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Note

Inhibition of HIV-1 Protease by Water-Soluble Lignin-Like Substance from an Edible Mushroom, *Fuscoporia obliqua*

Toshiaki ICHIMURA, Osamu WATANABE,* and Susumu MARUYAMA

National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, 1-1 Higashi, Tsukuba, Ibaraki 305, Japan
**Hokkaido Food Processing Research Center, Midorimachi, Bunkyodai, Ebetsu, Hokkaido 069, Japan*

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Activity that inhibited protease of human immunodeficiency virus type 1 was found in boiling water extracts of an edible mushroom, *Fuscoporia obliqua*. The active component was identified as a water-soluble lignin derivative of high molecular weight. Other polyphenols of low molecular weight and monomeric components of lignin did not inhibit the protease.

Key words: HIV-1 protease; lignin; mushroom

The human immunodeficiency virus type 1 (HIV-1) protease is a target of drug design for the treatment of acquired immnodeficiency syndrome.[1] The HIV-1 protease inhibitors, ABT-538, Ro 31-8959, and MK-639 are already in clinical use, but the emergence of resistant viral variants may limit their efficiency.[2] The anti-HIV effects of various crude drugs have been examined.[3] We are examining foodstuffs for an HIV-1 protease inhibitor. Edible mushrooms in particular seem likely to contain useful physiologically active agents. One edible mushroom, *Fuscoporia obliqua*, found in Europe, Siberia, and the northern part of Japan, is chill resistant. The sclerotium of this mushroom, called "charga", is used in the preparation of a beverage resembling tea at a region of Siberia. It has been reported that *F. obliqua* contains polysaccharides with antitumor activity.[4] Here, we describe isolation of a substance from *F. obliqua* that inhibits the activity of HIV-1 protease. Some characteristics of the substance were identified.

The sclerotium of *F. obliqua* harvested in Hokkaido was dried and ground to a powder. Fifty grams of the powder was extracted with 500 ml of boiling water for 10 min and filtered, and the extract was lyophilized. Then 60 mg of the lyophilized sample was dissolved in 6 ml of distilled water and added to 10 ml of Fractogel EMD 650 (Merck) equilibrated with distilled water. The gel was washed twice with distilled water and once with 1 M NaCl. A substance that inhibited HIV-1 protease activity was then extracted with 4.5 M NaCl. The solution was filtered with an ultrafiltration membrane (YM-10, Amicon Inc). The fraction of high molecular weight was put on a Superdex 200 column (26 × 600 mm, Pharmacia Biotech AB) equilibrated with 200 mM NaCl and eluted. Fractions with the most inhibitory activity were collected and used as the partially purified sample. Crude extracts of other edible mushrooms (listed in Table I) purchased in a market were also prepared by extraction with boiling water and were then lyophilized.

Inhibition of HIV-1 protease was measured as follows. An assay mixture containing 100 μl of buffer (100 mM sodium acetate, pH 4.9, 200 mM NaCl, 5 mM dithiothreitol, and 10% glycerol), 10 μl of substrate solution (His-Lys-Ala-Arg-Val-Leu-*p*-nitro-Phe-Glu-Ala-Nle-Ser-NH_2, 1 mg/ml in H_2O, Bachem Feinchemikalien AG), and 10 μl of a prepared sample was pre-incubated at 37°C for 5 min, and then 25 μl of a solution of recombinant HIV-1 protease (0.02 mg/ml, Bachem Feinchemikalien) was added. The tube containing the mixture was incubated at 37°C for 15 min and reaction was stopped by the addition of 10 μl of 10% trifluoroacetic acid. The reaction mixture was injected into a C_8 column for reverse-phase HPLC and eluted with a linear gradient of 5–65% acetonitrile containing 0.1% trifluoroacetic acid. The substrate and the cleaved substrate eluted from the column were detected at 300 nm by a UV monitor. Inhibition of trypsin,[5] pepsin,[6] papain,[7] carboxypeptidase A,[8] angiotensin I-converting enzyme,[9] and prolyl endopeptidase (from *Flavobacterium meningosepticum*)[10] was assayed under each standard condition with the appropriate synthetic peptide substrate (listed in Table II). The concentration of subfragments released from the substrates was also assayed by reverse-phase HPLC.

