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An Antibacterial Lectin from *Lampteromyces japonicus*

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화경버섯의 항세균성 렉틴

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ABSTRACT: A lectin was isolated from the fruiting bodies of *Lampteromyces japonicus* by preparative PAGE and named LJAP (*Lampteromyces japonicus* antibacterial protein). LJAP was a polymeric protein of more than one hundred kDa consisting of 17-kDa subunits. The amino acid analysis revealed a high content of serine, glycine, and acidic amino acids. LJAP has an excellent antibacterial activity for *Escherichia coli*, JM 109, K 12, HB 101, and JW 380. By the inhibition assay of the antibacterial activity, a glycoprotein, asialofetuin was confirmed as the best inhibitor. This is the first lectin isolated and characterized its antibacterial and agglutination activities from the family *Lampteromyces*.

KEYWORDS: ***Lampteromyces japonicus*, Antibacterial protein, Lectin, Antibacterial activity, Agglutination activity.**

Many antibacterial proteins have been isolated from microorganisms, plants and animals. Among them, relatively few studies have been conducted on antibacterial proteins from mushrooms; it is highly possible that antibacterial proteins which have unique properties will be found from mushrooms. Meanwhile, many mushrooms produce glycoproteins and some of them have been isolated and identified as lectins (Guillot *et al.*, 1983; Horejs and Kocourek, 1978; Kochibe and Matta, 1989; Presant and Kornfeld, 1972; Sage and Vazquez, 1967; Sage and Connett, 1969; Sueyoshi *et al.*, 1985; Sychrová *et al.*, 1985; Tsuda, 1979). The isolation or binding properties of some mushroom lectins were reported by Kawagishi *et al.* (Kawagishi and Mizuno, 1988; Kawagishi *et al.*, 1990; Kawagishi and Hori, 1991). However their biological functions are still unknown. In this paper we describe the screening of antibacterial proteins from 30 mushroom samples and also describe the isolation and properties of a protein which has both antibacterial and agglutination activities from the mushroom *Lampteromyces japonicus. Lampteromyces japonicus* protein was named LJAP.

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Materials and Methods

Materials

Fruiting bodies of 30 mushrooms were collected at Kwangchu, Kyungki-do, Korea, frozen upon collection, and stored at −20℃. Bacterial strains used for the assay of antibacterial activity were obtained from Genetic Engineering Research Institute of KIST. Toyopearl 55 S, CM-Toyopearl, DEAE-Toyopearl and AF-amino-Toyopearl were

obtained from Tosoh (Japan). Sephadex G-150 and Sepharose 4B were purchased from Pharmacia (Sweden). ZetaPrep 60 disk SP was obtained from Cuno (U.S.A.). YM-5 for ultrafiltration was product of Amicon (U.S.A.). Glycoproteins and lactulose for the inhibition test of antibacterial action were products of Sigma (U.S.A.). The other sugars for the test were D-forms and obtained from Nacalai Tesque (Japan). All the other chemicals were reagent grade.

Preparation of Affinity Adsorbents

Fetuin, asialofetuin, bovine submaxillary mucin (BSM), or asialo-BSM was conjugated to AF-amino-Toyopearl by following the instructions of the manufacturer.

Extraction of Antibacterial Materials from Mushrooms and Isolation of an Antibacterial Protein from *Lampteromyces japonicus*

All the procedures were done at 4℃ except for defrosting the fruiting bodies. The frozen fruiting bodies were defrosted at room temperature, immediately homogenized in a blender with saline, and extracted with stirring overnight. The resulting suspension was filtered with gauze and the filtrate was centrifuged (10,000×g) to remove insoluble residues. The supernatant was dialyzed, first against tap water then de-ionized water, and lyophilized. The lyophilized material was redissolved in distilled water and used for the screening test. Especially the lyophilized material obtained from mushroom *Lampteromyces japonicus* was used for preparative polyacrylamide gel electrophoresis (PAGE). After the electrophoresis, the gel was cut in thin slices, and each slice was homogenized in a small centrifuge tube and extracted with 10 mM phosphate-buffered saline, pH 7.4 (PBS) overnight. The suspension was centrifuged in the tube and then the supernatant obtained was filtered and tested the antibacterial activity. The active fraction was desalted by ultrafiltration with YM-5 and lyophilized, giving the purified antibacterial protein (LJAP).

