while the mutagenesis in TA100 by Trp-P-1 and B(α)P was reduced by 70.5–87.2%. Subfraction 1 was more inhibitory than subfraction 2 with regard to the mutagenic effects of 4NQO, Trp-P-1, and B(α)P. Subfractions 1 and 2 also had a strong antioxidant activity against DPPH radicals and were identified by MS, ^{1}H NMR and ^{13}C NMR analyses as 3β-hydroxy-lanosta-8, 24-dien-21-al and inotodiol, respectively. Thus, we show that the 3β-hydroxy-lanosta-8, 24-dien-21-al and inotodiol components of *Inonotus obliquus* bear antimutagenic and antioxidative activities.

1. Introduction

Recent studies have revealed the presence of natural bioactive materials in many different plant species worldwide and the antimutagenicity of these materials is currently under active investigation. In particular, the extracts of various mushrooms have been found to have anti-inflammatory, anti-tumor and anti-mutagenic properties [1,2].

The Chaga mushroom (*Inonotus obliquus*) is a white rot fungus that belongs to the Hymenochaetaceae family of Basidomycetes. Chaga mushrooms have been used in folk medicine for treating cancer in Russia, Western Siberia, Asia, and North America [3]. It has been shown that Chaga mushrooms contain many polyphenolic compounds and show various biological activities, including anti-bacterial [4], hepato-protective [5] and anti-tumor [6–8] properties.

In a previous study, we reported that the ethanol extract, ethyl acetate fraction, water fraction, and water-soluble and insoluble polysaccharides I and II of *Inonotus obliquus* have strong antimutagenic and antioxidative effects and inhibit genotoxicity [2,9]. We used crude fractions in our previous experiment [2]. We think that the crude fraction may have several bioactive compounds. We suggested that further studies are needed to determine whether the individual bioactive compounds in the ethyl acetate fraction could have the antimutagenic effects and further investigations characterizing the chemical structure of the individual bioactive components in the ethyl acetate fraction are needed.

Here, in an effort to identify the antimutagenic compounds in Chaga mushrooms, we subfractionated the ethyl acetate fraction of

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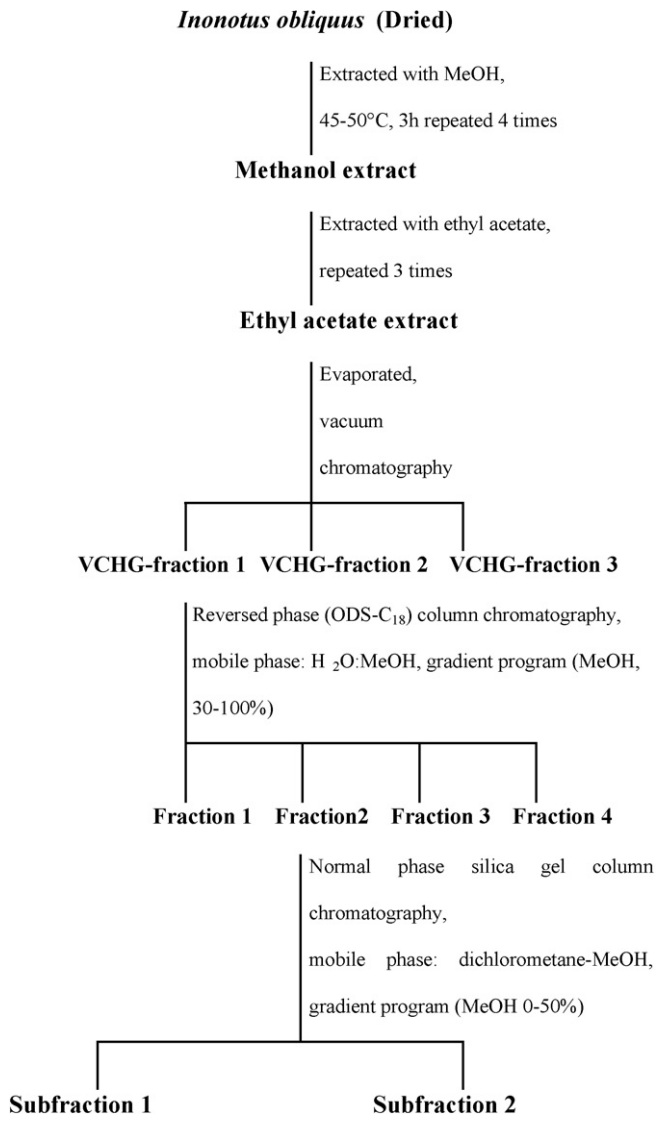

Fig. 1. Preparation of *Inonotus obliquus* subfractions.

these mushrooms by sequential vacuum chromatography, reversed phase (ODS-C_{18}) column chromatography, and normal phase silica gel column chromatography (Fig. 1). We used the subfractions for this study and the purity was confirmed by thin-layer chromatography (TLC), mass spectrometry (MS), ^{1}H nuclear magnetic resonance (^{1}H NMR) and ^{13}C nuclear magnetic resonance (^{13}C NMR) analyses (data not shown). The compounds 1 and 2 (subfractions 1 and 2) had a purity of over 99.5%. We tested the subfractions 1 and 2 each containing only a single compound for *in vitro* antioxidative and antimutagenic activities. The chemical structures of the compounds in the final subfractions bearing the antimutagenic property were characterized by MS, ^{1}H NMR and ^{13}C NMR analyses.

