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Research in the antioxidant of *Phellinus linteus* mycelia

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Phellinus linteus mycelia have many pharmacological effects, although their pharmacological efficacy principles have not been known yet. In the course of screening for biological activity of the extracts of *Phellinus linteus* mycelia, we found strong antioxidative activity in some fraction of water-insoluble. Therefore, we tried to isolate the active principle(s) from the extract. The isolation of the active compound was guided by superoxide anion radical scavenging activity. As a result, caffeic acid was isolated as an active compound. The IC_{50} of the compound was 3.05 μg/ml (16.9μM).

Key words : *Phellinus linteus*, electron spin resonance system, superoxide anion radical scavenging activity, caffeic acid

INTRODUCTION

Phellinus linteus is perennial fungus which is selectively parasitic on *Morus rubra*. This fungus is known as a Chinese medicine, Souou, and the mycelia of the fungus show antiallergy and antitumor activity [1]. However, the active principles of the mycelia have not been clarified yet. In the course of screening for biological activity of the extract of the fungus, we found strong antioxidative activity of the extract of the mycelia. We report here the isolation of the active compound from the fungus.

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MATERIALS AND METHODS

Chemicals. HPX was purchased from Sigma Chemical Co. (St. Louis, MO). XOD (1 U/mg, from cow milk)

was purchased from Boehringer Mannheim Corp. (Indianapolis, IN), and DMPO was from Labotec Co. (Tokyo, Japan). All other chemicals were the highest grade and used without purification.

P. linteus strain and culture conditions. The used strain of *P. linteus* was one of storage strains in IBI Co., LTD.. The components of the mycelium culture were as follows; glucose 4%, polypeptone 0.3%, yeast extract 0.3%, KH_2PO_4 0.05%, Na_2HPO_4 0.05% in distilled water. The culture medium was adjusted to pH 5.5 [2].

Superoxide anion radical scavenging activity measurements. Electron spin resonance spectra were recorded with a JES-FR30 ESR spectrometer (JOEL Co. Ltd., Tokyo, Japan). Instrument settings were as follows: magnetic field, 335.5 ± 5 mT; power, 4.0 mW; modulation frequency, 9.41 GHz; modulation width, 0.1 x 0.63 or 1 x 0.1 mT; sweep width, 0.5 mT; sweep time, 2 min; response time, 0.1 sec; and amplitude, 1 x 200. Electron spin resonance spectra were measured at 23 °C. The intensity of DMPO-OOH spin adducts that were generated from the reaction between DMPO and superoxide anion radical was expressed as a ratio of the signal intensity at the lowest magnetic field to that of Mn^{2+} in MnO used as an internal standard. A standard assay was performed using 130 μl of measuring sample in a quartz flat ESR cell. Fifty μl of 2 mM hypoxanthine (HPX), 50 μl of 0.4 units/ml xanthine oxidase (XOD), 30 μl of dimethyl sulfoxide (DMSO), 20 μl of 4.5 M 5,5-dimethyl-1-pyrroline N-oxide (DMPO), and 50 μl of the sample solution (5 mg/ml in DMSO), were added to the reaction cell, and the ESR signal of DMPO-OOH was measured [3].

Purification of caffeic acid. P. linteus mycelia were extracted with 100% ethanol, and the ethanol extract was separated by solvent partition between chloroform and water, and then ethyl acetate and water. The ethyl acetate soluble part was fractionated by using a silica gel flush column, and then a TOYOPEARL HW-40 (TOSOH Co.,Ltd.) column. The active fraction was purified by HPLC, giving compound **1** (caffeic acid).

RESULTS AND DISCUSSION

The exract of *P. linteus* was divided into chloroform-, ethyl acetate-, and water-soluble fractions. The radical scavenging activity of each fraction is shown in Fugure 1.

