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A Novel Lectin with Potent Immunomodulatory Activity Isolated from Both Fruiting Bodies and Cultured Mycelia of the Edible Mushroom *Volvariella volvacea*

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A novel lectin has been purified from the fruiting bodies as well as cultured mycelia of the edible mushroom *Volvariella volvacea*. The lectin, designated as VVL, was a homodimeric protein with a molecular weight of 32 kDa as demonstrated by gel filtration and SDS-PAGE. VVL had no carbohydrate moiety, and its hemagglutinating activity was inhibited by thyroglobulin but not by simple carbohydrates such as monomeric or dimeric sugars. The immunomodulatory activity of VVL was demonstrated by its potent stimulatory activity toward murine splenic lymphocytes. VVL was also found to markedly enhance the transcriptional expression of interleukin-2 and interferon-γ by reverse transcriptase-polymerase chain reaction. As revealed by its N-terminal amino acid sequence, VVL possessed a molecular structure distinct from other immunomodulatory proteins previously reported in the same fungus.

Lectins are proteins/glycoproteins that have been found in various organisms (1). They are currently attracting much interest primarily because they could participate in various important biological phenomena including cell growth regulation (2), cell adhesion (3,4), induction of programmed cell death (5) and immune response (6), suggesting that lectins are multi-functional.

Many lectins have been isolated and characterized from higher fungi (e.g. mushrooms) (7-12). Several of them which possess immunomodulatory and antitumor activities (13-15) are under clinical trial (16). Recently, some fungal immunomodulatory proteins defined by amino acid sequence similarity were isolated from the mushrooms *Ganoderma lucidium* (17), *Flammulina velutipes* (18) and *Volvariella volvacea* (19). They exhibited hemagglutinating activities. In this investigation, we purified a potent immunomodulatory protein from the fruiting bodies of *V. volvacea* and elucidated it as a new fungal lectin. To further characterize this lectin, a comparison of it with a lectin isolated from the cultured mycelia of the same fungus was made.

MATERIALS AND METHODS

Materials. The straw mushroom, *V. volvacea*, an edible mushroom, was purchased from a local market. DE-52 cellulose and CM-52 cellulose were obtained from Whatman Co. (Maidstone, Kent, U.K.). Mono S HR 5/5 and Superdex 75 HR 10/30 columns were the products of Pharmacia (Uppsala, Sweden). Chemicals for sequence analysis were purchased from Hewlett Packard (Palo Alto, CA, U.S.A.). All other chemicals were of reagent grade.

Strain and cultivation of mycelia. The strain of *V. volvacea* was provided by The Center for International Services for Mushroom Biotechnology at The Chinese University of Hong Kong. The culture medium used for the strain was made up by dissolving 25 g potato dextrose broth, 0.975 g L-asparagine, 0.52 g NH_4NO_3, 10 g glucose, 0.1 g yeast extract, 1 g KH_2PO_4, 0.4 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 13 mg $CaCl_2 \cdot 2H_2O$, 4.8 mg ferric citrate, 2.64 mg $ZnSO_4 \cdot 7H_2O$, 2 mg $MnCl \cdot 4H_2O$, 0.4 mg $CoCl_2 \cdot 6H_2O$, 0.4 mg $CuSO_4 \cdot 5H_2O$ and 2.5 mg vitamin B_1 in 1L distilled water. The pH of the medium was adjusted to 6.0. The *V. volvacea* mycelium was cultivated with shaking for 7 days at 30°C.

Isolation of VVL-F. All procedures were carried out at 4°C. Fresh fruiting bodies of *V. volvacea* (2 kg) were homogenized in 2 liters of 5% (v/v) acetic acid containing 0.1% (v/v) 2-mercaptoethanol with a Waring blender. The homogenate was kept for 3 h and then centrifuged at 10,000 g for 20 min. Solid ammonium sulfate was added to the supernatant to attain 95% saturation. After stirring for 1 h, the precipitate was collected by centrifugation at 20,000 g for 30 min, dissolved in and dialyzed against 10 mM sodium phosphate buffer, pH 8.2. After centrifugation of the dialysate, the supernatant was subjected to fractionation on a DE-52 cellulose column (2.5 cm × 22 cm) equilibrated with the same buffer. The hemagglutinating activity was eluted with the equilibration buffer (10 mM sodium phosphate

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Abbreviations used: VVL, *Volvariella volvacea* lectin; VVL-F, *Volvariella volvacea* lectin isolated from fruiting bodies; VVL-M, *Volvariella volvacea* lectin isolated from cultured mycelia; Con A, concanavalin A; IL-2, interleukin-2; IFN-γ, interferon-γ; RT-PCR, reverse transcriptase-polymerase chain reaction.

