韓 國 菌 學 會 誌 第 14 卷 第 4 號
Kor. J. Mycol. Vol. 14, No. 4, 265~271, 1986

Studies on Constituents of the Higher Fungi of Korea(LIV)

Studies on Toxic Component of *Auricularia polytricha*

Ha Won Kim, Eung Chil Choi and Byong Kak Kim

Department of Microbial Chemistry, College of Pharmacy, Seoul National University, Seoul 151, Korea

韓國産 高等菌類의 成分硏究(第54報)

털목이의 毒性 成分에 관한 硏究

金 河 元·崔 應 七·金 炳 珏

서울大學校 藥學大學 微生物藥品化學敎室

Abstract: To screen biologically active components of the higher fungi of Korea, the dried carpophores of *Auricularia polytricha* were extracted with water. The extract was examined for acute toxicity in ICR mice. A low molecular weight toxin of this fungus was purified by a acetone precipitation followed by cellulose, silica gel and Sephadex LH-20 column chromatography. Major symptoms of this toxin were decreasing of normal motility, eye extrusion, hair erection, shivering, trembling of head, paralysis, rapid running or moving before death and depression of respiration. The median lethal doses of the total extract were 1.28 g/kg and 4.31 g/kg by *i.p.* and *p.o.* administrations, respectively. The amounts of one mouse lethal unit of the total extract and final fraction that killed a 20 g mouse within 30 minutes were 28.5 and 12.0 mg/mouse, respectively.

Keywords: *Auricularia polytricha*, Acute toxicity test, Toxin, Median lethal dose, Mouse lethal unit.

The most poisonous mushroom that has been proved till now is the genus *Amanita*. The cyclic toxic peptides, amatoxins and phallotoxins, have been isolated from *Amanita phalloides*. Their chemical structures have been elucidated by Wieland *et al.* (1978). It was proved that these toxins were stable and can damage mainly liver and kidney. Various microassay methods were developed by using the actions of these toxins. Filter paper was used for the muscarine contents determination (Malone *et al.*, 1961). Chromatographic technique was used for the evaluation of toxic *Galerina* species (Tyler *et al.*, 1963). DNase I activity was employed for the assay of phallotoxins (Mullersman *et al.*, 1982).

Besides amatoxins and phallotoxins, another toxic components have been identified from *Amanita* species. Horgen *et al.* (1976) isolated amatoxins from *Amanita ocreata*. Virotoxins were isolated from *Amanita virosa* by Faulstich *et al.* (1980). Amaninamide, a toxin closely related to the family of amatoxins, was found exclusively in *Amanita virosa* mushroom by Buku *et al.* (1980).

Some toxic substances were isolated even in the edible mushrooms. Cardiotoxic protein, volvatoxin, which had hemolytic activity was isolated from the edible mushroom, *Volvariella volvacea* by Lin *et al.* (1973). They had also isolated another cardiotoxic protein, flamutoxin, from the edible mushroom, *Flammulina velutipes*, and proved that flamutoxin also had strong hemolytic

activity against human blood cells. Goose (1980) reported the case study of mushroom poisoning caused by *Tricholomopsis platyphylla* which had been considered an edible or at least a nonpoisonous species. Rubescenslysin, a protein derived from the edible fungus *Amanita rubescens*, was isolated and showed acute toxicity such as haemolysis, massive exudation of plasma with alveolar obstruction due to an increase in vascular permeability, cardiotoxicity and central nervous effects (Seeder *et vl.*, 1981).

Ukai and his associates continuously studied family *Auriculariaceae*. They isolated polysaccharides from the fruit bodies of *Tremella fuciformis* (Ukai *et al.*, 1972a). They showed that these polysaccharides had antitumor activity against sarcoma 180 (Ukai *et al.*, 1972b and 1974). In 1977, they revealed that those acidic polysaccharides were composed of alpha-1-3 linked D-mannopyranose backbone having highly branched points (Ukai *et al.*, 1977). Kiho and his colaborators reported that these acidic polysaccharides from *Tremella fuciformis* had O-acetyl groups at positions 4, 6 and both 4 and 6 of a part of the mannose residues (Kiho *et al.*, 1981).

Two kinds of acidic polysaccharides, molecular weights 300,000 and 370,000, had been isolated from the fruit bodies of *Auricularia auricular-judae* and they were constructed with alpha-1-3 linked D-mannopyranose residues as a backbone (Ukai *et al.*, 1982). Moreover, they reported that those polysaccharides had antitumor effects against sarcoma 180 in mice (Ukai *et al.*, 1983).

