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Research Note

Characterization of an Anti-gout Xanthine Oxidase Inhibitor from *Pleurotus ostreatus*

In-Taek Jang[1], Se-Hee Hyun[1], Ja-Won Shin[1], Yun-Hae Lee[2], Jeong-Hyun Ji[2] and Jong-Soo Lee[1,*]

[1]*Department of Biomedicinal Science and Biotechnology, Paichai University, Daejeon 302-735, Korea*
[2]*Mushroom Research Station, Gyeonggi-do Agricultural Research and Extension Services, Gwangju 464-873, Korea*

Abstract We selected *Pleurotus ostreatus* from among several edible mushrooms because it has high anti-gout xanthine oxidase (XOD) inhibitory activity. The maximal amount of XOD inhibitor was extracted when the *Pleurotus ostreatus* fruiting body was treated with distilled water at 40°C for 48 hr. The XOD inhibitor thus obtained was purified by Sephadex G-50 gel permeation chromatography, ultrafiltration, C_{18} solid phase extraction chromatography and reverse-phase high-performance liquid chromatography with 3% of solid yield, and its XOD inhibitory activity was 0.9 mg/mL of IC_{50}. The purified XOD inhibitor was a tripeptide with the amino acid sequence phenylalanine-cysteine-histidine and a molecular weight of 441.3 Da. The XOD inhibitor-containing ultrafiltrates from *Pleurotus ostreatus* demonstrated dose-dependent anti-gout effects in a Sprague-Dawley rat model of potassium oxonate-induced gout, as shown by decreased serum urated levels at doses of 500 and 1,000 mg/kg, although the effect was not as great as that achieved with the commercial anti-gout agent, allopurinol when administered at a dose of 50 mg/kg.

Keywords Anti-gout xanthine oxidase inhibitor, Edible oyster mushroom, *Pleurotus ostreatus*

Some mushrooms contain nutrients and minerals that can have health-stimulating properties and medicinal effects [1-3]. *Pleurotus ostreatus* is classified as part of the *Pleurotus* genus of the Pleurotus family. This mushroom is mainly found in Korea, Japan, and European countries. The medicinal effects of *P. ostreatus*, such as immunomodulatory, antitumor, antiviral, anti-inflammatory, antibiotic, and cholesterol-lowering effects, have long been known [4, 5].

Gout is a serious disease that has been growing in prevalence during the past years several years Western civilization [6]. The disease is characterized by abnormally high levels of uric acid in the body, resulting in the formation and deposition of urate crystals in the joints and kidneys [7]. Uric acid is the end product of purine metabolism in humans, and its overproduction by xanthine oxidase (XOD) from purine compounds or underexcretion can lead to hyperuricemia as gout.

XOD (EC 1.17.3.2) is a rate-limiting enzyme in the biosynthesis of uric acid and catalyzes the oxidation of hypoxanthine and xanthine to uric acid [8]. Therefore, XOD inhibitors can be potent therapeutic agents for the prevention of hyperuricemia by inhibition of uric acid biosynthesis [9]. Allopurinol is an XOD inhibitor used clinically for the treatment of gout. However, it can have side effects, such as hypersensitivity reaction, Stevens. Johnson syndrome, renal toxicity, and even fatal liver necrosis [10]. It is believed that XOD inhibitors from natural sources can be used as alternatives to allopurinol because of fewer potential adverse side effects [11]. Many XOD inhibitors have been isolated and characterized from plants [12, 13] and mushrooms [13-17]. However, to date they have not been used for the treatment of gout because of their low XOD inhibitory activity. This paper describes the characteristics of a new XOD inhibitor isolated from the fruiting body of *P. ostreatus*, which could be used as an anti-gout foodstuff or for the development of an alternative to allopurinol.

Six species of edible mushrooms including *Pleurotus eryngii*, *P. ostreatus*, *P. cornucopiae*, *P. salmoneo-stramineus*, *Lyophyllum cinerascens*, and *Lentinus lepideus* were obtained from the Mushroom Research Station, Gyeonggi-do Agricultural Research and Extension Service in Gwangju, South Korea. XOD, xanthine, allopurinol, and potassium oxonate a gout-inducing chemical were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex G-50 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and

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*Corresponding author
E-mail: biotech8@pcu.ac.kr

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acetonitrile was purchased from J. T. Baker (Phillipsburg, NJ, USA).

Seven-wk-old male Sprague-Dawley rats weighing 180~200 g were purchased from Orientbio Co., Seongnam, Korea. A uric acid assay kit was purchased from Abnova Corporation (Taipei, Taiwan). Unless otherwise specified, all chemicals and solvents were of analytical grade.