UV, FTIR, and NMR spectra were measured on a UV-260 spectrophotometer (Shimadzu Corp.), FTIR-

Table I. Effects of Extracts from Edible Mushrooms on HIV-1 Protease Activity

Extract origins	Enzyme activity (%)
Control	100
Fuscoporia obliqua	21
Pleurotus cornucopiae	93
Flammulina velutipes	114
Grifola frondosa	96
Agaricus bisporus	93
Lentinus edodes	98
Pleurotus ostreatus	97
Lyophyllum ulmarium	89
Pholiota nameko	136

Each assay mixture contained 70 μg of a lyophilized extract per milliliter. Distilled water was used instead of an extract solution for the control.

Abbreviations: *p*NA, *p*-nitroanilide; Z, benzyloxycarbonyl; Hip, *N*-benzoylglycine

Table II. Effects of the Extract of *F. obliqua* on Some Proteases

Enzyme	Substrate (final conc.)	Final conc. of extract (μg/ml)	Enzyme activity (%)
HIV-1 Protease	His-Lys-Ala-Arg-Val-Leu-*p*-nitro-Phe-Glu-Ala-Nle-Ser-NH_2 (0.05 mM)	2.5[a]	50
Trypsin	*N*-benzoyl-DL-Arg-*p*NA (0.5 mM)	100	92
Pepsin	Z-His-Phe-Phe-OEt (0.05 mM)	200	104
Papain	pGlu-Phe-Leu-*p*NA (0.5 mM)	200	101
Carboxypeptidase A	*p*-Hydroxybenzoyl-Ala-Phe (0.5 mM)	200	40
Angiotensin I-converting enzyme	Hip-His-Leu (5 mM)	100	105
Prolyl endopeptidase[b]	Z-Gly-Pro-*p*NA (0.4 mM)	1.4[a]	50

[a] IC_{50}; [b] from *F. meningosepticum*.

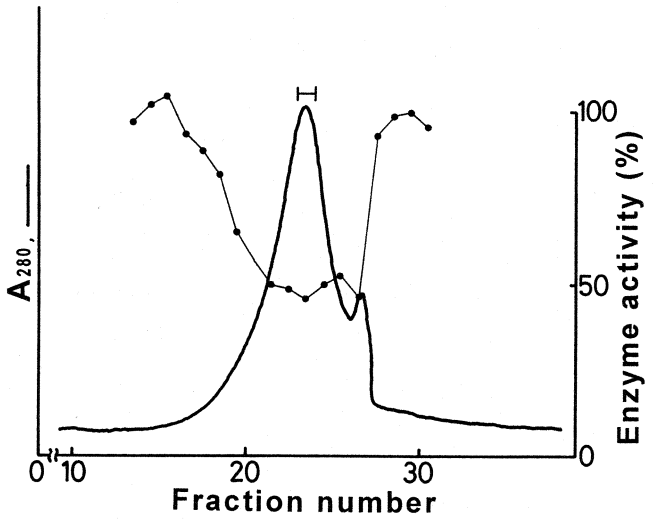

Fig. 1. Elution Pattern of a Substance(s) Inhibiting HIV-1 Protease by Gel Filtration Chromatography.

The thick line shows absorbance at 280 nm, and the thin line is the activity of HIV-1 protease. Each fraction was 12 ml. The fractions marked with a horizontal line were collected.

4200 infrared spectrophotometer (Shimadzu), and JNM-EX270 FT NMR system (JEOL).

Inhibition of HIV-1 protease by extracts from several edible mushrooms is summarised in Table I. Inhibitory effect was indicated by remaining enzyme activity that is the ratio of protease activities measured with and without inhibitor. The extract of *F. obliqua* had a strong inhibitory effect, unlike all of the other mushrooms tested. Inhibition of proteases by the extract is shown in Table II. The typical proteases, trypsin, pepsin, and papain were not inhibited, although prolyl endopeptidase was strongly and carboxypeptidase A was a little inhibited.