The reports on *Auricularia polytricha* began to appear in 1980. Hammerschmidt (1980) made a case report that a patient who had taken large quantity of this fungus bled for three days after a dental extraction. He found this mushroom contained heat stable platelet aggregation inhibitor. Makheija and Bailey (1981) postulated adenosine as the antiplatelet component. Agarwal and his coworkers (1982) showed that another component besides adenosine was responsible for the inhibitory activity. In 1981, Hokama reported that the extracts of *Auricularia polytricha* and *Cortinellus shiitake* had a strong inhibitory component of platelet aggregation. Hokama and his colabolators (1983) found a blastogenic inhibitory factor from some mushrooms including *Auricularia polytricha.*

The high molecular weight toxins, auratoxin I and II, had been isolated from the edible mushroom, *Auricularia polytricha*, in our laboratory (Kim *et al.*, 1984). However, no report on low molecular weight toxin from *Auricularia polytricha* has yet been found except blastogenic inhibitory factor (Hokama *et al.*, 1983). We found some acute toxicities on mice when injected orally or intraperitoneally. This paper reports on acute toxicities and properties of low dalton toxic components from *Auricularia polytricha.*

Materials and Methods

Materials

The dried carpophores of *Auricularia polytricha* (the family *Auriculariaceae*), black tree fungus or Jew's ear, used in this work were kindly provided by Agricultural Science Institute at Suwon, Gyeongi Province, where they cultivated.

Extraction and Isolation

The dried fruit bodies (100 g) of *Auricularia polytricha* were extracted with distilled water for 12 hours at 40°C twice. The water extracts were combined, filtered and concentrated. Same volume of chloroform was added to the water concentrate and extracted. The residual water layer, H_2O layer-1, was successively extracted with ethyl acetate. The H_2O layer-2 was concentrated and was added cold acetone (−20°C) and resulting precipitates were discarded. After evaporation of the supernatant to dryness, it was sub-

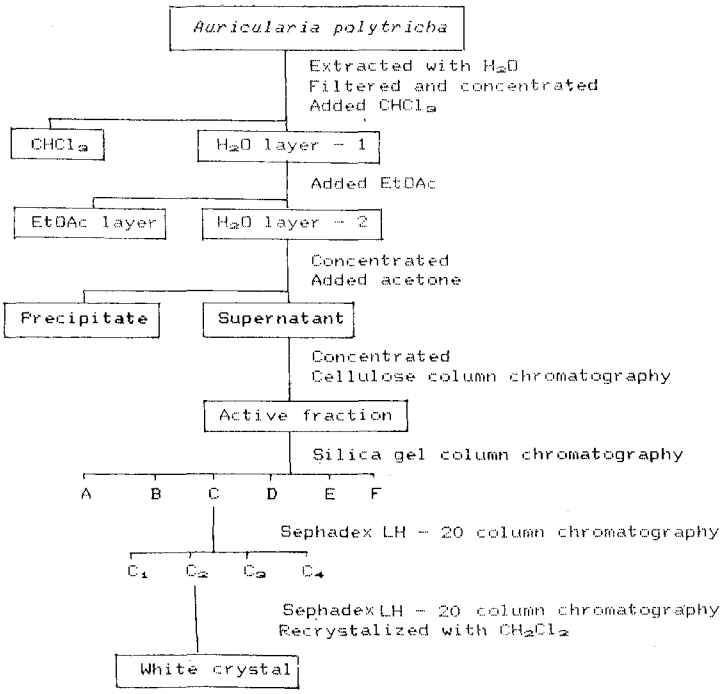

Fig. 1. Extraction and isolation of low molecular weight toxin from *Auricularia polytricha.*

jected to cellulose column chromatography (Avi cel, E. Merck), eluting with ethyl acetate—methanol—water—ammonia water mixture (10 : 10 : 2 : 0.2, v/v). Active fraction was collected and applied it to silica gel chromatography (als finer 230 mesh, E. Merck) with chloroform—methanol—ammonia water (15 : 3 : 0.05, v/v). Thin layer chromatography (Silica gel 60 F-254) and ninhydrine reagent were used for the fractions, resulting A (150 ml), B (31 ml), C (200 ml), D (160 ml), E (270 ml) and F (300 ml). Among them, C and E fractions showed high toxicities on ICR strain mice. After evaporation, C fraction was applied to Sephadex LH-20 column chromatography eluting with methanol. By TLC and toxicity tests with ICR mice, C1, C2, C3 and C4 fraction were obtained and C2 fraction showed the highest toxicity. Further purification was carried out by Sephadex LH-20 chromatography with methanol-chloroform (1 : 1, v/v). Active fraction was obtained and recrystalized with dichloromethan (Fig. 1).