Dried fruiting bodies of each of the different species of mushroom (30 g) were pulverized and then added to 1 L of distilled water or 95% ethanol. The extracts were obtained by soaking for 24 hr at 40°C with stirring. The extracts were centrifuged at 5,000 ×*g* for 20 min and filtered through Whatman No. 41 filter paper (Whatman, Maidstone, UK). The supernatant filtrates were lyophilized and used as water or ethanol extracts.

The XOD inhibitory activities of the extracts were determined as follows. XOD (0.1 U/mL; 100 μL) was added to a mixture of 0.1 M potassium phosphate buffer (0.6 mL; pH 7.5) and 100 μL of the *P. ostreatus* extract (20 mg/mL) and 200 μL of 1 mM xanthine. The mixture was allowed to react for 5 min at 37°C, and the reaction was stopped by addition of 200 μL of 1 N HCl. Protein was removed by centrifugation of the reaction mixture at 12,000 rpm for 10 min, and the amount of uric acid present was determined by spectrophotometry by measuring absorbance at 292 nm.

Purification of XOD inhibitor from *P. ostreatus*. The lyophilized extract was dissolved in distilled water and applied to a Sephadex G-50 column (3.0 × 35 cm), equilibrated with distilled water, and eluted with distilled water at a flow rate of 1.5 mL/min. The active fractions obtained were then applied to a C18 solid-phase extraction column (Sep-Pak C18 Cartridges; Waters Co., Milford, MA, USA) and equilibrated with 5% acetonitrile. Acetonitrile step gradient elution was carried out in water at 5%, 25%, 50%, and 100% (v/v) acetonitrile. The active fraction was then applied to an analytical reverse phase high-performance liquid chromatography column (RP-HPLC; Vydac 218TP54, C18 column, 5 μm, 4.6 × 250 mm; Discovery Science Co., Deerfield, IL, USA) equilibrated with 5% acetonitrile. A linear acetonitrile elution gradient was carried out with 0.1% trifluoroacetic acid in water from 5% to 25%. Active fractions of the purified XOD inhibitor were collected and immediately lyophilized.

***In vivo* hyperuricemic action and statistical analysis.** Male Sprague-Dawley rats (age, 7 weeks; weight, 180~200 g) were maintained on a 12-hr light/dark cycle in a temperature- and humidity-controlled room for 1 wk. All rats were randomly distributed into experimental groups (n = 5/group). The uricase inhibitor potassium oxonate was used to induce hyperuricemia in the rats [18, 19]. To increase serum urate levels, the rats were injected intraperitoneally with potassium oxonate (280 mg/kg) 1 hr before the *P. ostreatus* extract test samples were administered. Then, various concentrations of *P. ostreatus* (1,000 mg/kg and 500 mg/kg) and the anti-gout agent allopurinol (50 mg/kg) were administered orally. Blood was obtained from the rats by cardiac puncture, and was allowed to clot for approximately 1 hr at room temperature. Then, it was centrifuged at 3,000 rpm for 10 min to obtain serum, which was stored at −20°C until use. Serum uric acid levels were determined using a uric acid assay kit.

Each experiment was performed at least three times, and all quantitative data were expressed as mean ± standard deviation (SD) values.

Table 1. Xanthine oxidase inhibitory activities of water extracts from fruiting bodies of various species of edible mushrooms

Water extracts	Xanthine oxidase inhibitory activity (%)
Pleurotus eryngii extracts	29.2 ± 1.0
Pleurotus ostreatus extracts	78.3 ± 0.4
Pleurotus cornucopiae extracts	40.2 ± 0.7
Pleurotus salmoneo-stramineus extracts	67.2 ± 0.7
Lyophyllum cinerascens extracts	10.3 ± 0.4
Lentinus lepideus extracts	13.6 ± 1.0

Extracts produced by 48-hr extraction at 40°C.

XOD inhibitory activities of the extracts obtained from mushrooms. XOD inhibitory activities of the water extracts of the fruiting bodies of six species of edible mushrooms were determined (Table 1). The water extract from *P. ostreatus* had the highest XOD inhibitory activity (78.3%), and the XOD inhibitory activity of the extract of *P. salmoneo-stramineus* was also high (67.2%). The other mushroom species had low XOD inhibitory activity (< 50%). The XOD inhibitory activity of *P. ostreatus* was higher than that of the edible mushroom *Agaricus brasiliensis* (72.9%) [17]. In contrast, the 95% ethanol extracts had very low (< 10%) XOD inhibitory activity or not detectable activity (data not shown).

The effect of temperature and time on the extraction of the XOD inhibitor from *P. ostreatus* was evaluated at temperatures ranging from 30°C to 60°C and extraction times ranging from 24 to 72 hr. Water extracts obtained at 30°C and 40°C showed higher XOD inhibitory activities (72.2~83.2%) than those of the extracts obtained at 50°C and 60°C (17.0~41.7%). The maximal XOD inhibitory activity (83.2%, IC_{50} 12.2 mg) of the *P. ostreatus* fruiting body was demonstrated by the extracts obtained with distilled water at 40°C for 48 hr (Fig. 1).