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buffer, pH 8.2), and the active fractions were pooled and applied to a CM-52 cellulose column (2.5 cm × 22 cm) which had previously been equilibrated with 10 mM sodium acetate buffer, pH 4.8. After exhaustive washing with the buffer, the adsorbed materials containing hemagglutinating activity were eluted with a linear gradient of 0-0.3 M NaCl in the acetate buffer. The active fractions were combined and applied to a Mono S HR 5/5 column with an FPLC system. The purified lectin, which was named VVL-F, was obtained by elution from the column with 10 mM sodium acetate buffer, pH 5.2, followed by dialysis against distilled water and then lyophilization.

Isolation of VVL-M. The culture broth was filtered through cheesecloth. The mycelia were washed free of culture broth with distilled water. The subsequent extraction and purification procedures for VVL-M were the same as those of VVL-F.

Hemagglutination test. Hemagglutinating activity was measured as described previously (12). Agglutination of 2% erythrocytes in phosphate-buffered saline and inhibition of the agglutination by sugars and glycoproteins were done in microtiter U-plates at room temperature. The titer is defined as the reciprocal of the end-point dilution causing hemagglutination. Inhibition is expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of hemagglutination of the lectin at a titer of 3.

SDS-PAGE. SDS-PAGE (15%, w/v) was performed in a Bio-Rad mini protein gel apparatus according to the method of Laemmli (20). The gel was visualized after staining with Coomassie Brilliant Blue R-250 or periodic acid-Schiff technique for detecting carbohydrate (21). Protein concentration was determined with the method of Lowry *et al.* (22) using bovine serum albumin as a standard.

Gel filtration. Gel filtration for measuring the molecular weight (MW) of native lectin was carried out on a Superdex 75 HR 10/30 column with an FPLC system. Standard protein markers used included cytochrome C (MW 12.4 kDa), chymotrypsinogen A (MW 25 kDa), ovalbumin (MW 45 kDa), and bovine serum albumin (MW 67 kDa).

Amino acid sequence analysis. The N-terminal amino acids of VVL-F and VVL-M were determined by automated Edman degradation. Microsequencing was performed using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system.

Assay for mitogenic activity. Three BALB/c mice (8 weeks old) were killed by cervical dislocation and the spleens were aseptically removed. Spleen cells were isolated by pressing the tissue through a sterilized 100-mesh stainless steel sieve and resuspended to 5×10^6 cells/ml in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 units penicillin/ml, 100 μg streptomycin/ml and 200 mM L-glutamate. The cells (5×10^5 cells/50 μl/well) were seeded into a 96-well culture plate and serial concentrations of VVL and Con A in 50 μl of the medium were added. After incubation of the cells at 37°C in a humidified atmosphere of 5% CO_2 for 42 h, 10 μl [^{3}H]-thymidine (0.25 μCi, Amersham, UK) was added, and the cells were incubated for a further 6 h under the same conditions. The cells were then harvested with an automated cell harvester onto a glass filter, and the radioactivity was measured with a Beckman model LS 6000SC scintillation counter. All reported values are the means of triplicate samples.