When the extract of *F. obliqua* was further purified as mentioned above, the elution profile during Superdex 200 gel filtration chromatography measured by UV absorption at 280 nm was as shown in Fig. 1. The intensity of inhibition rose in proportion with the absorbance. The fraction of the eluate that most strongly inhibited the protease (which was at the main peak of UV absorption, fraction No. 23) was collected, desalinated, and used as the partially purified sample. This fraction was examined by gel filtration chromatography on a TSK-gel G2000SWXL column (Tosoh) equilibrated with 200 mM NaCl and calibrated with protein molecular markers (Boehringer Mannheim GmbH). A symmetrical single peak broader than the peaks of the marker proteins was seen at the 30 kDa region on the chromatogram; the peak may not arise from a single substance.

Fraction 23 was examined spectroscopically for identification of its main component. The UV profile had one intense absorption peak at 208 nm accompanied by a shoulder near 280 nm, and endo-absorption was found throughout the UV range. An infrared spectrum obtained using KBr tablet method had several absorption peaks that we assigned to aromatic C-H and primary alcohol (1030 cm^{-1}), syringyl units (1126 cm^{-1}), guaiacyl rings (1225, 1270, and 1328 cm^{-1}), methyl C-H (1462 cm^{-1}), the aromatic skeleton (1422 and 1594 cm^{-1}) and so on. An infrared spectrum of lignin alkali (Aldrich Chemical Co.) was a very similar. After the solvent of the fraction was replaced with D_2O, an ^1H NMR spectrum at 270 MHz was taken with homogated decoupling pulses to eliminate the signal of H_2O. The shift values were expressed on the δ scale with reference to sodium trimethylsilyl propanesulfonate. The spectrum had several broad signals that probably originated from protons of polymer molecules. The most intense signal at about 4.3–3.5 was matched with the contribution of C-H protons adjacent to the oxygen, for instance, methoxy groups. Blunt signals at about 8–7 and 7–6 probably from aromatic-ring and aliphatic double-bond protons, respectively, were also observed. The overall profile of this spectrum showed characteristics of lignin. The results together suggested that fraction 23 contained mostly water-soluble lignin derivatives.

Compounds related to lignin and other polyphenols were examined for the ability to inhibit HIV-1 protease to confirm the chemical species of the active components in fraction 23 (Table III). Lignin alkali (Aldrich) as well as the extract from *F. obliqua* and the fraction 23 of the extract strongly inhibited HIV-1 protease activity. These results show that the inhibitory substance in the extract was lignin derivative(s). Coniferyl alcohol and sinapyl alcohol, which are constituent units of lignin, had no inhibitory activity, nor did the other polyphenols. Hence, the polymerized form of water-soluble lignin derivatives seemed to be needed for inhibition.

Water-soluble lignin inhibits the cytopathic effect of HIV.[11] One lignin derivative inhibits HIV reverse transcriptase.[12] In this study, it was concluded that the activi-

Table III. Effects of Polyphenols on HIV-1 Protease Activity

Polyphenol	Final conc. (μg/ml)	Enzyme activity (%)
Control	0	100
Extract of *F. obliqua*	2.5[a]	50
Fraction 23 of *F. obliqua* extract	1.4[a]	50
Lignin alkali (Aldrich)	4.0[a]	50
(±)-Catechin	70	103
(+)-Catechin	70	102
(−)-Epigallocatechin	70	86
(−)-Epigallocatechin gallate	210	83
(−)-Epicatechin	70	114
trans-Cinnamic acid	70	106
Cinnamyl alcohol	70	106
Coniferyl alcohol	70	109
Sinapyl alcohol	70	117

[a] IC_{50}.

ty of HIV-1 protease was inhibited by a lignin-like substance. Results of gel filtration of a mixture of the extract and the protease suggested adsorption of the lignin derivative on to the protease (data not shown), which mechanism may account for its effects.

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