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Toxic Components of *Auricularia polytricha*

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To find biologically active components of the higher fungi of Korea, the carpophores of *Auricularia polytricha*, a well-known edible mushroom, were extracted with 0.14 M NaCl solution. The extract was successively fractionated by adding ammonium sulfate at various concentrations, and the respective precipitates were separated by centrifugation, then dialyzed and freeze-dried. When a dose of 60 mg/kg of each was injected i.p. into ICR mice, the fraction which precipitated at 20% ammonium sulfate showed the highest toxicity, killing seven out of seven mice within two days. The fraction obtained at 40% ammonium sulfate showed the second highest toxicity. The two fractions were named auritoxin I and II after the genus name. However, they were shown to have nearly identical composition by physicochemical and instrumental analysis. The chemical anlaysis of auritoxin showed 93.9% polysaccharide and 6.8% protein. The polysaccharide moiety was found to have 12.3% α-linkage and 87.7% β-linkage and to be a heteromannoglucan consisting of 45.1% glucose, 43.9% mannose and 11.0% xylose. The protein moiety contained ten amino acids. The molecular weight of the toxin was 1.5×10^6 dalton by Sepharose CL-4B gel filtration. The median lethal doses of auritoxin in mice were 56.4, 157.2 and 454.6 mg/kg by i.p., s.c. and p.o. administrations, respectively. The signs of intoxication were convulsion during the first 30 minutes after the injection, coma or sleeping within an hour, tremor, lacrimation, nasal bleeding, congestion, and death in 24 hours. Among the various organs, the spleen was found to be enlarged remarkably. Human platelet aggregation was inhibited by the addition of auritoxin. The activity of malic dehydrogenase *in vitro* was inhibited by the toxin.

Key words: *Auricularia polytricha*, Toxicity, Auritoxin, Glycoprotein, Polysaccharide, Median lethal dose, Platelet aggregation, Malic dehydrogenase

INTRODUCTION

It is well known that the mushrooms of the genus *Amanita* have strong toxic components such as amatoxins and phallotoxins (Wieland, 1968; Hatfield, 1975; Preston, 1975; Horgen and Ammirati, 1976; Wieland and Faulstich, 1978; Faulstich *et al.*, 1980; Buku *et al.*, 1980; Mullersman and Preston, 1982; Perez-Silva and Alfonso, 1983; Wieland, 1986). However, some mushrooms known as edible for a long time were found to have toxicity. Calvacine is a basic glycoprotein isolated from the edible giant puffball, *Calvacia giganitea*. It has strong antitumor activity *in vitro* (Roland *et al.*, 1960). It proved to be too toxic for medical use (Stemberg *et al.*, 1963). Lin and his associates reported cardiotoxic proteins, volvatoxin A1 and volvatoxin A2, from the edible mushroom *Volvariella volvacea* in 1973 (Lin *et al.*, 1973). They isolated another cardiotoxic protein, flammutoxin, from the edible mushroom, *Flammulina velutipes* in the next year (Lin *et al.*, 1975). Rubescenslysin, a cytolytic protein derived from the edible fungus, *Amanita rubescens*, was reported by Seeger *et al.* in 1981 (Seager *et al.*, 1981).

The fungi of the genus *Auricularia* have been widely used as food or tonic agent in the Orient, particularly in China and Korea. Two famous species are *A. auricula* and *A. polytricha*. Ukai and his associates reported in 1982 that acidic heteroglycans isolated from the carpophores of *A. auricula* had molecular weights of 300,000 and 370,000 (Ukai *et al.*, 1982). In 1983, they found that these components had antitumor activity.