Assay of Antibacterial Activity

Antibacterial activity was assayed essentially as described by Okada and Natori (1983). Briefly, *E. coli* K 12 (594, str^r) grown in antibiotic medium (Difco) was collected in the exponential phase of growth and suspended in 10 mM phosphate buffer (pH 6.0) containing 130 mM NaCl (buffer A) at a density of 2.5×10^8 cells/m*l*. In practice, A_{650} was adjusted to 0.3. Sample (LJAP) was diluted serially with buffer A containing 0.2% (w/v) bovine serum albumin, and diluted sample (200 μ*l*) was incubated with antibiotic medium (190 μ*l*) and *E. coli* suspension (10 μ*l*) in a test tube at 37℃ for 160 min with shaking. The mixture was then rapidly chilled, and its A_{650} was measured. One unit of antibacterial activity is defined as the amount causing 50% inhibition of bacterial growth relative to the control.

The method of Hultmark *et al.* (1980) was used for qualitative demonstration of the presence of antibacterial activity (for screening test). For this, samples (the lyophilized materials of 30 mushroom extracts) were electrophoresed in polyacrylamide gel under nondenaturing conditions. The gels were then incubated in medium containing 0.2 M phosphate buffer, pH 7.4. They were then overlaid with the same medium containing 0.7% agar and 2.5×10^6 cells/m*l* of the bacteria. This layer was overlaid by another layer of agar without bacteria, and the gel was incubated at 37℃ for 18∼20 h. Materials having antibacterial activity gave clear spots due to inhibition of bacterial growth on the gel.

Erythrocytes

Human blood was collected in 3% sodium citrate. The erythrocytes were washed three times with PBS and suspended at a concentration of 3% in the buffer.

Enzyme Treatment of Erythrocytes

A 3% suspension of erythrocytes in PBS (10 m*l*) was treated with Pronase P (7 mg) for 30 min at 47℃, then washed three times with the buffer and suspended at a concentration of 3% in the buffer.

Hemagglutination Test

Agglutination of 3% erythrocytes and inhibition

of the agglutination by sugars and glycoproteins were done in microtiter U-plates. The titer was defined as a reciprocal of the end-point dilution causing hemagglutination. Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of hemagglutination of titer 4 of LJAP.

PAGE

Electrophoresis under nondenaturing conditions was carried out in 10% (w/v) polyacrylamide slab gels at pH 7.0~9.5. Preparative PAGE was done in 7.5% gel at pH 9.5. SDS-PAGE was done by the method of Laemmli (1970). Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100℃. Gels were stained with Coomassie Brilliant Blue. The molecular weight standards (Pharmacia, Sweden) used were phosphorylase b (M_r 94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400).

Gel Filtration for Molecular Weight Estimation

Gel filtration for measuring the molecular weight of native form of LJAP was carried out on a column (1.5×100 cm) of Toyopearl 55 S or Sephadex G-150 equilibrated with PBS containing 0.1% SDS. Standard proteins (Serva Feinbiochemica, U.S.A.) used were aldolase (Mr 160,000), bovine serum albumin (67,000), egg albumin (45,000), myoglobin (17,800) and blue dextran was used for determination of the void volume of the column.

Amino Acid Analysis

Amino acids were analyzed with a Hitachi model 835 amino acid analyzer after hydrolysis of LJAP in 6 M HCl at 110℃ for 20 h in a sealed evacuated tube.

Results and Discussion

Bacterial strains used for the assay of antibacterial activities of saline extracts which were obtained from 30 mushroom samples are listed in Table 1. The results of the antibacterial activity assay are shown in Table 2. *Lampteromyces japonicus* has the strongest antibacterial activity against the all bacteria used in this assay.

Table 1. Bacterial strains used for the assay of antibacterial activities of saline extracts from 30 mushroom samples.

