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Isolation and Characterization of a Lectin from Edible Mushroom, *Volvariella volvacea*

Jung-Yaw LIN and Tze-Bin CHOU

Institute of Biochemistry, College of Medicine, National Taiwan University, Taipei, Taiwan

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A lectin was purified from edible mushroom, *Volvariella volvacea* by extraction with 5% cold acetic acid in the presence of 0.1% 2-mercaptoethanol, followed by ammonium sulfate fractionation, and DEAE-C-52 and CM-C-52 column chromatographies. The molecular weight was measured to be 26,000, and the lectin consisted of two non-identical subunits as demonstrated by gel filtration and polyacrylamide gel electrophoresis.

The lectin does not contain half-cystine, methionine, or histidine. The LD_{50} of the lectin is 17.5 mg per kg body weight of mice. The lectin has a moderate inhibitory effect on the growth of tumor cells.

Lectins of plant origin are very useful in studying cell membrane structure, and they have been used for the synthesis of chimeric proteins with the A chain of ricin for inhibiting the growth of tumor cells (*1*). Both activities of lectins are due to their characteristic of specific affinities toward glycoproteins or glycolipids.

In a previous investigation, a hemolytic protein, volvatoxin A, was isolated from *Volvariella volvacea* (*2*). This report describes the isolation of a new antitumor protein, *Volvariella volvacea* lectin, and the chemical, physical, and biological properties of the lectin.

MATERIALS AND METHODS

The edible mushroom, *Volvariella volvacea*, was purchased from a local market. TPCK-trypsin, chymotrypsinogen A and ovalbumin were obtained from Worthington Co., U.S.A. Bovine serum albumin and cytochrome *c* were products of Sigma Chemical Co.

Hemagglutination—Red blood cells from humans (O type) and various animals were washed with 0.01 M phosphate-buffered saline, pH 7.4. Various amounts of lectin were added to the washed red blood cells, and the reaction mixtures were incubated at 37°C for 2 h. The degree of hemagglutination was assessed according to the pattern formed by agglutinated cells on the bottom of tubes (*3*).

Determination of Protein—Quantitative determination of protein was performed according to the method of Lowry *et al.* (*4*).

Hemolysis—Degree of hemolysis was measured as described by Henden and Frankel-Conrat (*5*).

Determination of Molecular Weight—The mo-

Abbreviations: VAG, *Volvariella volvacea* lectin; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; MSH, 2-mercaptoethanol.

lecular weight of lectin was determined by SDS-polyacrylamide gel electrophoresis and by gel filtration with a Sephadex G-75 column. SDS-polyacrylamide gel electrophoresis was performed according to the method of Weber and Osborn (*6*). A 10% separation gel was used for these purposes. Staining was carried out with 0.15% Coomassie Brilliant Blue in 10% acetic acid. Gel filtration was carried out according to the method of Andrews (*7*).

Amino Acid Analysis—Protein was hydrolyzed with constant-boiling HCl in evacuated, sealed tubes at 105°C for 24 h. The amino acid compositions in the hydrolysates were measured with a Beckman amino acid analyzer (model 120C) (*8*). Tryptophan was determined by the spectrophotometric method of Edelhoch (*9*).

Tryptic Map Analysis—VAG (20 mg) was digested with trypsin by using a pH Stat (Radiomer) at pH 8.6 and 37°C. The reaction was terminated by addition of 6 M acetic acid to pH 4. The digests were analyzed by paper electrophoresis at pH 1.9 (formic acid : acetic acid : water, 1 : 4 : 45, v/v) followed by paper chromatography using BAWP as a solvent system (butanol : acetic acid : pyridine : water, 75 : 50 : 15 : 60, v/v) (*10*). The tryptic peptides were detected with 0.05% ninhydrin in ethanol.

Disc Polyacrylamide Gel Electrophoresis—The disc polyacrylamide gel electrophoresis was performed by a modification of the method of Gabriel (*11*). Beta-alanine buffer, pH 4.6, and Tris-glycine buffer, pH 8.5, were used as tray buffers. In both cases, 6% separation gel in 8 M urea was used.