Reports on the toxicity of the fungus *A. polytricha* began to appear in 1980. Hammerschmidt found that this mushroom, when eaten as food, could produce transient inhibition of platelet aggregation and a mild hemorrhagic diathesis (Hammerschmidt, 1980). In 1981, Makheja and Bailey (1981) postulated that adenosine

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was the antiplatelet component of this fungus. In 1982, however, Agarwal *et al.* (1982) showed another compound besides adenosine was responsible for the inhibitory activity. In 1981, Hokama and Hokama (1981) reported that extracts of *A. polytricha* and *Lentinus edodes* had a strong inhibitory activity on platelet aggregation. In 1983, Hokama *et al.* (1983) found a blastogenic inhibitory factor in this mushroom. In the course of our screening biologically active components of Korean higher fungi, the acute toxicity of *A. polytricha* in mice was noticed. However, no literature report of its acute toxicity in animals has yet been found. This paper reports on the toxic components of this edible mushroom and their properties.

EXPERIMENTAL METHODS

Materials

Dired, cultivated carpophores of *A. polytricha* (family Auriculariaceae) were kindly provided by Agricultural Science Insitute at Suwon, Gyeong Gi Province.

Extraction and Purification

The carpophores (100 g) of *A. polytricha* were rehydrated in 0.14 M NaCl (pH 4.0) for 12 hours at 4℃ and then homogenized with a blender. They were extracted twice at 40℃ for 30 hours. After centrifugation, the supernatant was adjusted to 20% of saturation with ammonium sulfate at 4℃ for 30 hours. After centrifugation, the supernatant was adjusted to 20% of saturation with ammonium sulfate at 4℃. After 48 hours, the precipitate was separated by centrifugation, dissolved in distilled water, and freeze-dried. A dark brownish powder, ppt-20, was obtained. Deproteination of the crude product was carried out by the modified method of Sevag, and the resulting material was named auritoxin I, after the genus name of the mushroom. The supernatant from ppt-20 was adjusted to 40% of saturation with ammonium sulfate, and processed in the same way to yield finally the material named auritoxin II.

Characterization of Auritoxin

Molecular weights of auritoxin I and auritoxin II were measured by Sepharose CL-4B gel filtration using 0.01 M sodium phosphate buffer (pH 6.8). Auritoxin I (12.0 mg), auritoxin II (20.2 mg) and standard dextrans (15.0 mg) were separately applied to the column and optical densities of the fractions were measured at 625 nm after anthrone reaction.

In order to characterize auritoxins, various color reactions, such as anthrone reaction, phenol sulfuric acid reaction, Molish reaction, iodine reaction, tryptophan test, biuret reaction, ninhydrin reaction and Lowry-Folin reaction were carried out. To determine the identity and content of monosaccharides of auritoxins I and II, 5.0 mg of the toxins and 5.0 mg each standard monosaccharides were methanolyzed at 90± 5℃ for 20 hours. The toxin samples were then filtered and the insoluble residues methanolyzed again. Each methanolysate was analyzed by HPLC. Total polysaccnaride content of the auritoxin was determined by the anthrone method, using the constituent sugars as standards.

The identity and content of amino acids was determined by hydrolyzing 20 mg samples in 6 N HCl for 24 hrs at 100℃ and separating the components on an automatic amino acid analyzer (Hitachi A.A. analyzer 835) using Hitachi ion exchanger resin column (No. 2619F). Total protein content was measured by the Lowry-Folin method, using bovine serum albumin as a standard. Elemental analysis, IR spectrum and proton NMR were run on a Perkin-Elmer elemental analyzer, Perkin-Elmer FT-IR and Varian FT-90A NMR, respectively. In order to elucidate linkage configurations of the polysaccharide, 1.0 mg of auritoxin was treated with 100 units of α-glucosidase (tye VI, Sigma Chem. Co.) and 100 units of β-glucosidase (Sigma Chem. Co.) according to the modified method of Nanba (Nanba *et al.*, 1987). The diffusates were freeze-dried and analyzed by GLC (Hewlet-Packard 5890 GC) using HP-1 capillary column (10 m×0.53 mm). In addition, Congo red maximum absorption shift was measured by using visible spectrophotometer (LKB Ultrospec 4050) to confirm β-configuration according to the modified method of Ogawa (Ogawa *et al.*, 1972).