Species	Strains
Salmonella typhimurium	ATCC[1] 29629[T][3]
Enterobacter aerogenes	ATCC 13048[T]
Escherichia coli JM 109	ATCC 26521[T]
Escherichia coli K 12	NCIB[2] 10218[T]
Escherichia coli HB 101	ATCC 33694[T]
Escherichia coli JW 380	ATCC 19219[T]
Serratia marcescens	ATCC 27117[T]
Micrococcus luteus	ATCC 4698[T]
Staphylococcus aureus	ATCC 65389[T]

[1]ATCC: American Type Culture Collection, Rockville, Md., USA.
[2]NCIB: National Collection of Industrial Bacteria, NCIMB Ltd., Aberdeen, Scotland, UK.
[3]T: Type Strain.

The antibacterial activity and agglutination activity of the saline extract of the mushroom *Lampteromyces japonicus* could not be recovered completely by salting out with ammonium sulfate even at 100% saturation. Therefore the extract was put directly through affinity chromatography on a fetuin, an asialofetuin, a BSM, or an asialo-BSM column, since hemagglutination by the extract was inhibited by these four glycoproteins. All materials which had activities were adsorbed to the columns, but any conditions used [0.2 M NH_4OH, 1 M NaOH, 3 M KSCN, 1 M AcOH, 0.1 M glycine-HCl (pH 3.0), asialofetuin (10 mg/ml), fetuin (10 mg/ml), asialo-BSM (10 mg/ml), or BSM (10 mg/ml)] could not elute the protein at all. We also attempted other chromatographies for purification of the protein and those attempts gave similar results; the protein was adsorbed to DEAE-Toyopearl, CM-Toyopearl, Toyopearl 55 S, Sepharose 4B, and Sephadex G-150 columns, and a ZetaPrep-SP disk at various pH. No activities were recovered under the conditions mentioned above.

Table 2. Antibacterial activities of the saline extracts obtained from 30 mushroom samples.

Mushrooms	Antibacterial activities								
	Salmonella typhimurium	*Enterobacter aerogens*	*Escherichia coli* JM 109	*Escherichia coli* K 12	*Escherichia coli* HB 101	*Escherichia coli* JW 380	*Serratia marcescens*	*Micrococcus luteus*	*Staphylococcus aureus*
Hygrophorus eburneus	−	−	−	−	−	−	−	−	−
Hygrophorus pratensis	−	−	−	−	−	−	−	−	−
Hygrophorus subviolaceus	−	−	−	−	−	−	−	−	+
Lampteromyces japonicus	+	+	++	++	++	++	+	+	+
Clitocybe candicans	−	−	+	+	+	+	−	−	−
Tricholomopsis rutilans	−	−	−	−	−	−	−	−	−
Tricholoma matsutake	−	−	−	−	−	−	−	−	−
Flammulina velutipes	−	−	−	−	−	−	−	−	−
Panus rudis	−	−	−	−	−	−	−	−	−
Pleurotus ostreatus	−	−	−	−	−	−	−	−	−
Russula adusta	−	−	−	−	−	−	−	−	−
Volvariella volvacea	−	−	−	−	−	−	−	−	−
Agaricus bisporus	−	−	−	−	−	−	−	−	−
Paxillus atrotomentosus	−	−	−	−	−	−	−	−	−
Stecchernium achraceum	−	−	−	−	+	+	−	−	−
Lenzites betulina	−	−	+	−	−	−	−	−	−
Microporus affinis	−	−	−	−	−	−	−	−	−
Daedaleopsis tricolor	−	−	−	−	−	−	−	+	−
Hirschioporus abietinus	−	−	−	−	−	−	−	−	−
Cryptoderma citrinum	+	−	−	−	−	−	−	−	−
Calvatia craniiformis	−	−	−	−	−	−	−	−	−
Agaricus placomyces	−	−	−	−	−	−	−	−	−
Psathyrella hydrophila	−	−	−	−	−	−	−	−	−
Stropharia rugosoannulata	−	−	−	−	−	−	−	−	−
Inocybe lacera	−	−	+	+	−	−	−	−	−
Cortinarius cinnanomeus	−	−	−	−	−	−	+	−	−
Phylloporus bellus	−	−	−	−	−	−	−	−	−
Suillus pictus	−	−	−	−	−	−	−	−	−
Cantharellus cibarius	−	−	−	−	−	−	−	−	−
Auricularia auricula-judae	−	−	−	−	−	−	−	−	−

*Symbols: +; positive, ++; strong positive, −; negative

Table 3. Purification of LJAP from the fruiting bodies (800 g) of *Lampteromyces japonicus*.

