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Identification of Mushroom Brown Blotch Causing Agent from *Pseudomonas tolaasii* Culture Broth

Chul-Jin Park, Sung-Ki Oh and Uck-Han Chun
Department of Food Science and Technology,
KyungHee University, Suwon, Korea

Abstract : The toxic substance causing blown blotching on the mushroom cap was extracted and purified from the culture broth of *P.tolaasii*. Purification and identification of the toxic compound was carried out with the silica gel chromatography, mass spectrum and NMR and appeared to be an aminobenzene in amylamine group. The purified toxic substance showed UV spectrum at 234nm and melting point at 76℃, respectively (Received May 9, 1994; accepted September 22, 1994).

Introduction

Commercially produced mushrooms are after downgraded because of unsightly brown blotching which develops on the mushroom caps. Brown blotch disease of the cultivated mushroom, *Agaricus bisporus*, caused by *Pseudomonas tolaasii*, is a serious problem to the mushroom.[1,3] *Pseudomonas tolaasii* has been isolated from causing peat of healthy and diseased mushroom beds, compost of diseased mushroom beds and from soils around a mushroom farm. It was not isolated from fresh peat or compost from healthy mushroom beds. *Pseudomonas tolaasii* is competing with other pathogens, *P. agarici* or *P. gingeri* for nutrients contained in mushrooms.[6,7] *Pseudomonas tolaasii* is also detected on mushroom compost and mushroom cap without visible symptoms.[3] The symptoms of the disease are dark brown, often wet and sunken lesions on the caps and stalks which render the crop unsaleable.[1,3] Nair and Fahy[4] also reported that the symptoms of disease such as sunken lesions and blotch were found within 6 hrs when the culture broth of *P. tolaasii* was dropped on the mushroom cap. Toxic substance causing such diseases was reported to be produced during the log phase of *P. tolaasii* culture and not to increase after log phase.

In this study, *P. tolaasii* was grown in liquid culture medium and extracted and purified in an attempt to identify the secreted chemical which could be responsible for the blotching.

Materials and Methods

Organisms and cultivation

Pseudomonas tolaasii was isolated from the blotch diseased mushroom, *Agaricus bisporus*, cultivated on the mushroom farm located in Kyungki province in Korea. Isolation and screening of *P. tolaasii* were followed by Wong and Preece.[7] Media compositions for *P. tolaasii* were as following; NH_4Cl 1.0 g/*l*, $MgSO_4 \cdot 7H_2O$ 0.5 g/*l*, $(NH_4)_2SO_4$ 0.05 g/*l*, $CaCl_2$ 0.05 g/*l*, yeast extract 5.0 g/*l*, and glucose 2.0 g/*l*. *P. tolaasii* was grown at 25℃ for 3~4 days.

Isolation and purification of toxic substances

The crude culture of *P. tolaasii* was centrifuged at 2,500×g for 20 min (Sorval, U.S.A). The supernatant obtained by centrifugation was applied to a column (4.5×60 cm) packed with Amberite XAD-2

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*Corresponding author : U.-H. Chun

resins(20~50 mesh, 500 g). Resins were activated prior to use by extracting with acetone overnight and washing with distilled water.

The column was eluted with 2 liters of methanol. The eluted methanol was concentrated to 100 m*l* *in vacuo* and 100 m*l* of distilled water was added into the concentrated methanol layer. The mixture was extracted with 200 m*l* of ethyl acetate (EtOAc) for three times. The EtOAc extracts were combined together and evaporated in a rotary evaporator. The residue was redissolved in 5 m*l* of methanol and transferred to a 10 m*l* vial. The solvent in a vial was evaporated to dryness under a gentle flow of nitrogen gas on the steam bath and stored in the refrigerator until use.

The pellet obtained by centrifugation was extracted twice with 200 m*l* of EtOAc. The extracts were filtered through Whatman No. 1 filter paper. The EtOAc extract was concentrated *in vacuo* at 40℃ and transferred to a 10 m*l* vial and evaporated again to dryness as mentioned above.

Each crude extract obtained from supernatant and pellet was bioassayed with mushroom cap and analyzed by TLC.[8,9]

The crude extracts (8 g) were obtained from 10 *l* of culture broth of *P. tolaasii.* The extract was dissolved in 10 m*l* of chloroform and applied onto a silica gel 60 (70~230 mesh, 150 g, E. Merk) in a 250 m*l* Buchner funnel with frited disc (ϕ65 mm, porosity 40~60μ, Pyrex) for vacuum chromatography.

Elution was performed stepwise with chloroform-methanol (vol/vol) in ratio of 99 : 1, 97 : 1, 9 : 1, 8 : 2, 7 : 3, 5 : 5 and methanol under vacuum. All fractions were monitored by TLC and antimushroom activity.