Toxicities of Auritoxin

ICR female mice weighing 18-20 g were used for toxicity tests. They were supplied from the Experimental Animal Farm of Seoul National University. Median lethal doses of the toxins were measured by the method of Litchfield-Wilcoxon. The effects of auritoxin on weights of various organs such as spleen, liver and kidney were determined after 6 days. The effects of auritoxin on the ADP-induced human platelet aggregation were measured by Born method (Born, 1962) using a Chronolog aggregometer. Platelet rich plasma was diluted to $2.3\text{-}2.5\times10^{8}$ cells/ml with platelet poor plasma. The mixture of the platelet plasma and toxin was preincubated in a cuvette for one minute and platelet aggregation was induced by 5×10^{-5} M ADP solution. Light trasmission was measured for 4-5 minutes. The percent inhibition of aggregation was obtained by the following equation: % Inhibition of aggregation=100×(% aggregation in control−% aggregation with sample)/% aggregation in control.

In order to determine whether auritoxin could influence the activity of key enzymes, the effects of auritoxin on the activity of leucine aminopeptidase, α-amylase, β-amylase, esterase, malic dehydrogenase, lactic de-

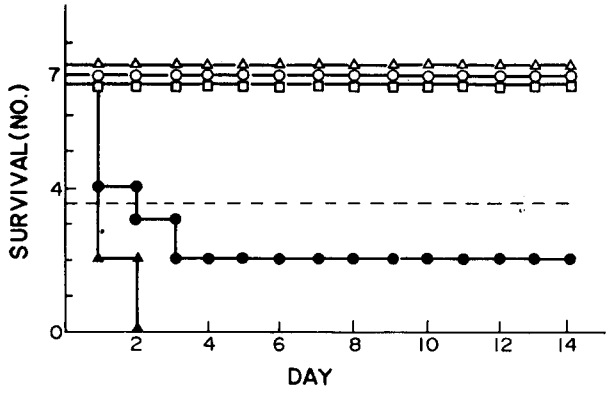

Fig. 1. Acute toxicity in mice of the fraction of *Auricularia polytricha*. A dose of 60 mg/kg in 0.1 ml was *i.p.* injected. △—△: control, ○—○: ppt 60-80, □—□: ppt 40-60, ●—●: ppt 20-40, ▲—▲: ppt 20

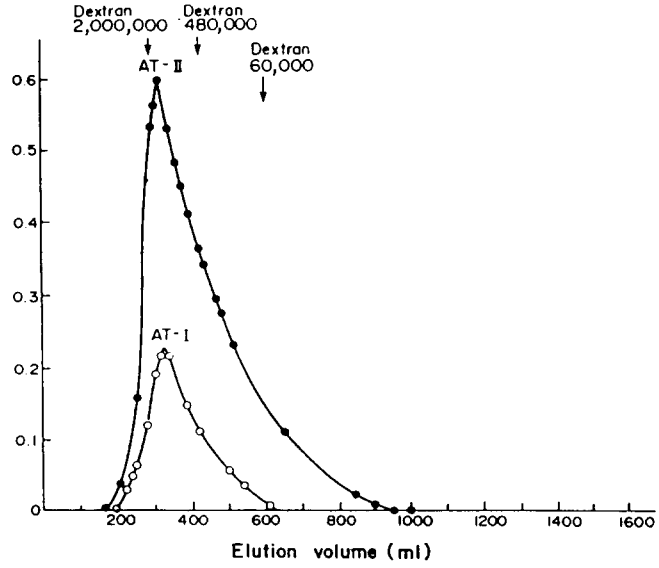

Fig. 2. Elution diagram of auritoxin I (AT-I) and auritoxin II (AT-II) for the determinations of homogeneity and molecular weight.
Sample: AT-I (12.0 mg) and AT-II (20.2 mg) in 4.5 ml phosphate buffer solution, Eluent: phosphate buffer solution (pH 6.8 and ionic strength 0.044 g·ions/l), Column: bed height 87.0 cm, 840 ml and flow rate 0.25 ml/min, Assay: Anthrone test.

hydrogenase and alkaline phosphatase were measured *in vitro*.