RNA extraction and RT-PCR. Spleen cells from BALB/c mice were isolated as described above, and resuspended at 10^7 cells/60-mm culture dish. The cultures were treated with various concentrations of VVL and Con A at 37°C in a humidified atmosphere of 5% CO_2 for 4 h. Total cellular RNA was isolated according to the method of Chomczynski and Sacchi (23). Oligo(dT)-primed cDNAs were prepared in a volume of 20 μl from 1 μg of total RNA using MMLV reverse transcriptase. The primers used to amplify the cDNA fragments of interleukin-2 (IL-2) and interferon-γ (IFN-γ) for analysis of gene expression were: IL-2 forward 5′-ATGTACAGCATGCAGCTCGCATCCTGTGTCGAC-3′, IL-2 reverse 5′-CTGCTTGGGCAAGTAAAATTTGAAGGTGAGC -3′; IFN-γ forward 5′-CTTCTTGGATATCTGGAGGAACTGGCAAAA-3′, IFN-γ reverse 5′-CTCAAACTT- GGCAATACTCATGAATGCATC-3′ (24). For internal control, β-actin was employed by using the primers: forward 5′-ATGGATGATGATATCGCCGCG-3′, and reverse 5′-CTA- GAAGCATTTGCGGTGGACGATGGAGGGGCC-3′ (25). PCR was carried out in a 50 μl mixture using the PCR Reagent System (10198-018, Life Technologies, USA) and 30 cycles of exponential amplification were performed. The cycling parameters were as follows: denaturation at 94°C for 1 min (5 min before the first cycle), annealing at 55°C for 1 min for both IFN-γ and β-actin but 60°C for IL-2, extension at 72°C for 2 min, with an additional 7 min final extension at 72°C. The amplified products were analyzed by 1.2% (w/v) agarose gel electrophoresis, and their intensities were visualized by ethidium bromide staining under UV.

RESULTS

Purification of VVL. Erythrocytes from Wistar rats were employed for monitoring the hemagglutinating activity in different fractions during purification. Hemagglutinating activity was detected neither in crude extracts of *V. volvacea* mycelia and fruiting bodies nor in the ammonium sulfate precipitate (Table 1), which contained substances causing cell lysis in the hemagglutination assay. After DE-52 cellulose column chromatography, five peaks were observed (Fig. 1A). The first three peaks were eluted by 10 mM sodium phosphate buffer, pH 8.2, and the last two peaks were eluted by 0.3 M NaCl in the same buffer. Peak A displayed the highest specific hemagglutinating activity. The active fractions were collected for further purification on a CM-52 cellulose column. The activity was eluted with a linear gradient of 0-0.3 M NaCl in 10 mM sodium acetate buffer, pH 4.8 (Fig. 1B). It showed minor contamination with other proteins in SDS-PAGE (data not shown). Using a Mono S column with an FPLC system, purified VVL-F was obtained by elution with 10 mM sodium acetate buffer, pH 5.2. It was well resolved from other contaminating proteins that were adsorbed on the column and eluted with a linear gradient of 0-0.075 M NaCl in the same buffer (Fig. 1C). A summary of the purification of VVL-F is shown in Table 1. In a typical purification experiment, about 65 mg of purified VVL-F was obtained from 2 kg of the edible mushroom. A lectin, VVL-M, was also isolated from the cultured mycelia of the same fungus according to the method used for the isolation of VVL-F (data not shown).

Characterization of VVL. Purified VVL-F migrated as a single band in SDS-PAGE corresponding to a molecular weight of 15.5 kDa, regardless of the presence or absence of 2-mercaptoethanol (Fig. 2, lane 2 vs. lane 3), indicating that the lectin was not disulfide-linked multimers. VVL-M also appeared as a band with a molecular weight of 15.5 kDa corresponding to the position of VVL-F in SDS-PAGE. The profile did not change under reducing or non-reducing conditions (Fig. 2, lane 4 vs. lane 5). In FPLC-gel filtration on a Superdex 75 column, both VVL-F and VVL-M showed a molecular

TABLE 1

Purification of VVL from 2 kg of Straw Mushroom (*Volvariella volvacea*)

Step	Total protein (mg)	Specific hemagglutinating activity (units/mg)[a]	Total hemagglutinating activity (units)	Recovery of activity (%)
95% $(NH_4)_2SO_4$ precipitate	4355	nd	nd	nd
DE-52 cellulose chromatography	263	754	1.98×10^5	100
CM-52 cellulose chromatography	88	1912	1.68×10^5	85
FPLC (Mono S)	66	2404	1.59×10^5	80

[a] Calculated as the inverse of the minimum concentration producing a positive reaction in the hemagglutination assay. nd, not detected.

weight of 32 kDa in 0.05 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.0. These results indicate that native VVL-F or VVL-M is most likely a non-covalently associated homodimeric protein. Staining of the VVL-F and VVL-M bands after SDS-PAGE with periodic acid-Schiff reagent yielded negative results, indicating that VVL-F and VVL-M are devoid of carbohydrate.