Fraction	Total protein[1] (mg)	Total antibacterial activity[2] (units)	Specific antibacterial activity (units/mg protein)	Recovery of antibacterial activity[3] (%)	Total agglutination activity (titer)[4]	Specific agglutination activity (titer/mg protein)	Recovery of agglutination activity[3] (%)
Saline extract	1.480	1.640	1.1	100	2870	1.9	100
Eluate from PAGE	1.1	520	472.7	31.7	1456	1323.6	50.7

[1] Protein was determined by the method of Lowry *et al* (1951).
[2] Antibacterial activities were measured as described in the methods.
[3] Based on the saline extract.
[4] Titer defined as the reciprocal of the end-point dilution showing the hemagglutination with Pronase-treated type A erythrocytes in PBS.

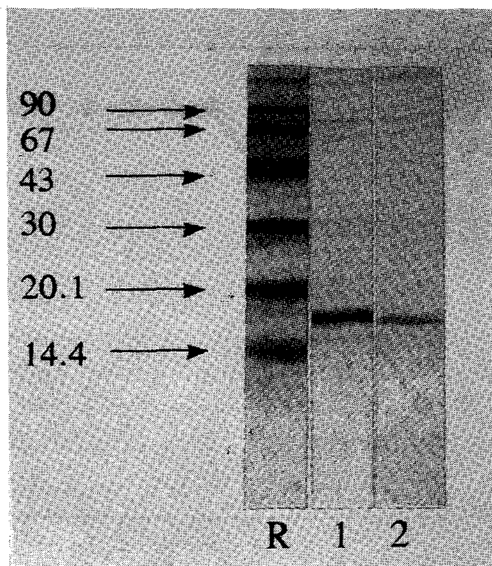

Fig. 1. SDS-PAGE of LJAP in the presence (lane 1) and absence (lane 2) of 2-mercaptoethanol. Lane R shows molecular mass (in kDa) of marker proteins.

Only 0.1% SDS could elute the protein from the adsorbents or matrices. After all we succeeded in purification by using preparative PAGE although the recovery of the antibacterial activity (32%) was not satisfactory. After electrophoresis the gel was extracted with PBS and the extract was desalted by ultrafiltration with YM-5, lyophilized, giving the purified protein (Table 3).

The purified protein obtained by elution from PAGE gel yielded a band corresponding to the molecular mass of 17-kDa on SDS-PAGE both in reducing and non-reducing conditions as shown in Fig. 1. Since the SDS-eluted protein from affinity columns gave the same band on SDS-PAGE (data not shown), we concluded that this protein (LJAP) obtained from the preparative PAGE was a purified lectin. The size homogeneity of this lectin was confirmed again by gel filtration. The lectin appeared as a sharp single peak shown in gel filtration on a Toyopearl 55 S (Fig. 2) or Sephadex G-150 column using PBS containing 0.1% SDS as a running buffer. The antibacterial and agglutination activities of the lectin were not affected in the buffer. However, when PBS containing 1% SDS was used as a running buffer, the lectin was inactivated and the molecular mass of the inactivated one was estimated at 1.0 or 2.0-kDa by gel filtration using the two matrices, respectively. Therefore it was assumed that the activities and the native form of the lectin were retained at concentration of 0.1% SDS and the native lectin occurred as a polymer of more than one hundred kDa having no disulfide bond between each subunit of 17-kDa. Similar multimeric lectins are known; a hemagglutinin from the coelomic fluid of the sea urchin *Anthocidaris crassispina* which has a