Lethality—The lethality of lectin was determined according to the method of Reed and Mueuch (*12*).

Evaluation of Tumor Growth—Sarcoma 180 cells were used to determine the antitumor activity of lectin. Tumor inoculation was carried out by injection of 0.2 ml of ascitic fluid containing 2×10^7 cells. A single dose of lectin was injected ip 1 h after tumor transplantation. The growth of Sarcoma 180 tumor was evaluated by determining the increase of life span (*13*).

RESULTS

Purification of VAG—A mixture of 2 kg of edible mushrooms (*Volvariella volvacea*) and 2 liters of cold 5% acetic acid containing 0.1% 2-mercaptoethanol was homogenized with a blender. The homogenate was kept at 4°C for 3 h, and then centrifuged at 10,000 rpm for 10 min with a Sor-

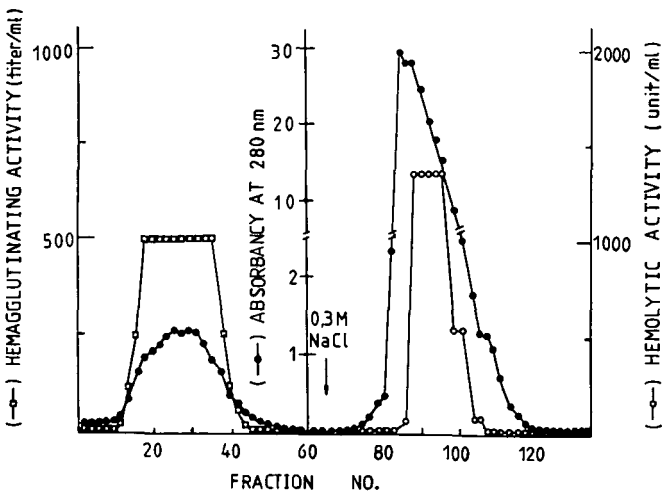

Fig. 1. Chromatography of crude extract on a DEAE-C-52 column. The supernatant of the dialysate from an ammonium sulfate fractionation was applied to a DEAE-C-52 column (4×25 cm) and eluted with 5 mM Tris buffer, pH 8.2 (60 fractions) followed by 0.3 M NaCl in 0.01 M Tris buffer, pH 8.2. The active fractions of hemagglutinating activity were pooled for the following experiments. Fractions of 15 ml each were collected.

vall RC-2B refrigerated centrifuge. Solid ammonium sulfate was added to the supernatant at 4°C to make 95% saturation. The precipitate was pelleted by centrifugation at 10,000 rpm for 10 min, and dialyzed against 5 mM sodium acetate containing 0.1% 2-mercaptoethanol and 1 mM EDTA at 4°C for 48 h with three changes of the buffer. The supernatant of the dialysate was subjected to fractionation by anion exchange column chromatography with a DEAE-C-52 column (4× 25 cm). The hemagglutinating activity was eluted with the equilibrium buffer, but it was found to be heterogeneous (Fig. 1). The peak containing hemagglutinating activity was further fractionated by cation exchange column chromatography with a CM-C-52 column (2× 1.2 cm). The results are shown in Fig. 2. The peak eluted with 0.2 M NaCl had hemagglutinating activity, and it was pooled, dialyzed against distilled water at 4°C and lyophilized. The result of a typical purification of VAG is summarized in Table I. About 95 mg of purified VAG was obtained from 2 kg of the edible mushrooms.

Amino Acid Composition—The amino acid contents of a hydrolysate of VAG are summarized in Table II. No half-cystine, methionine, or histidine residue was detected in the hydrolysate. The lectin contained an unusually large amount