N-terminal amino acid sequence of VVL. The sequences of the first 30 N-terminal amino acids in VVL-F and VVL-M were the same, indicating that they are the same lectin, being designated as VVL. Alignment of the N-terminal sequence of VVL with the C- and N-terminal sequences of Fip-*vvo*, which was isolated from the same fungus by Hsu *et al.* [19], to obtain maximal sequence homology is presented in Fig. 3. The first eleven N-terminal amino acids of VVL were identical to the C-terminal (100-110) sequence of Fip-*vvo*. The N-terminal (12-30) sequences of VVL and Fip-*vvo* were identical. The sequence, WKSTDLTQLLFFI, which represents the C-terminal (111-112) and N-terminal (1-11) amino acids of Fip-*vvo*, was missing from the N-terminal sequence of VVL. Thus the sequence of VVL was equivalent to an addition of the C-terminal (100-112) sequence in front of the N-terminal sequence of Fip-*vvo* followed by a deletion of a 13-amino acid fragment equivalent to its C-terminal (111-112) and N-terminal (1-11) sequences.

Hemagglutination reaction. VVL exhibited a hemagglutinating activity toward red blood cells of three rat species (Wistar, Sprague-Dawley and Lewis) at concentrations higher than 13.1 nM. With red blood cells from hamsters and rabbits, the minimal concentration of VVL required for hemagglutination was 5.3 nM and 10.6 nM respectively. VVL had the highest hemaggluti-

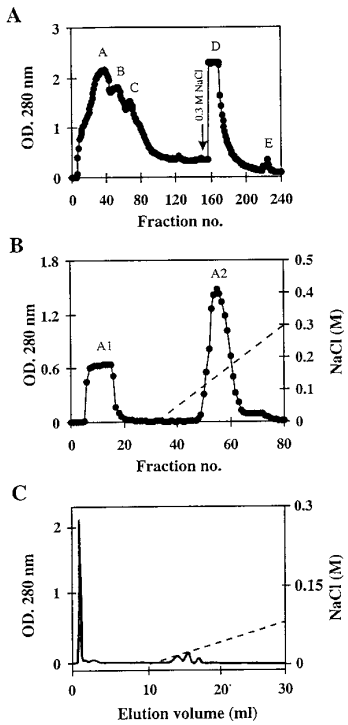

FIG. 1. Purification of VVL by Ion-exchange chromatography. (A) Elution profile of VVL from a DE-52 cellulose column. (B) Elution profile of VVL from a CM-52 cellulose column. (C) Mono S column chromatography of VVL. Conditions are described in MATERIALS AND METHODS. Fractions (10 ml) were collected (A, B) and monitored by absorbance at 280 nm. The active fractions were detected by hemagglutination assay.

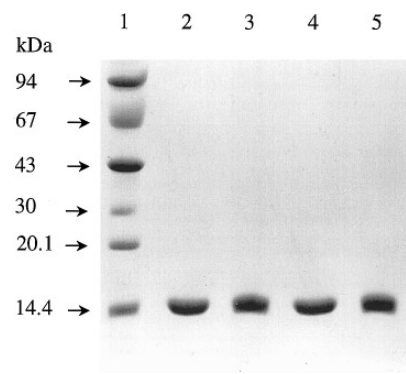

FIG. 2. SDS-PAGE of VVL-F and VVL-M. Purified VVL-F (lanes 2 and 3) and VVL-M (lanes 4 and 5) were analyzed by SDS-15%PAGE. Lane 1, molecular mass markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa); lanes 2 and 4, in the presence of 2-mercaptoethanol; lanes 3 and 5, in the absence of 2-mercaptoethanol.