Determination of LD_{50}

1) LD_{50} by Intraperitoneal Injection

ICR female mice (18~22 g) were supplied from the Experimental Animal Farm of Seoul National University. Median lethal does and 95% confidence limits of H_2O layer-2 fraction were determined by Litchfield and Wilcoxon method. In the preliminary test maximum and minimum doses were obtained.

2) LD_{50} by Oral Administration

Median lethal does and 95% confidence limits of H_2O layer-2 fraction for oral administration were determined by Litchfield and Wilcoxon method by using ICR female mice (18~22 g). Each group was 10 mice.

Dose-death Time Relationship

1) Establishment of Mouse Lethal Unit

The dose-death time curve was obtained by plotting the mean death time of three mice against the mouse lethal unit (=MLU). An

amount of sample which can kill mice in 15~30 min was defined as one MLU. Total amounts of the toxin can be calculated from the amount equivalent to one MLU. Death time was determined when the injected mouse was completely ceased muscle movement and breath.

2) Sample Administration

ICR mice(18~22 g) and H_2O layer-2 fraction were used for this experiment. Each group was three mice and injected intraperitoneally at the dose of 19.0, 28.5, 47.5, 57.0, 85.5 and 114.0 mg/mouse.

Results

Physicochemical Properties of the Toxic Component

The water extract of the *Auricuraria polytricha* was shaken with chloroform several times and subsequently with ethyl acetate. Its toxic activity was detected in water layer. After removing high molecular weight fraction by acetone pecipitation method, its toxicity was still shown in the supernatent. Further purification by cellulose and silica gel column chromatography resulted in the toxicity of C fraction. For further purification, repeated Sephadex LH-20 column chromatography was adopted. As the result, only C2 fraction showed toxicity. C2 fraction was concentrated and dissolved in dichloromethan and stored in a refrigerator to yield white crystals in needle or plate form. However, there occurred always coprecipitation with minor impurity. The toxic crystal was readily soluble in water but showed decreasing solubility in warm methanol, ethanol and ethyl acetate. Its melting point was 200~205°C.

Physiological Properties of the Toxic Component

During fractionation and isolation, when injected intraperitoneally or orally, the general symptoms of the toxic fraction were decreasing of normal motility and hair erection in low concentration. However, in high concentration that can kill mouse, its symptoms were shivering, trembling of head, eye extrusion, paralysis, rapid running or moving before death and depression of respiration. When one MLU was administered intraperitoneally, the mouse began to show paralysis. This mouse was observed rolling down to the bottom of slanted plate without any resistance. The injected mouse could not stand, thus lying on the ground, decreased in breath and finally died. Death time was taken when muscle movement and breath were completely stopped. However, heart stroke was continued for one minute after its death (Table I). All the toxic fractions of *Auricularia polytricha* showed only acute toxicity, and if death did not occur within a certain time, the mouse would survive with no apparent effects.

Table I. Major symptoms of low dalton toxin of *Auricularia polytricha* in mice.

Dose	Symptoms
Low dose*	Decreasing of normal motility Hair erection
High dose**	Decreasing of normal motility
	Hair erection
	Shivering
	Trembling of head
	Eye extrusion
	Paralysis
	Rapid running or moving before death
	Depression of respiration
	Death

* Doses that did not cause death.
** Doses that caused death finally.

Table II. LD_{50} of the water extract of *Auriculraia polytricha.*

Animal	Route of administration	LD_{50}(mg/kg)
Mouse	*i.p.*	1245.1(1115.6~1389.6)
	p.o.	4180.0(3991.0~4378.0)

Determination of LD_{50}

1) LD_{50} by Intraperitoneal Injection

Median lethal dose of H_2O layer-2 fraction was determined by Litchfield and Wilcoxon method. As shown in Table II, LD_{50} (*i.p.*) was 1.25 g/kg and its 95 % confidence limits were 1.12 and 1.39g/kg.

2) LD_{50} by Oral Administration

By the method of Litchfield and Wilcoxon, median lethal dose of H_2O layer-2 fraction was determined by using ICR female mice. LD_{50} (*p.o.*) was 4.18 g/kg and its 95% confidence limits were 3.99 and 4.3 g/kg.