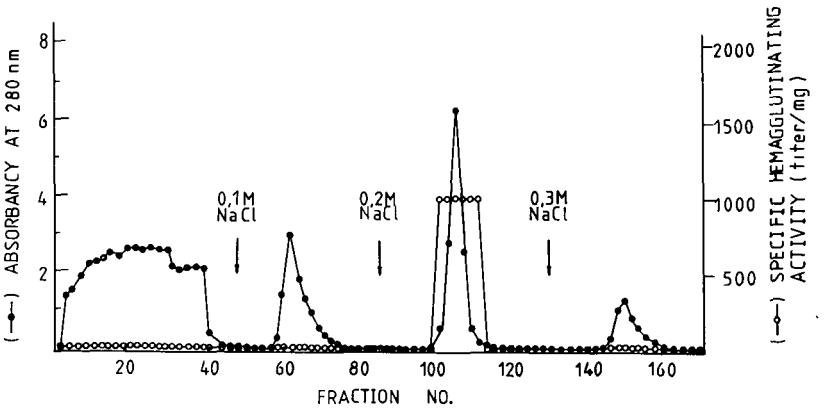

Fig. 2. Chromatography of partially purified VAG on a CM-C-52 column. The active fractions obtained from the DEAE-C-52 column were applied to a CM-C-52 column (2×1.2 cm). The column was eluted with 0.1 M, 0.2 M, 0.3 M NaCl in 0.01 M acetate buffer, pH 4.0. Fractions of 4 ml each were collected.

TABLE I. Purification of VAG from 2 kg of mushrooms (*Volvariella volvacea*).

Fraction	Total protein (mg)	Total hemagglutinating activity (titer)	Specific hemagglutinating activity (titer/mg)	Protein recovery (%)	Hemagglutinating activity recovery (%)
Crude extract with acetic acid [a]	5,522	— [c]	— [c]	100	— [c]
95% A.S. precipitate [b]	4,448	— [c]	— [c]	80	— [c]
DEAE-C-52 column chromatography	280	1.43×10^5	512	5	100
CM-C-52 column chromatography	95	9.70×10^4	1,024	1.7	67.5

[a] From 2 kg of mushrooms. [b] Ammonium sulfate. [c] The hemagglutinating activity could not be detected because this fraction showed a strong hemolytic activity.

TABLE II. Amino acid composition of VAG. Values are the means of three different analyses.

Amino acid	Residues per mol of protein [a]	Integer
Lys	13.8	14
His	—	—
Arg	6.0	6
Asp	30.0	30
Thr	14.4	14
Ser	13.2	13
Glu	20.4	20
Pro	9.0	9
Gly	16.8	17
Ala	12.6	13
1/2 Cys	—	—
Val	15.0	15
Met	—	—
Ile	11.4	11
Leu	18.0	18
Tyr	19.8	20
Phe	10.4	10
Trp [b]	3.4	3
Total		213

[a] The molecular weight of VAG was assumed to be 26,000. [b] Measured spectrophotometrically.

of tyrosine residues.

Molecular Weight—VAG gave a single band on disc polyacrylamide gel electrophoresis at pH 4.6, but it gave two bands at pH 8.5 (Fig. 3). The molecular weight of the lectin was determined to be 26,000 ± 2,000 by gel filtration with a Sephadex G-75 column (Fig. 4A), but it was found to be 13,000 ± 1,000 by SDS-polyacrylamide gel electrophoresis (Fig. 4B).

Tryptic Peptide Map Analysis—The results of tryptic peptide map analysis are shown in Fig. 5. There are fourteen lysine and six arginine residues in one molecule of VAG with a molecular weight of 26,000, but only ten tryptic peptides were detected. This suggests that VAG consists of two non-identical subunits each with a molecular weight of 13,000 as demonstrated by disc gel electrophoresis at pH 8.5, and SDS-polyacrylamide gel electrophoresis.

Hemagglutinating Activity—The hemagglutinating activities of VAG were tested with red blood cells from different animals. The results are summarized in Table III. The red blood cells from cat or dog have the highest titer (8,192 titer/mg) of hemagglutinating activity for VAG while those from duck or chick have the lowest titer (32 titer/mg) or no hemagglutinating activity at all.