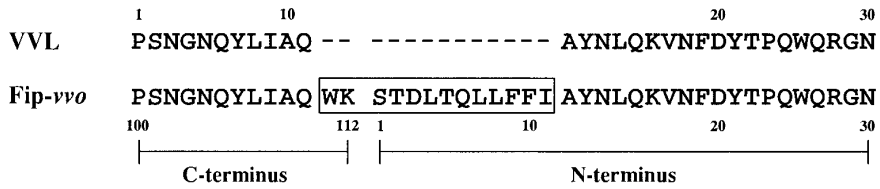

FIG. 3. Comparison of N-terminal amino acid sequence of VVL with the C- and N-terminal amino acid sequences of Fip-*vvo* [19] arranged adjacent to each other in order to achieve maximal sequence homology with VVL. The sequence of amino acids enclosed in a rectangle in Fip-*vvo* is missing from the VVL sequence.

nating activity toward red blood cells from cats and dogs: the minimal VVL concentration required for hemagglutination was 2.6 nM and 1.3 nM respectively. VVL was found to be quite stable over a wide range of pH (2 to 11) and temperatures (40 to 90°C). Half of the activity was lost at pH 11 (after 24 h) and at 90°C (after 30 min). The hemagglutinating activity of this lectin was not affected by demetalization with EDTA, nor after addition of 2 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ or $ZnSO_4$ to the demetalized lectin. The hemagglutinating activity of VVL was not inhibited by any of the following saccharides at concentrations up to 0.4 M: D-glucose, D-galactose, L-fucose, α-lactose, D-mannose, sucrose, D-glucosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetylneuraminic acid. However, among the 4 glycoproteins (egg albumin, thyroglobulin, human chorionic gonadotropin and heparin) tested, thyroglobulin was found to be able to inhibit hemagglutination at the minimal concentration of 93 nM.

Immunomodulatory activities of VVL. The proliferative response of murine splenic lymphocytes to VVL was studied, and Con A was used as a positive control (Fig. 4). Both VVL and Con A induced lymphoblastic proliferation and the maximal uptake of [^{3}H]-thymidine was achieved by VVL and Con A at around 10 nM and 100 nM respectively. VVL was thus about 10-fold more potent than Con A in lymphocyte activation. The maximal uptake of [^{3}H]-thymidine in response to 5 nM VVL and 5 nM Con A acting in concert was not different from that to 10 nM VVL (data not shown).

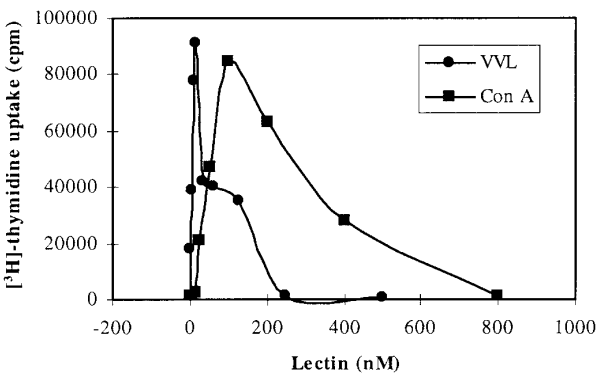

FIG. 4. Stimulatory effects of VVL on proliferation of murine splenic lymphocytes. Spleen cells were cultured for 42 h with various doses of VVL and Con A and pulsed with [^{3}H]-thymidine for the last 6 h as indicated in Materials and Methods. Each point, expressed in cpm/well, is the mean of triplicate measurements.

Induction of cytokine gene expression by VVL. The induction of cytokine gene expression by VVL was demonstrated by using murine splenic lymphocytes. The results of RT-PCR revealed that VVL significantly stimulated both IL-2 and IFN-γ gene expression in 4h, and the peak induction was between 25-50 nM. On the other hand, 400 nM of Con A was required to obtain the equivalent expression intensity for both genes, further indicating that VVL was about 10-fold more potent than Con A (Fig. 5).