Dose-death Time Relationship

1) Determination of Mouse Lethal Unit

H_2O layer-2 fraction was completely evaporated and dissolved in distilled water. When it was injected at the doses of 95, 47 and 28.5 mg/mouse intraperitoneally into ICR female mouse, death was observed in 3, 10 and 13 minutes, respectively. Therefore one MLU which was defined as the amount required to kill in 15-30 minutes was 28.5 mg/mouse. Thus 667 MLU was contained in the dried fruit bodies (100 g) of *Auricularia polytricha.*

2) Dose-death Time

When each aliquote of 19.0, 28.5, 47.5, 57.0, 85.5 and 114.0 mg of the H_2O layer-2 fraction was injected into each group which consisted of three ICR female mice (18~22 g), their death times were 60.0, 11.6, 9.3, 6.0 and 5.3 minutes, respectively (Fig. 2). One MLU of C fraction and final white crystal were 25.0 and 12.0 mg/mouse, respectively (Table III).

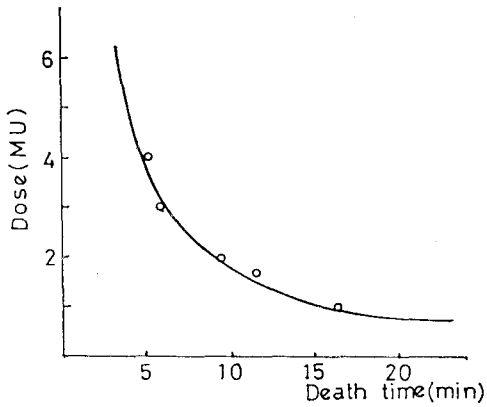

Figure 2. Dose-death time curve of the water extract from *Auricularia polytricha.* One mouse lethal unit (28.5 mg/mouse, *i.p.*) was defined to kill mouse in 15-30 min.

Table III. Mouse lethal units of the fractions that were obtained from the extract of *Auricularia polytricha.*

Fractions	Injection route	MLU (mg/mouse)*
H_2O layer-2	*i.p.*	28.5
C fraction	*i.p.*	25.0
Final fraction	*i.p.*	12.0

* One mouse lethal unit (MLU) was defined as the amount required to kill mice in 15~30 minutes.

Discussion

We had already reported the presence of high molecular weight toxins, auratoxin I and II, from *Auricularia polytricha* (Kim *et al.*, 1984). Their general symptoms of intoxication were convulsion during the first 30 minutes after injection, then sleeping within an hour, tremor, lacrimation, nasal and ophthalmic bleeding, congestion in ears and claws and death in 24 hours. However, the low molecular weight toxin which was studied in this paper showed different pattern, *i.e.*, decreasing of normal motility, heir erection, shivering, trembling of head, eye extrusion, paralysis, rapid running or moving before death and depression of respiration. Besides the differences of symptoms, the auratoxins can not be dialyzed against visking tube, but the low molecular weight toxin can be dialyzed. Thus the latter toxic component can be regarded as clearly different from the former. The possibility of the presence of toxic component was reported by Hokama *et al.* (1983) who had found a potent low dalton blastogenic

inhibitory factor that could pronounce progressive killing of blood mononuclear cells.

The active white crystal in this paper was mixture of four or five compounds and there was no benzene group by IR and NMR spectrophotometer. Thus the active component could have tertiary or quarternary amine group, for it was detected by ninhydrine reagent throughout the isolation procedure. The amounts of one MLU of H_2O layer-2 fraction, C fraction and final white crystal were 28.5, 25.0 and 12.0 mg/mouse. There was no decrease in the amount of one MLU through purification. The reasons can be thought in two aspects. First, there can be various toxic compounds in the total extract and thus high toxicity was shown. Second, the toxic compound could have lost some of its original toxicity during isolation by the heat or light. The median lathal doses of *i.p.* and *p.o.* were 1.25 and 4.18 g/kg and there was 650~700 MLU in 100 g of the dried fruit bodies of *Auricularia polytricha*. Therefore it can be concluded that this fungus has strong toxic compounds.

From these data, the extract and its fractions showed severe toxicity when injected into ICR female mice intraperitoneally or orally. Thus, as this fungus is used as food or tonic, it can evoke unknown toxicity when the extract of this mushroom was taken like other tonics. It is desirable to establish the microassay methods of this toxin and to elucidate its structure.

적 요

털목이버섯의 저분자 독성물질로부터 다음과 같은 결론을 얻었다. 털목이버섯 열수추출액의 LD_{50}은 복강 및 경구투여시 각각 1.25와 4.18 g/kg이었다. 건조 털목이버섯 100 g에는 650~700 mouse lethal unit(=MLU)이 함유되어 있었다. 열수총추출액, C분획 및 최종성분의 1 MLU는 각각 및 28.5, 25.0 및 12.0 mg/mouse 이었다. 독성물질의 일반적인 증상은 행동둔화, 체모기립, 전율, 두부진동, 안구돌출, 사지마비, 사망직전의 급격한 다리운동 및 호흡마비 등이었다.