RESULTS

Isolation of Toxic Components

Four fractions were isolated from the carpophores of *A. polytricha*. Each fraction was administered to seven ICR female mice intraperitoneally at a dose of 60 mg/kg in an injection volume of 0.1 ml of the

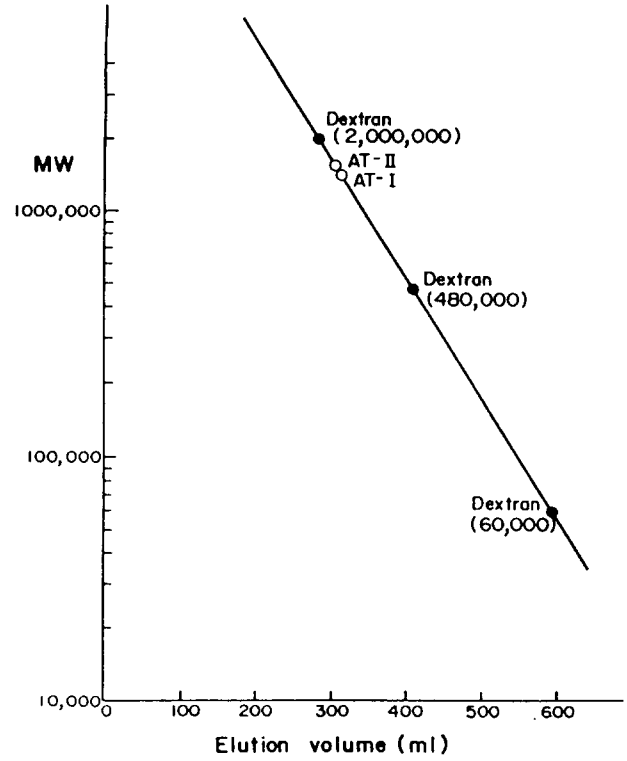

Fig. 3. Determination of molecular weights of auritoxin I (AT-I) and auritoxin II (AT-II) by gel filtration on Sepharose CL-4B wit standard dextrans. The elution volume (ml) was plotted agains the logarithm of molecular weight of polysaccharide.

aqueous solutions of the precipitates. The results of the acute toxicity test of ppt 20, ppt 20-40, ppt 40-60 and ppt 60-80 are shown in Fig. 1. Here, the numbers refer to the ammonium sulfate concentrations, expressed as a percent of saturation, at which the precipitates formed. Of the four fractions, ppt 20 showed the highest toxicity, killing all seven mice within two days after the single intraperitoneal injection. The second fraction, ppt 20-40, had the second highest toxicity, killing five of seven mice within three days. Precipitation 20 and ppt 20-40 were named crude auritoxins I and II, respectively. These were further purified until a single peak in the Sepharose CL-4B column appeared.

Characterization of Auritoxin

The molecular weights of the toxins were determined by Sepharose CL-4B gel filtration together with standard dextrans. Optical densities (O.D.) of the eluted fraction were measured at 625 nm after anthrone reaction. Auritoxins I and II were eluted at 315 ml and 306 ml by Sepharose CL-4B gel filtration (Fig. 2). Their molecular weights were calculated by plotting O.D. against the logarithm of molecular weights of the polysaccharides. Molecular weights of auritoxins I and II are the same, 1.5×10^6 dalton (Fig. 3).