Active fraction was further purified by a silica gel column (4×60 cm, Kieselgel 60, 70~230 mesh, 200 g, E. Merk) chromatography with solvent systems of chloroform-methanol (97 : 3, 200 m*l*; 95 : 5, 200 m*l*; 9 : 1, 200 m*l*; 3 : 1, 200 m*l*; 1 : 1, 200 m*l*; methanol, 500 ml, v/v). Fractions were collected by a fraction collector (Vision Science, Korea) and monitored by TLC. Active portions were collected and concentrated *in vacuo*.

Preparative LC

The freeze dried active fraction was isolated in sufficient quantity with the preparative HPLC column for structural determination. The detector was a Waters model 450 with wavelength at 254 nm. The reverse phase column was Delta Pak C18-100α semi-prep column (Waters, Japan). The solvents for preparative HPLC of the active fraction was 70% methanol. Preparative seperations were performed at a flow rate of 5.0 m*l*/min.

Physicochemical analysis of toxic substance

UV absorption spectra were recorded with Hitachi UV-visible spectrophotometer (Hitachi 200). Melting points were determined in the open-end capillary with the Electrothermal melting point apparatus (Griffin & George, S31-855/000, U. K.). Fast atom bambardment mass spectrometry was performed on a mass spectrometer (model HP5871A GC/MS, 70eV). Proton nuclear magnetic resonance spectrum was obtained with Bruker AM200 (200 MHz).

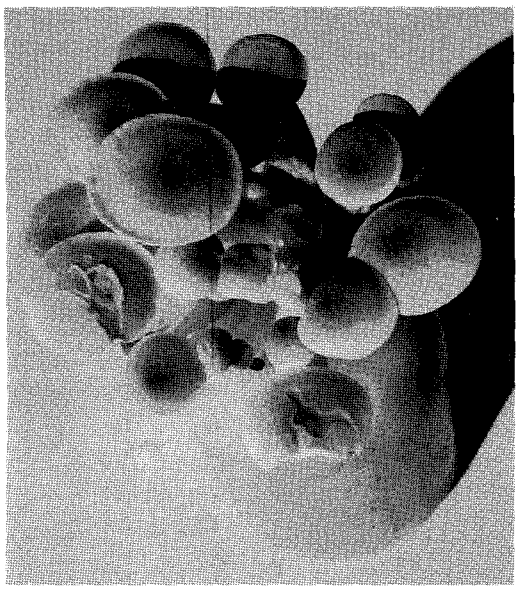

Fig. 1. Toxic activity of crude extract by *P. tolaasii* isolate on the mushroom cap.

Results and Discussion

Toxicity test of *P. tolaasii* culture

Toxicity was tested with crude extract from *P. tolaasii* culture broth by using paper disc method. The paper disc soaked in the supernatant obtained from centrifuged culture broth was applied on the agar media inoculated with mushroom. Mushroom hyphae were not grown around the paper disc due to the toxicity of supernatant of culture broth while the crude extract obtained from the pellet did not show any toxic effect against mushroom hyphae (data not shown). When the crude extract was applied to the cap of mushroom, the symptoms of blotch and sunken lesions quickly (Fig. 1).

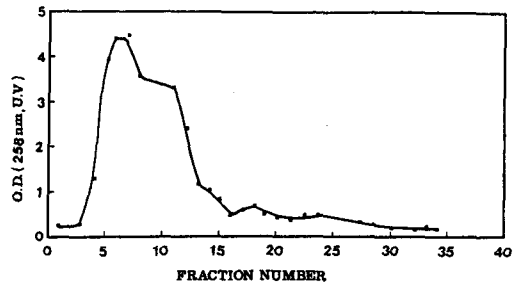

Fig. 2. Chromatogram of silica gel column.

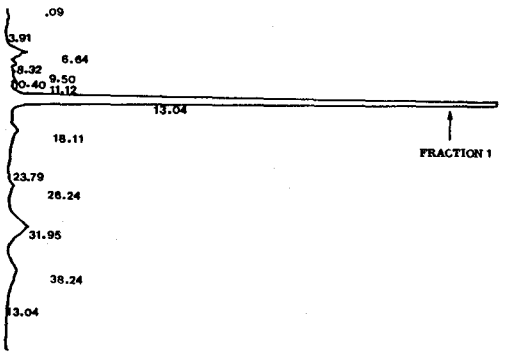

Fig. 3. Prep LC chromatogram of active fraction 1.

Purification and crude extract

A vacuum chromatography was performed with crude extract by using $CHCl_3$-CH_3OH as a gradient and activity was shown at the $CHCl_3$-CH_3OH, 97 : 3. The fraction was combined together and used for the silica gel column chromatography. Fig. 2 shows that the activity was found at the fractions 5-15.