References

Agarwal, K.C., Russo, F.X. and Parks, R.E. (1982): *Thromb. Haemostas* **48**:162.

Buku, A., Wieland, T., Bodenmuller, H. and Faulstich, H. (1980): *Experimentia* **36**:33.

Faulstich, H. Buku, A., Bodenmuller, H. and Wieland, T. (1980): *Biochem.* **19**:3334.

Goose, R.D. and Shoop, C.R. (1980): *Mycologia* **72**: 433.

Hammerschmidt, D.E. (1980):*N. Engl. J. Med.* **302**: 1191.

Hanelin, L.G. and Moss, A.A. (1975):*Am. J. Roentgenol. Radium Ther. Med.* **125**:782.

Hatfield, M. (1975): *Lloydia* **3**:36.

Hokama, Y. and Hokama, J.L.R.Y. (1981):*Res. Comm. Chem. Pathol. Pharmacol.* **31**:177.

Hokama, Y., Cripps, C., Hokama, J.L.R.Y., Sato, M.A.L. and Kimura, L.H. (1983); *Res. Comm. Chem. Pathol. Pharmacol.* **41**:157.

Horgen, P.A. and Ammirati, J.F. (1976): *Lloydia* **39**:368.

Kiho, T., Hara, C. and Ukai, S. (1981): *Chem. Pharm. Bull.* **26**:225.

Kim, H.W., Choi, E.C. and Kim, B.K. (1984): *Kor. J. Microbiol.* **22**:223.

Lin, J.Y., Jeng, T.W., Chen, C.C., Shi, G.Y. and Tung, T.C. (1973): *Nature* **246**:524.

Lin, J.Y., Lin, Y.J., Chen, C.C., Wu, H.L., Shi, G.Y. and Jeng, T.W. (1974): *Nature* **252**:235.

Lin, J.Y., Wu, H.L. and Shi, G.Y. (1975): *Toxicon.* **13**:323.

Makheja, A.N. and Bailey, J.M. (1981): *N. Engl. J. Med.* **304**:175.

Malone, M.H., Robichaud, R.C., Tyler, V.E. and Brady, L.R. (1961): *Lloydia* **24**:204.

Mengs, U. and Trost, W. (1981): *Arch. Toxicol.* **48**:61.

Mullersman, J.E. and Preston, J.F. (1982): *Anal. Biochem.* **119**:266.

Nozoe, S. and Kasano, G. (1981): *Pharmacy* **17**:723.

Ott, J., Wheaton, S. and Chilton, W.S. (1975): *Physiol. Chem. Phys.* **7**:381.

Preston, J.F., Stark, H.J. and Kimbrough, J.W. (1975): *Lloydia* **38**:153.

Seeger, R., Odental, K.P. and Mengs, V. (1981): *Toxicon.* **19**:409.

Tyler, V.E., Brady, L.R. and Benedict, R.G. (1963): *Lloydia* **26**:154.

Ukai, S., Hirose, K. and Kiho, T. (1972a): *Chem. Pharm. Bull.* **20**:1347.

Ukai, S., Hirose, K., Kiho, T., Hara, C., Irikura, T., Tanechika, T. and Hasegawa, Y. (1972b): *Chem. Pharm. Bull.* **20**:2293.

Ukai, S., Hirose, K., Kiho, T. and Hara, C. (1974): *Chem. Pharm. Bull.* **22**:1102.

Ukai, S., Hirose, K., Kiho, T. and Hara, C. (1977): *Chem. Pharm. Bull.* **25**:338.

Ukai, S., Morisaki, S., Koto, M., Kiho, T., Hara, C. and Hirose, K. (1982): *Chem. Pharm. Bull.* **30**:635.

Ukai, S., Kiho, T., Hara, C., Morita, M., Goto, A., Imaizumi, N. and Hasegawa, Y. (1983): *Chem. Pharm. Bull.* **31**:741.

Wieland, T. and Faulstich, H. (1978): *Crit. Rev. Biochem.* **5**:185.

Wieland, T., Beijer, B., Seeliger, A., Dabrowski, J., Zanotti, G., Tonelli, A.E., Gieren, A., Dederer, B., Lamm, V. and Hadicke, E. (1981): *Liebigs Ann. Chem.* 2313.

Yamaura, Y., Maezawa, H., Takabatake, E. and Hashimoto, T. (1981): *J. Food Hyg. Soc. Jpn.* **22**: 203.

Yamaura, Y., Maezawa, H., Takabatake, E. and Hashimoto, T. (1981): *J. Food Hyg. Soc. Jpn.***23**: 314.

〈Received October 20, 1986;
Accepted November 4, 1986〉