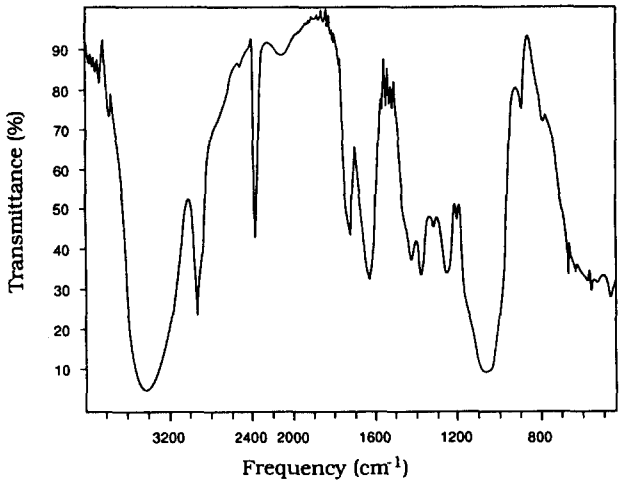

Fig. 4. IR spectrum of auritoxin I.

Table I. Color reactions of 1% aqueous auritoxin solution

Reaction	Color	Result
Anthrone reaction	Greenish blue	Saccharide confirmed
Phenol-sulfuric acid reaction	Brown	Saccharide confirmed
Molish reaction	Purple ring	Saccharide confirmed
Iodine test	*	—
Tryptophan test	Purple ring	Saccharide confirmed
Biuret reaction	Purple blue	Peptide confirmed
Ninhydrine reaction	Weak purple	Peptide confirmed
Lowry-Folin reaction	Dark blue	Peptide confirmed

*No color change

Elemental analysis of auritoxins I and II showed that the mole ratio of C:H:O:N of the both toxins was 6.0:9.4-9.8:6.2:0.01-0.04. IR spectrograms of auritoxins I and II showed O-H stretching, C-H stretching and C-O stretching at frequencies 3400 cm^{-1}, 2900 cm^{-1} and 1640 cm^{-1}, respectively (Fig. 4). These absorptions are common to all polysaccharides.

Proton NMRs of auritoxins I and II were measured. Both of the toxins showed anomeric proton peaks at 4.3, 4.9 and 5.1 ppm. Peaks due to the protein were detected at 2.1, 3.6 and 3.9 ppm. The results of elemental analysis, IR and NMR spectra showed that auritoxin I and II were almost identical.

Various color reactions were carried out on auritoxin I and II. The results are summarized in Table I. They show that the major constituents of the toxin are polysaccharide and a peptide. The sugar content of the polysaccharide moiety of auritoxin was 45.1% glucose, 43.9% mannose and 11.0% xylose by HPLC analysis using a carbohydrate analysis column.

In determining total polysaccharide content of the toxin four different standard sugars were used and compared, i.e., glucose alone, a mixture of glucose-xylose-mannose, xylose or mannose as standard sugars. The calibration curves of the four standards showed

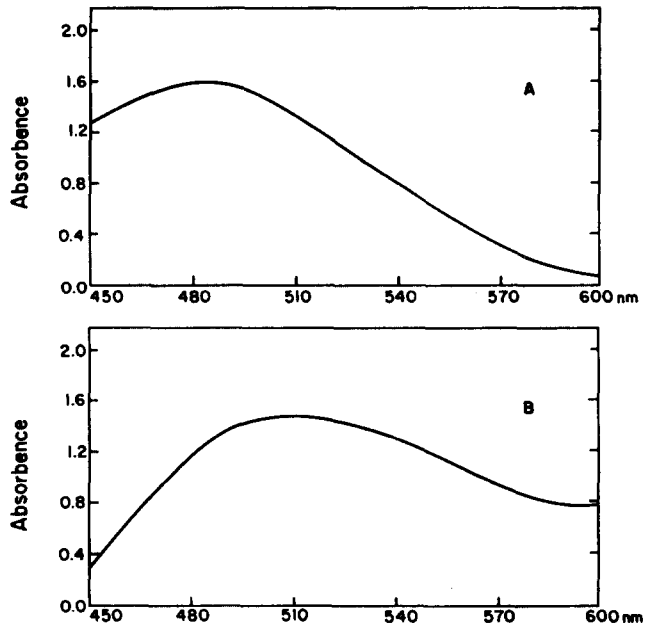

Fig. 5. Visible absorption spectra of congo red in 0.1 M NaOH solution in the absence (A) and in the presence (B) of auritoxin at room temperature. Absorption maximum of congo red shifted 490 to 510 nm.