Preparative LC

The active material obtained from the silica gel ch-

Table 1. Physicochemical properties of toxic substance produced by *P. tolaasii* isolate

Properties	Parameters
Appearance	pale yellow
Melting point (℃)[a]	76
UV maxima (nm)[b]	234

[a]Melting point was determined with open capillary tube.
[b]The scanning range of absorption maxima was 190 nm to 400 nm.

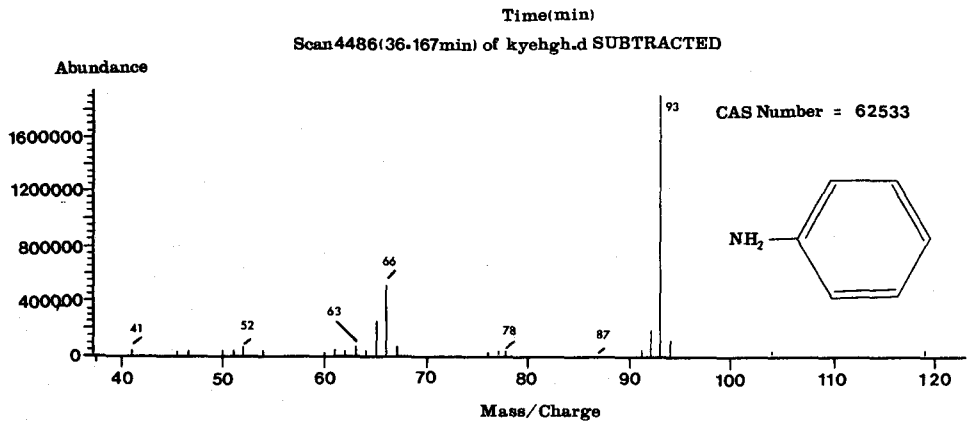

Fig. 4. Mass spectrum of toxic substance produced by *P. tolaasii* isolate.

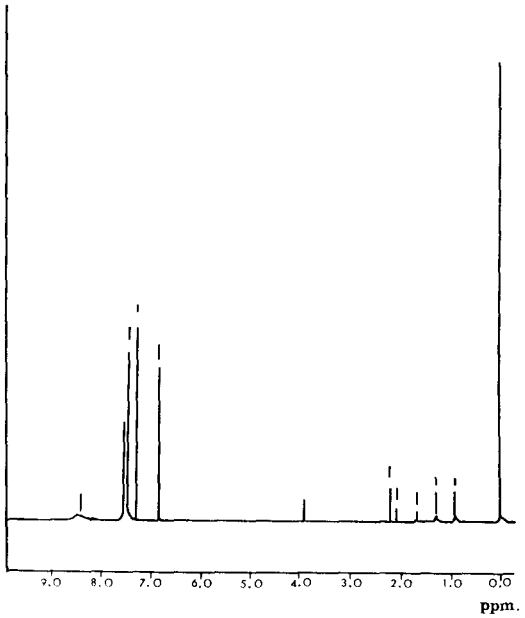

Fig. 5. ^{1}H-NMR spectrum of toxic substance produced by *P. tolaasii* isolate.

romatography was again run on the preparative LC. As shown in Fig. 3, major peaks appeared at the retention times of 6.5, 12.76, 12.19, 25.26 and 30.56 and the fractions from each peaks were investigated for activity. Only at retention time of 12.76, the activity was seen and the fraction was collected in large amount for the physicochemical analysis.

Physicochemical properties of the purified toxic substance

The physicochemical properties of the purified toxic substance were investigated and the data are summarized in Table 1. The melting point and UV absorption maxima of the active fraction were found to be 176℃ and 234 nm, repectively.

Feb-MS

The fast atom bambardment on this active material was carried out to determine the structure of component. As can be seen in Fig. 4, molecular ion(M^+) peak was found at m/e +93 and this material showed similarity to aminobenzene when compared to mass library.

NMR

Further analysis on the active material with ^{1}H-NMR spectrum (Fig. 5) showed it to have multiple signals at δ7.4~7.6 ppm. These signals were thought to be protons in the aromatic ring. The signal appeared at δ8.4 ppm was estimated to be a proton of the NH-group. The signals shown around δ7.3 ppm and δ0.9~2.2 ppm might be due to the moiety of $CHCl_3$ and other impurities.

Acknowledgements

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Pseudomonas tolaasii 배양액으로부터 독성물질의 동정

박철진 · 오성기 · 전억한(경희대학교 식품가공학과)

초록 : 버섯의 cap에 갈변병을 유발하는 독성물질을 *P. tolaasii* 배양액에서 추출, 정제하였다. 독성물질의 정제는 silica gel chromatography, mass spectrum과 NMR을 사용하였다. 정제된 독성물질은 amylamine group의 aminobenzene인 것으로 밝혀졌으며, UV spectrum으로 분석한 결과 234 nm에서 확인되었고, 융점은 76℃ 이었다.