Purification and characterization of the XOD inhibitor. Purification of the water extracts of *P. ostreatus* was performed by Sephadex G-50 gel filtration chromatography and the XOD inhibitory fraction had an IC_{50} of 10.4 mg/mL. After ultrafiltration of the active fraction using the Centriprep YM-50, 30, and 3 ultrafiltration units (Millipore Co., Billerica, MA, USA), we obtained active filtrates of less than 3 kDa

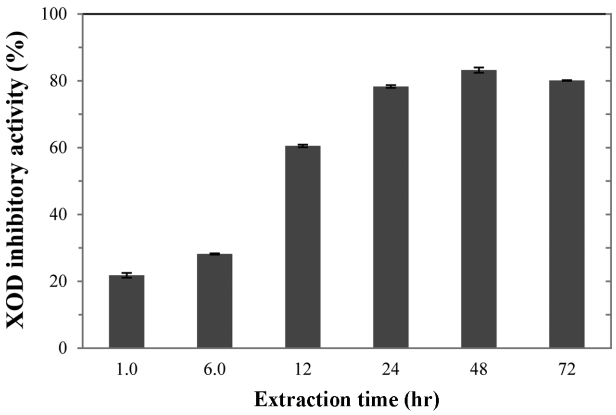

Fig. 1. Effect of extraction time on the xanthine oxidase (XOD) inhibitory activity of water extract from the *Pleurotus ostreatus* fruiting body.

by ultrafiltration (IC_{50}, 1.9 mg/mL). The active sub-3 kDa filtrates were subjected to C18 solid-phase elution with an acetonitrile gradient ranging from 5% to 100%. The eluates from 5% acetonitrile had the highest XOD IC_{50} (1.5 mg/mL). The active extracts from the 5% acetonitrile elution were subjected to RP-HPLC using a Vydac protein/peptide reverse-phase 218T P54 column, and purified XOD inhibitor was obtained at a yield of 3.0% (w/w) and an XOD IC_{50} of 0.9 mg/mL (13.6-fold purification) (Table 2, Fig. 2).

The purified XOD inhibitor was analyzed by LC-MS/MS and a tripeptide with the amino acid sequence phenylalanine-cysteine-histidine was obtained (Fig. 3). The molecular weight of the purified XOD inhibitor was estimated to be 441.3 Da.

Anti-gout action of the XOD inhibitor. The anti-gout action of the ultrafiltrates from *P. ostreatus* was evaluated in normal rats and potassium oxonate-treated hyperuricemic rats.

As shown in Fig. 4, the serum uric acid level in the non-hyperuricemic rats (normal rats; administered distilled water) was 2.4 ± 0.8 mg/dL, while that in the potassium oxonate-treated hyperuricemic rats that received no XOD inhibitor was 8.3 ± 0.14 mg/dL. When hyperuricemic rats were administered *P. ostreatus* extracts at an oral dose of 100 mg/kg or 500 mg/kg, uric acid levels decreased to 6.35 ± 0.5 mg/dL and 7.75 ± 0.4 mg/dL respectively, but these decreases were not statistically significantly. Allopurinol treatment significantly decreased serum urate levels to values nearing the range in normal rats. While the degree of the effect of the *P. ostreatus* extract was not as high as that of allopurinol, these results suggest that the decrease in serum uric acid levels was caused by the XOD inhibitory activity of the *P. ostreatus* extract. Based in these results, we conclude that the extract obtained from *P. ostreatus* is a potent bioactive agent for the development of foods or drugs beneficial for the treatment of gout.

Table 2. Summary of the purification steps for the xanthine oxidase inhibitor from *Pleurotus ostreatus*

Purification steps	Xanthine oxidase inhibitory activity (IC_{50}; mg/mL)	Solid yield (w/w, %)	Purification fold (times)
Water extract	12.2	100	1.0
Sephadex G-25	10.4	82.0	1.2
Ultrafiltration (3 kDa)	1.9	21.5	6.4
C_{18} solid-phase extraction	1.5	8.2	8.1
RP-HPLC	0.9	3.0	13.6

RP-HPLC, reverse phase high-performance liquid chromatography column.