Table II. Contents of the amino acids in the protein moiety of auritoxin

Amino acid	Mole %
L-Valine	35.40
L-Lysine	13.34
L-Histidine	13.20
L-Methionine	8.70
L-Leucine	5.03
L-Isoleucine	4.83
L-Phenylalanine	4.56
L-Cysteine	2.81
L-Arginine	2.69
L-Tyrosine	2.63

Ammonia was detected.

that the polysaccharide contents were 73.3%, 93.9%, 112.8% and 196.5%. The best result was obtained with the mixture of glucose, xylose and mannose.

Total protein content of auritoxin was measured by Lowry-Folin method using bovine serum albumin as a standard protein. The protein content was 6.8%. Ten amino acids were detected by automatic amino acid analyzer (Table II). Thus, auritoxin is composed of 93.9 % polysaccharide and 6.8% protein. The polysaccharide linkage configuration was determined by using α-glucosidase and β-glucosidase. One mg of auritoxin released 0.019 mg and 0.135 mg glucose by the α- and β-glucosidase treatments, respectively. Thus the polysaccharide moiety of auritoxin consisted of 12.3% α-linkage and 87.7% β-linkage. Auritoxin also shifted maximum absor-

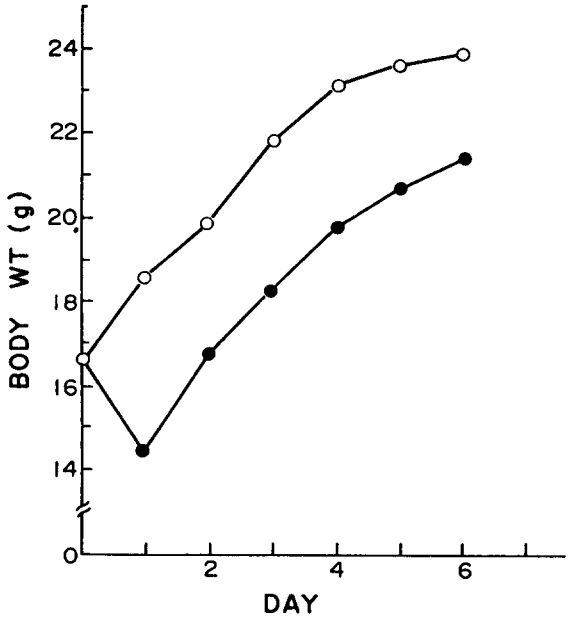

Fig. 6. Effects of auritoxin on the body weight of ICR female mice. A dose of 30 mg/kg in 0.05 ml was i.p. injected. Each group was 10 mice and one mouse of the treated group was died after one day.
○—○: control, ●—●: treated

ption peak of Congo red to long wave number, i.e., 490 nm to 510 nm (Fig. 5). These data support the conclusions that the saccharides of auritoxin are mainly linked through the β-configuration.

Toxicities of Auritoxin

Median lethal doses of auritoxin by i.p., s.c. and p.o. administrations in ICR female mice were performed by Litchfield-Wilcoxon method. Their values were 56.4, 157.2 and 454.6 mg/kg by i.p., s.c. and p.o. administrations, respectively. And their 95% confidence limits were 47.6-66.8, 126.6-195.3 and 316.4-653.3 mg/kg, respectively.