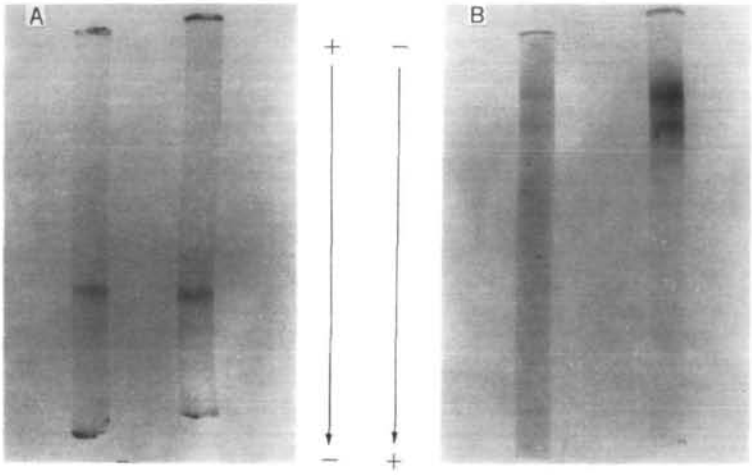

Fig. 3. Disc polyacrylamide gel electrophoresis of purified VAG. The purified VAG (about 20 μg after the step of CM-C-52 column chromatography) was applied to 6% separation gel in 8 M urea in 10% β-alanine buffer, pH 4.6, (A) or in 0.1 M Tris-glycine buffer, pH 8.5, (B). The patterns shown on the left and right sides in (A) and (B) are for VAG after and before MSH reduction, respectively. MSH reduction was carried out by treating VAG (1 mg/ml) with 5% MSH at 37°C for 2 h.

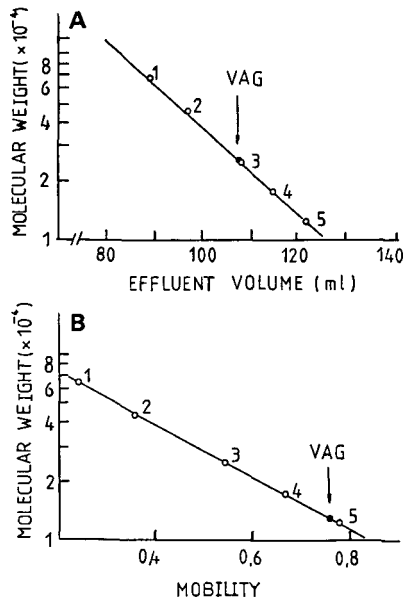

Fig. 4. Estimation of the molecular weight of VAG by gel filtration on Sephadex G-75 and by SDS-polyacrylamide gel electrophoresis. (A) Gel filtration: The Sephadex G-75 column (1.5×93 cm) was equilibrated with 0.01 M phosphate buffer, pH 7.4 and eluted with the same buffer. The flow rate was 30 ml/h, and fractions of 2 ml each were collected. Molecular weight markers used were; 1, bovine serum albumin (mol. wt. 67,000); 2, ovalbumin (mol. wt. 45,000); 3, chymotrypsinogen A (mol. wt. 25,000); 4, myoglobin (mol. wt. 17,800); 5, cytochrome *c* (mol. wt. 12,400). (B) SDS-polyacrylamide gel electrophoresis: The experiment was carried out as described in "MATERIALS AND METHODS." Molecular weight markers used were the same as those in gel filtration.

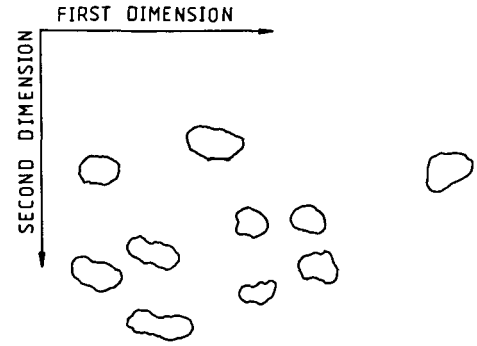

Fig. 5. Tryptic map analysis was carried out according to the method described in "MATERIALS AND METHODS." The first dimension is paper electrophoresis at pH 1.9 followed by a second dimension of paper chromatography using BAWP (butanol : acetic acid : pyridine : water, 75 : 50 : 15 : 60, v/v) as the solvent system.

TABLE III. Hemagglutinating activity of VAG.