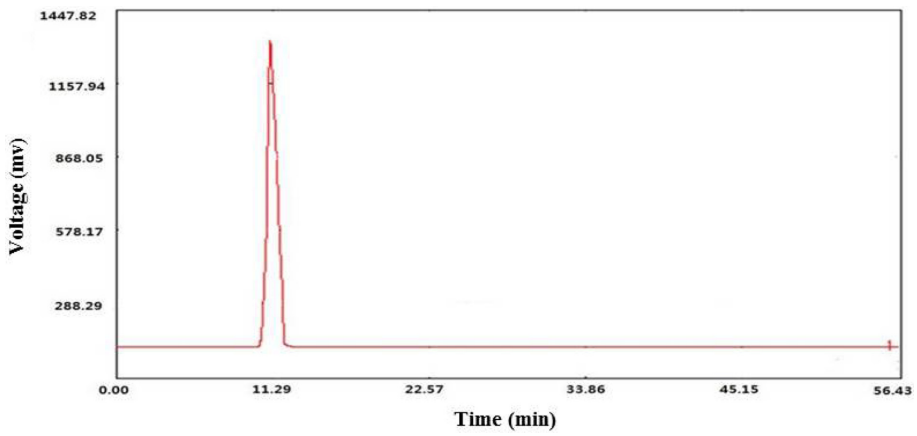

Fig. 2. Reverse-phase high-performance liquid chromatography chromatograph of the xanthine oxidase inhibitory active fraction from C_{18} solid-phase extraction chromatography.

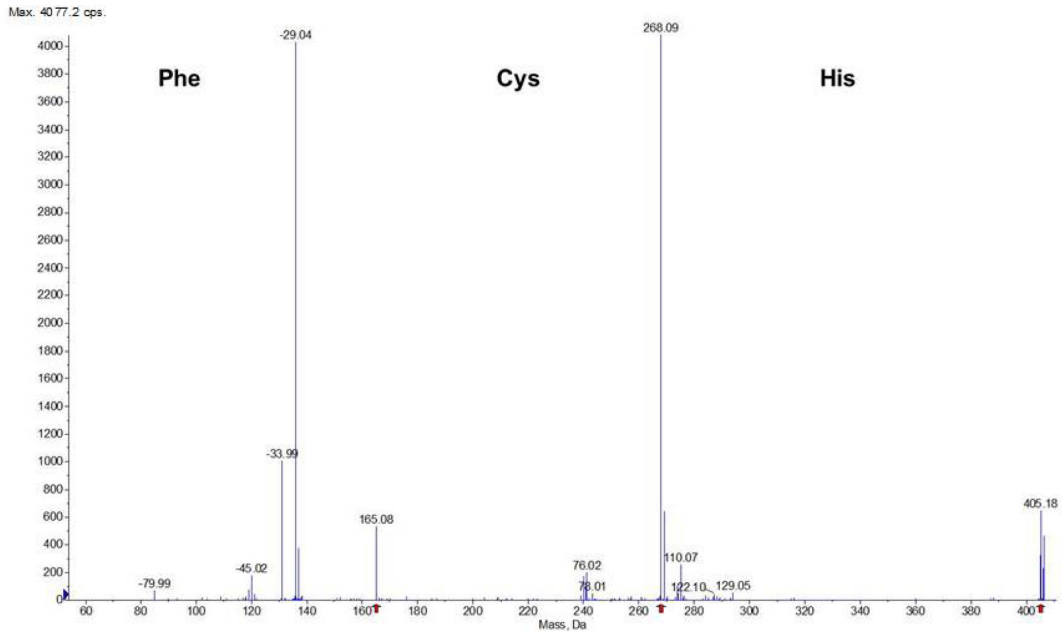

Fig. 3. Molecular mass and amino acid sequence of the purified xanthine oxidase inhibitory peptides, determined using LC-MS/MS.

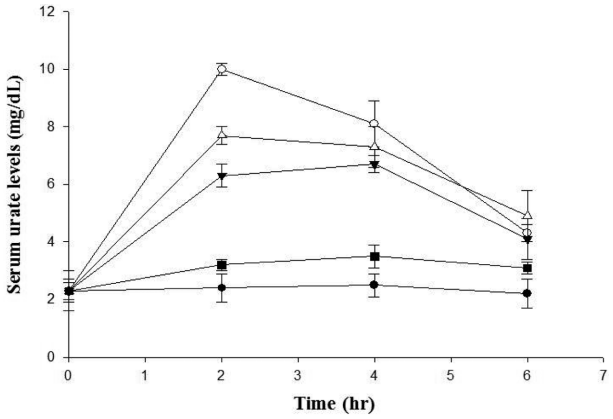

Fig. 4. Anti-gout activity of xanthine oxidase inhibitor-containing ultrafiltrates from *Pleurotus ostreatus* in normal and potassium oxonate-induced gout rats. Different symbols on the bars indicate significant difference ($p < 0.05$), as determined by Duncan's multiple range test. -●-●-, normal rats; -■-■-, allopurinol-administered rats (50 mg/kg, $p < 0.05$); -○-○-, potassium oxonate-treated rats with no xanthine oxidase inhibitor treatment; -▼-▼-, rats treated with *P. ostreatus* water extract (1,000 mg/kg, NS); -△-△-, rats treated with *P. ostreatus* water extract (500 mg/kg, NS). NS, not significant by Duncan's multiple range test.

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