When a dose of 30 mg/kg of auritoxin was injected intraperitoneally into ten female ICR mice, one mouse died after one day. The body weights of the remainder decreased rapidly at first but began to increase after one day (Fig. 6). On the sixth day after injection, the average body weight of the injected group was 90.1% of that of the control group. When changes in percentage were expressed on the basis of the ratio of each organ weight to the body weight, those of the spleen, liver, and kidney were 183.0%, 121.2% and 99.5%, respectively (Table III).

The typical signs of intoxication by auritoxin were convulsion during the first 30 minutes after i.p. injection, then coma within an hour, various other signs such as tremor, lacrimation, nasal and opthalmic bleeding, congestion and death were observed in 24 hours.

Effects of auritoxin on human platelet aggregation

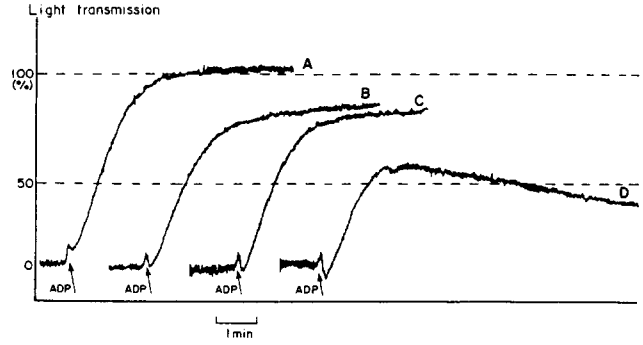

Fig. 7. Effects of auritoxin I and II on the ADP induced human platelet aggregation.
A: control, B: auritoxin I (100 μg/ml), C: auritoxin II (100 μg/ml), D: auritoxin I (1200 μg/ml)

Table III. Effects of auritoxin of *A. polytricha* on the various organs of ICR mouse

	Control (g)	Treated (g)	Weight change (%)
Body weight (At day 0)	16.62± 0.27	16.70± 0.32	
Body weight (After 6 dyas)	23.90± 0.27	21.54± 0.68 ($p<0.01$)	90.1
Spleen/kg body wt	6.70± 0.45	12.32± 0.82 ($p<0.001$)	183.0
Liver/100g body wt	6.66± 0.19	8.07± 0.41 ($p<0.01$)	121.2
Kidney/100g body wt	1.89± 0.06	1.88± 0.08 (NS)	99.5

Values are mean± SD one group was 10 mice.
NS: not significant

$$\text{Weight change (\%)} = \frac{\text{weight of treated group}}{\text{weight of control group}} \times 100$$

were measured with a Chronolog aggregometer. One hundred μg/ml auritoxin I and 100 μg/ml auritoxin II inhibited platelet aggregation 18.5% and 21.5%, respectively. These aggregation patterns were monophasic and primary aggregation (Fig. 7). Effects of auritoxin on the activity of key enzymes such as leucine aminopeptidase, α-amylase, β-amylase, esterase, malic dehydrogenase, lactic dehydrogenase (LDH) and alkaline phosphatase were examined *in vitro*. Among the enzymes tested, auritoxin inhibited the activity of LDH dose-dependently. At concentration of 250, 500 and 1000 μg/ml, auritoxin inhibited LDH activity 87.0%, 83.0% and 76.2%, respectively, relative to that of the controls.

DISCUSSION

We isolated high-molecular weight toxins, auritoxins I and II, from *Auricularia polytricha*, which is one of the favorite edible mushrooms in the Asia. Auritoxins I and II had the same molecular weights, 1.5×10^6 dalton. Elemental analysis of these toxins showed simi-

lar values. The IR and proton NMR spectra of auritoxins I and II also gave similar patterns. These results indicate that auritoxins I and II are almost identical.

Auritoxin gave positive responses, except in the iodine test in the polysaccharide color tests. These data indicate that auritoxin differs structurally from starch. An accurate total polysaccharide content was obtained by calibration with an appropriate mixture of the constituent sugars: glucose, xylose and mannose. Such a technique is necessary because different monosaccharides show different optical density values after reaction with anthrone (Norris and Ribbons, 1971). Thus in case of heteropolysaccharides, the exact polysaccharide content can be obtained by the calibration curve of a mixture of constituting monosaccharides.