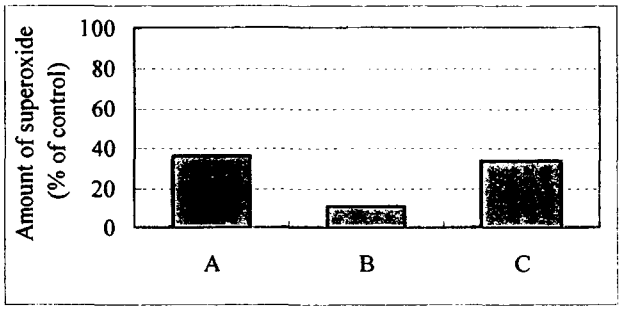

Figure 1. Superoxide anion radical scavenging activities of *P.linteus* mycelia extracts. A: chloroform, B: ethyl acetate, C: water.

Since the activity of the ethyl acetate fraction was the strongest among three fractions (11.5%, Figure 1), this fraction was separated by silica gel flush chromatography (Figure 2). The active fraction was further separated by column chromatography using Toyopearl HW40 and finally HPLC, giving compound **1** as yellow crystals. All the spectroscopic evidences of **1**

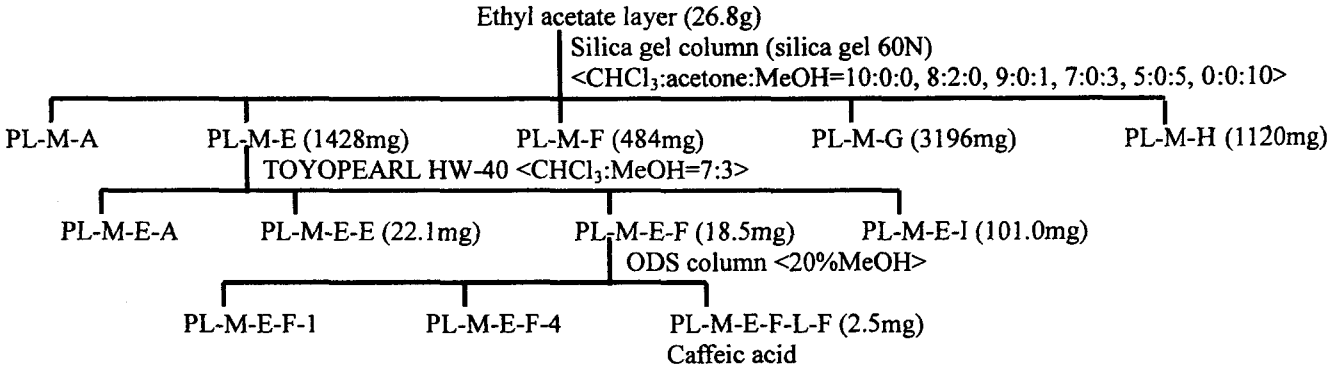

Figure 2. Chromatographic fractionation of *P. linteus* mycelium extracts

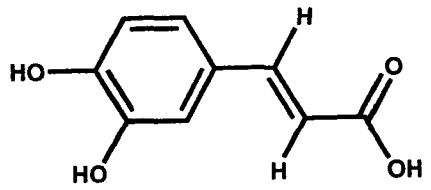

Figure 3. Structure of caffeic acid (**1**).

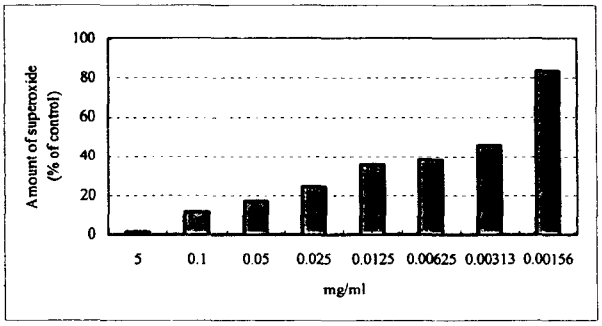

Figure 4. Superoxide anion radical scavenging activities of caffeic acid.

was identical with those of caffeic acid (Figure 3). The IC_{50} of superoxide anion radical scavenging activity of caffeic acid was 3.05 μg/ml (16.9 μM) (Figure 4).

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