DISCUSSION

A lectin (VAG) from the edible mushroom *V. volvacea* has been isolated by Lin *et. al.* (26). More recently, they purified an immunomodulatory protein (Fip-*vvo*) from the fruiting bodies of the same fungus based on the purification procedure of VAG with an additional step involving Mono S column chromatography with an FPLC system (19). Fip-*vvo,* which has a molecular weight of 26 kDa, is homodimeric and composed of two identical subunits. Its N-terminus is blocked. The molecular weight, amino acid composition, structure and hemagglutination activity of Fip-*vvo* were similar to those of VAG. In this study, we also purified and characterized a lectin, VVL, from the same fungus using the isolation procedure for Fip-*vvo.* However, VVL could not be adsorbed on the Mono S column using the equilibration buffer (10 mM sodium acetate, pH 5.2) for Fip-*vvo.* When the pH of equilibration buffer was lowered to 4.5, VVL was found to be bound to the column, but it could not be well separated from other contaminating proteins (data not shown). This result showed that VVL had hydrophobic groups different from those of Fip-*vvo,* thereby inducing different surface charges and affecting the binding ability. On the other hand, VVL exhibited a larger molecular mass (32 kDa) than that of Fip-*vvo* (26 kDa). The N-terminus of VVL was not blocked, and a comparison of the N-terminal sequences of VVL and Fip-vvo indicates that VVL is a product of a gene distinct from, yet closely related

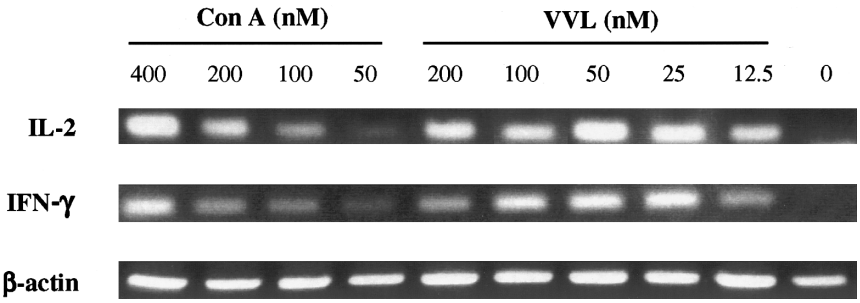

FIG. 5. Induction of IL-2 and IFN-γ gene expression following the treatment of mouse spleen cells with VVL. Expression was analyzed by RT-PCR of total RNA extracted from splenic lymphocytes. Equal amounts of cDNA were loaded, as confirmed by the equal intensities of ethidium bromide staining of β-actin.

to, the gene encoding Fip-*vvo*. This gene may be a mutant of the Fip-*vvo* gene formed by linking of the C-terminal (100-112) residues to the N-terminus of Fip-*vvo* and subsequent omission of the C-terminal (111-112) and N-terminal (1-11) amino acids (Fig. 3). To further characterize the lectin (VVL-F) isolated from the fruiting bodies of *V. volvacea*, we also purified a lectin (VVL-M) from the cultured mycelia of the same fungus and identified that VVL-M and VVL-F were the same lectin (VVL) by comparing their chromatographic behavior, N-terminal amino acid sequences, molecular weights and biological activities. Lectins from same plant species were also found to have structurally different subunits (27). It would be interesting to investigate the relationships between subunit structures and biological roles of lectins.

Many properties of VVL suggest that it is a lectin. Although the carbohydrate- binding specificity of VVL could not be demonstrated, thyroglobulin, not simple carbohydrates, is able to inhibit the hemagglutination, implying that VVL binds to an oligosaccharide. Many legume lectins display a higher affinity for oligosaccharides than for monosaccharides, and some seed and vegetable lectins are only known to bind to oligosaccharides (28). Lectins, such as those of *P. vulgaris*, that display a high affinity for a complex oligosaccharide structure, are not readily inhibited by simple carbohydrates (28).

VVL was a potent mitogen as shown by its ability to stimulate the uptake of [^{3}H]-thymidine in cultured murine lymphocytes as some lectins do. The proliferative response induced by VVL was similar to that induced by Fip-*vvo* and other immunomodulatory proteins, LZ-8 (17) and Fip-*fve* (18) from mushrooms; however VVL was 9-fold more potent than LZ-8 in activating lymphocytes. Moreover, VVL was shown to affect the levels of several cytokine mRNAs. It was observed that the expression of IL-2 and IFN-γ was markedly enhanced in VVL-treated splenic lymphocytes. Compared with Fip-*vvo*, VVL was more potent in inducing the gene expression of IL-2 and IFN-γ. It is known that mouse T helper (Th 1) cells produce IL-2 and IFN-γ. VVL up-regulated the mRNAs of IL-2 and IFN-γ, suggesting that the Th 1 T-cell subset was affected (29).

It is concluded that VVL from *V. volvacea* is a structurally novel fungal lectin which possesses potent immunomodulatory activities.

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