2. Materials and methods

2.1. Materials

D-biotin, 4-nitroquinoline-1-oxide (4NQO), N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), β-NADP, glucose-6-phosphate (G-6-P), gentamycin sulfate, benzo(α)pyrene [B(α)P], 3-amino-1,4-dimethyl-5H-pyrido-(4,3-b)indol (Trp-P-1), and L-histidine were purchased from Hwa-Kwang Chemical (Japan). Nutrient broth was purchased from Difco Laboratory (USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid, and α-tocophenol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The rat liver enzyme S-9 (1254 Aroclor induced in 0.154 M KCl) was purchased from Molecular Toxicology Inc. (Boone, NC28607, USA).

2.2. Extraction of inonotus obliquus and isolation of its active antimutagenic compounds

The *Inonotus obliquus* specimen used in this experiment was imported from Russia, dried, powdered, and stored at −20 °C. The powdered specimen (500 g) was extracted twice with 3 L of methanol (99.8%) at 45–50 °C for 3 h. The methanol fraction (45 g) was then rotary evaporated, emulsified, dissolved in water, and extracted three times with ethyl acetate (H_2O:EtOAc, 5:7, v/v). The ethyl acetate fraction (12 g) was obtained after rotary evaporation and the dried extract was reconstituted in methanol. This stock solution was subjected to vacuum chromatography (N–2N, Eyela, Tokyo, Japan) with silica gel (60 g, 230–400 mesh) mixed with dichloromethane. The mobile phases used were A, dichloromethane and B, methanol, with a gradient of increasing methanol (0–100%) in dichloromethane and re-equilibration of the column with 100% A for 3 min prior to the next run (Fig. 1). The success of the fractionation was confirmed by TLC. Of the three ethyl acetate extract fractions, the most bioactive fraction, namely, VCHG-fraction 1, was selected and subjected to reversed phase (ODS-C_{18}) column chromatography using a reversed phase column (ODS-C_{18}) containing LiChroprep RP-18 (25–40 μm) mixed with H_2O:MeOH (1:30, v/v) in a gradient program ranging from 30% methanol to 100% methanol (mobile phase A: methanol; mobile phase B: ultra pure water). Four fractions were isolated, of which fraction 2 contained the most bioactivity. Fraction 2 was further fractionated by chromatography with a normal phase silica gel column (2.4 cm × 15 cm) containing silica gel (200 g, 70–230 mesh; Merck Co.) mixed with dichloromethane eluted by a mobile phase gradient ranging from 100% dichloromethane to 50% methanol. The flow rate was 1.0 mL/min (Fig. 1). This resulted into two subfractions designated as subfractions 1 and 2, both of which showed bioactivity. The compounds in subfractions 1 and 2 were crystallized and analyzed by determining their MS (JEOL), ^{1}H NMR and ^{13}C NMR (Bruker DRX 500 MHz) spectra.

2.3. Confirmation for the ames test

The LT-2 TA98 and TA100 histidine-demanding auxotrophs of *Salmonella typhimurium* were obtained from B.N. Ames, California (USA). The histidine requirement, presence of the rfa and uvrB mutations, and R-factor genetics of these strains were determined according to the method of Maron and Ames [10].

2.4. Metabolic activation system

The rat liver enzyme S-9 was prepared at +4 °C immediately before use and maintained at this temperature until it was added to the overlay agar. The final composition of the S-9 mixture was as follows: $MgCl_2$–KCl salt solution (1.65 M KCl + 0.4 M $MgCl_2$) (0.2 mL), 0.2 M sodium phosphate buffer pH 7.4 (5 mL), 0.1 M nicotinamide adenine dinucleotide (0.4 mL), 1 M glucose-6-phosphate (G-6-P) (0.05 mL), S-9 fraction (1 mL), sterile distilled water (3.35 mL).

2.5. Mutagenicity assay

The mutagenicity of the *Inonotus obliquus* extract subfractions was assayed according to the method of Ames using *Salmonella typhimurium* strains TA98 and TA100 [10]. A solution of 0.5 mL of S-9 mixture, 0.1 mL of overnight-cultured *Salmonella typhimurium* TA98 or TA100, and 0.1 mL of the *Inonotus obliquus* specimen was prepared and preincubated at 37 °C for 20 min. Subsequently, 2 mL of molten top agar supplemented with L-histidine and D-biotin at 45 °C was added to the mixture. The mixture was then gently mixed and poured onto minimal glucose agar plates. The plates were inverted and incubated at 37 °C for 48 h. To test the mutagenicity of the subfractions from *Inonotus obliquus* extracts, each experiment was performed at least in duplicate. Colony counting occurred after 48 h and the inhibition ratio was calculated by using the following formula:

$$\text{Inhibition ratio}(\%) = \left[\frac{(A-C)}{(A-B)}\right] \times 100$$

where *A* is the number of histidine revertants induced by the mutagen alone, *B* is the number of revertants induced in the presence of sample solution and solvent (negative control), and *C* is the number of histidine revertants induced by the mutagen in the presence of the *Inonotus obliquus* sample.

2.6. Antimutagenicity assay

The antimutagenicity of the *Inonotus obliquus* subfractions was assayed by using the Ames test as described above except mutagen was added before the preincubation step. The mutagens used were the directly acting mutagens MNNG and 4NQO, and the indirectly acting mutagens Trp-P-1 and B(α)P, which require the S-9 mixture for metabolic activation. In the assay, 50 μL of the *Inonotus obliquus* subfractions at different concentrations were added to 50 μL of direct or indirect