RBC species	Specific hemagglutinating activity (titer/mg)
Human A type	64
B type	64
O type	64
AB type	128
Albino rat	1,024
Swiss mouse	1,024
Guinea pig	512
Long Evans rat	2,048
Chicken	0
Duck	32
Cat	8,192
Dog	8,192
Frog	64

The titer for VAG to agglutinate Sarcoma 180 cells was 128 per mg. The hemagglutinating activity of VAG was quite stable to high temperature. At 1 mg/ml, VAG is stable up to 80°C (2 h incubation). However, 25% of the hemagglutinating activity was lost when VAG was treated at 90°C for 30 min. The hemagglutinating activity of VAG (1 mg/ml) was also quite stable at extreme pH. It was fully active at pH 2 or 11 or 24 h. The hemagglutinating activity of VAG (1 mg/ml) was not inhibited by any of the following sugars (0.3 M) at room temperature: D-glucose, D-galactose, D-mannose, L-fucose, D-xylose, D-arabinose, lactose, maltose, raffinose, sucrose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-galactosamine. It is likely that VAG has affinity for a complex carbohydrate structure on the cell surface (*14*).

Lethality—The LD_{50} of VAG was determined to be 17.5 mg per kg body weight of mice. The lethality of VAG is much lower than that of volvatoxin A, which causes hemolysis (*2*).

Antitumor Activity—In these experiments, 12 mice were used in each group, and the survival time was expressed as mean ± S.D. The life span

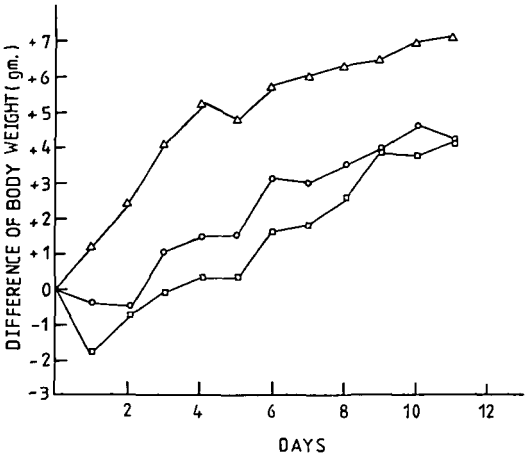

Fig. 6. Effect of VAG on the growth of experimental tumor. The experiments were carried out as described in "MATERIALS AND METHODS." △, control group; ○, 85 μg VAG/mouse; □, 175 μg VAG/mouse.

of the control group after inoculation of Sarcoma 180 cells was 12.5±5 days. The test group given 85 μg of VAG showed a 63.2% increase of life span, while the group inoculated with 175 μg of VAG showed a 109.7% increase. The body weight changes of the control and test groups are shown in Fig. 6. VAG retarded the growth of Sarcoma 180 cells.

DISCUSSION

In this work, 2-mercaptoethanol was used during the preparation of VAG to inactivate peroxidases which were very active in the aqueous homogenate of *Volvariella volvacea* and affected the yield of VAG.

Several lectins have been purified from mushrooms. That from *Flammulina veltipes* has a molecular weight of 20,000, and can be dissociated into two non-identical subunits with molecular weights of 12,000 and 8,000 (*14*). The lectin from *Agricus campestris* has a molecular weight of 64,000 and a four-chain structure of the A_2B_2 type was proposed (*15*). Two lectins have been partially purified from *Agricus bisporus*, and one of them was found to have a molecular weight of 58,500 (*16*). The molecular weight of VAG was found to 26,000 by gel filtration at pH 7.4, but on SDS-polyacrylamide gel electrophoresis, the molecular weight of VAG decreased to 13,000. The probability that VAG consists of two non-identical subunits was raised by the amino acid composition data coupled with tryptic peptide map analysis and the result of disc gel electrophoresis at pH 8.5.

The hemagglutinating activity of VAG is similar to that of lectin isolated from *Flammulina veltipes* but quite different from those of lectins from *Agricus campestris* and *Agricus bisporus*, because no simple specific sugar can inhibit the hemagglutination caused by VAG or the *Flammulina* lectin. Though the lethality of VAG to mice is very low in comparison with that of other toxic proteins (*17*), VAG has a moderate inhibitory effect on the growth of Sarcoma 180 cells.

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