The polysaccharide linkage modes of auritoxin were found to be β-linkage mainly. Nanba and his coworkers reported that glucose was detected only in the hydrolysate by β-glucosidase (Nanba *et al.*, 1987). However, there are many reports that polysaccharides have both α- and β-linkages in their molecules, such as PS-K of *Coriolus versicolor* (Tsukagoshi and Ohashi, 1974), polysaccharide of *Grifola frondosa* (Michell and Scurfield, 1967) and galactomannan of *Cordyceps sinensis* (Kiho *et al.*, 1986). It was reported that β-glucan has characteristic peaks at 890 cm^{-1} and 1640 cm^{-1} in its IR spectrum (Michell and Scurfield, 1967). The IR spectrum of auritoxin showed the same peaks in these regions. Thus, this is additional evidence that auritoxin had a polysaccharide of β-linkage.

Auritoxin was obtained by the ammonium sulfate precipitation method which does not require heat treatment. However it was found later that auritoxin was very heat stable.

Toxicities of polysaccharides, such as polygalactan, λ-carrageenans, were reported by Thomson and Home (1976). Toxic lectin from tora bean was reported by Hayashi *et al.* (1985). The median lethal dose of tora bean lectin was 240 mg/kg. Thus auritoxin was more toxic than tora bean lectin. Among the various organs of the mice, the spleen was enlarged remarkably by auritoxin. Steele and Lowes (1979) also reported toxicity on spleen by the injection of carrageenan. The signs of intoxication by the administration of auritoxin were convulsion, coma, tremor, lacrimation, nasal bleeding, congestion. These signs were quite different from those of amatoxins, pallotoxins, volvatoxin or flammutoxin, but similar with those of *Tricholomopsis platyphylla*, which showed sweating, dizziness, convulsion and spasm after eating it as food (Goos and Shoop, 1980). Auritoxin also inhibited MDH activity, which plays important roles in TCA cycle.

In the United States and some parts of Europe, physicians often encountered patients complaining of numbness at the back of the neck, headache, palpitation, lacrimation and other unpleasant symptoms after their eating foods at Chinese restaurants (Kwok, 1968). These unusual symptoms are therefore called "Chinese restaurant syndrome". The physicians attributed it to monosodium glutamate that is abundantly used as an artificial seasoning in Chinese foods, or to hypernatremia due to salt intake. However, we suggest that auritoxin may be involved in causing the syndrome.

As far as we know, this is the first report on the acute toxicity of the protein-bound polysaccharide of the famous edible mushroom, *Auricularia polytricha*, when it was injected into mice. Since the mushroom has been consumed as a food without apparant toxic manifestations by humans for so many years, our results can be at least a warning to the public for its unusual properties. Further studies on the exact toxic mechanisms of auritoxin are in progress.

CONCLUSION

High molecular weight toxic compound (1.5×10^3 kDa), auritoxin, was isolated from *Auricularia polytricha* which is well known edible mushroom. Auritoxin was composed of 93.9% polysaccharide and 6.8% protein. The polysaccharide moiety was found to have 87.7% β-linkage and 12.3% α-linkage and to be a heteromannoglucan which consisted of 45.1% glucose, 43.9% mannose and 11.0% xylose. The protein moiety contained ten amino acids. The median lethal doses of auritoxin were 56.4, 157.2 and 454.6 mg/kg by *i.p.*, *s.c.* and *p.o.* administrations, respectively. Major signs of the intoxication were convulsion during the first 30 minutes after the injection, coma or sleeping within an hour, and tremor, lacrimation, nasal bleeding, congestion and death in 24 hours. Among the various organs of the mice, the spleen was found to be enlarged remarkably. Human platelet aggregation was inhibited by the addition of auritoxin. The activity of malic dehydrogenase *in vitro* was inhibited by the toxin.

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