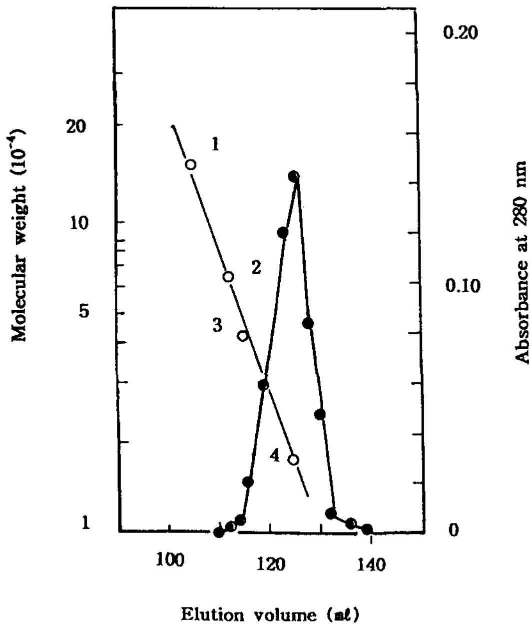

Fig. 2. Gel filtration of LJAP on a Toyopearl 55 S column (1.5×100 cm) equilibrated with PBS containing 0.1 % SDS. The molecular weight markers used were: 1 aldolase (M_r 160,000), 2 bovine serum albumin (67,000), 3 egg albumin (45,000), 4 myoglobin (17,800).

300-kDa molecular mass and consists of 13-kDa subunits (Giga *et al.*, 1985). The molecular masses of native forms of all the known lectins from mushrooms are below 100-kDa. Such a high molecular mass lectin is without precedent in mushroom lectins. Amino acid composition of the lectin is presented in Table 4. These data show a high content of serine, glycine, and acidic amino acids. The hydrophilicity of the lectin, which was not salted out by ammonium sulfate even at 100% saturation, might be explained in terms of the higher content of hydrophilic amino acids of the protein, but there is discrepancy between a high content of hydrophilic amino acids and non-specific hydrophobic interaction with chromatographic adsorbent and matrices; these might be due to localization of hydrophobic amino acids in its primary structure. It is necessary to determine the complete primary structure to verify this speculation.

Table 4. Amino acid composition of LJAP from *Lampteromyces japonicus*

Amino acid	mol %	Amino acid	mol %
Asx	8.6	Ile	2.0
Thr	6.8	Leu	4.3
Ser	18.3	Tyr	2.0
Glx	13.9	Phe	3.0
Gly	18.2	Lys	2.2
Ala	8.4	His	2.3
Val	2.0	Arg	7.8
Met	0.0	Pro	0.2

Table 5. Inhibition of the antibacterial activity of LJAP by glycoproteins.

Inhibitor	Minimum inhibitor concentration* (μg/ml)
Asialofetuin	1.09
Fetuin	5.88
Asialo-BSM	71.2
BSM	76.5
1-Acid glycoprotein	121

* Minimum concentration required for inhibition of 3 units antibacterial activity dose of LJAP.

The antibacterial activity of LJAP was inhibited only by five glycoproteins (Table 5): asialofetuin was the best inhibitor, and the second was fetuin; asialo-BSM, BSM, and α_1-acid glycoprotein had very weak inhibitory activity on antibacterial action of the lectin. And also, asialofetuin and fetuin had strong inhibitory activity on hemagglutination by the lectin. The following mono- and oligosaccharides have no effect on the antibacterial activity up to a concentration of 0.2 M: glucose, galactose, mannose, ribose, N-acetylglucosamine, N-acetylgalactosamine, lactose, lactulose, and N-acetylneuraminic acid. Lectins are classified into two groups according to their carbohydrate specificity: first, exolectins and second, endolectins. Endolectins will recognize complex oligosaccharides and the agglutination by which can be inhibited only

by specific sugar sequences. According to this definition LJAP can be classified as endolectins. This is the first lectin isolated and characterized its antibacterial and agglutination activities from the mushroom *Lampteromyces japonicus*.

적 요

화경버섯의 자실체로부터 PAGE로 한 렉틴을 정제하고, LJAP(*Lampteromyces japonicus* antibacterial protein)라 이름 붙였다. LJAP는 17-kDa의 서브유닛트가 모여서 된 백 kDa이 넘는 회합 단백질이였다. 이 단백질은 아미노산 분석결과 세린, 글리신, 산성 아미노산의 함량이 높았다. LJAP는 대장균중 특히 JM 109, K 12, HB 101, JW 380에 대한 항세균작용이 뛰어났다. 한편, 항세균활성은 당단백질인 asialofetuin에 의하여 크게 억제되었다. 본 당단백질은 화경버섯에서 처음으로 얻어진 항세균활성과 적혈구 응집활성을 가진 렉틴이다.

감사의 말

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