Isolation of a Galactomannan That Enhances Macrophage Activation from the Edible Fungus *Morchella esculenta*

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The edible mushroom *Morchella esculenta* is among the most highly prized and morphologically recognizable fungi in the world. We describe the isolation from a polar extract of *M. esculenta* carpophores of a high-molecular-weight galactomannan, about 1.0 million Da, that exhibits immunostimulatory activity. At 3.0 μg/mL the galactomannan polysaccharide increased NF-kappa B directed luciferase expression in THP-1 human monocytic cells to levels 50% of those achieved by maximal activating concentration (10 μg/mL) of lipopolysaccharide. This galactomannan comprises about 2.0% of the dry fungal material weight, and its glycosyl components include mannose (62.9%) and galactose (20.0%).

INTRODUCTION

The genus *Morchella* (Morchellaceae) is characterized by a hollow fruit body consisting of a pitted cap with an intergrown stipe (*1*). Members of the genus *Morchella*, commonly known as morels, are among the most highly prized edible mushrooms in the world (*1, 2*) and are among the few documented fungi used by North American natives as food (*3*). *Morchella* species are also used in traditional Chinese medicine to treat indigestion, excessive phlegm, and shortness of breath (*4*).

In our search for biologically active natural products from higher fungi, we evaluated carpophores of the edible mushroom *Morchella esculenta* Fries, and discovered strong immunostimulatory activity present in the crude extract. Because immune-enhancing activity has not been reported from these mushrooms, our purpose was to isolate and characterize the compound(s) responsible for this biological activity.

MATERIALS AND METHODS

Fungal Material. Specimens were collected in Lake of the Woods County, Baudette, Minnesota, from April 25 to May 15, 2000. The genus *Morchella* is divided into two species concepts that are distinguishable largely on the basis of color: the dark-colored species is *M. elata* and the light-colored species is *M. esculenta*. The light-colored mushrooms collected were identified as *M. esculenta*. A voucher specimen (MISS accession 62892) is housed in the Pullen Herbarium (MISS), Department of Biology, The University of Mississippi.

Materials and Reagents. Bacterial lipopolysaccharide [LPS] (*E. coli*, serotype 026:B6), pullulan standards (0.1 and 1.66 million Da), and dextran standards (0.58, 2, and 5−40 million Da) were obtained from Sigma Chemical Co. (St. Louis, MO). THP-1 human monocytes were obtained from American Type Culture Collection (Rockville, MD). LucLite luciferase reporter gene assay kit was purchased from Packard (Downers Grove, IL). NF-kappa B plasmid construct (pBIIXLUC) was a gift from Dr. Riccardo Dalla-Favera and contained two copies of NF-kappa B motif from HIV/IgK (*5*).

Extraction and Isolation. Approximately 1 kg of frozen material was lyophilized to dryness (94.22 g). The lyophilized material was exhaustively extracted using three consecutive solvent systems: 95% EtOH (at room temperature), then 95% EtOH/H$_2$O 4:1 (at room temperature), then hot H$_2$O. The 95% EtOH/H$_2$O extract (11 g) was subjected to crude fractionation by reversed-phase vacuum liquid chromatography using 10-g Alltech C$_{18}$ Sep Pak devices. Four fractions were eluted successively with water, methanol, ethyl acetate, and diethyl ether. The water fraction was further fractionated by open column chromatography with Sephadex LH-20 using a methanol/water (1:1) mobile phase; the early eluting fractions from this were the most active. Final purification of the high-molecular-weight galactomannan polysaccharide from the active Sephadex column fractions was accomplished using HPLC size-exclusion chromatography (SEC).

The HPLC−SEC system (Waters Associates, Milford, MA) consisted of a model 600E system controller, UK6 injector, model 600 solvent delivery system, model 401 differential refractometer, and a model 3396A Hewlett-Packard integrator. Isolation was performed at a flow rate of 1 mL/min using HPLC grade water and a Shodex OHpak SB-806 HQ SEC column (300 mm × 8.0 mm i.d.) maintained at 30 °C with a temperature control module (Waters). This column has an estimated pullulan exclusion limit of about 20 million Da.

Structural Characterization. The molecular weight of the purified active polysaccharide was estimated by comparison of its retention time using the HPLC−SEC system with the retention times of high-

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molecular-weight dextran and pullulan standards. A salt solution mobile phase (0.9% NaCl) was used for estimation of molecular weight because, for this polysaccharide, the presence of charged sugar residues or intermolecular interactions (e.g., aggregation) would cause erroneous values to be obtained if using a pure water mobile phase.

Glycosyl composition and glycosyl linkage analyses were performed by the Complex Carbohydrate Research Center at The University of Georgia. The glycosyl composition was determined using GC−mass spectrometry analysis of the TMS-methyl glycosides (*6*). Glycosyl linkage analysis was performed using the NaOH/MeI procedure (*7*). Coomassie blue protein determinations were used to estimate the amount of protein present within purified galactomannan preparations (*8*).

Macrophage Assay. Macrophage activation was measured using a luciferase reporter gene assay in THP-1 human monocytic cells. This assay measures immunostimulatory activity as indicated by increased expression of a NF-kappa B-driven luciferase reporter. THP-1 cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10% v/v) and amikacin (60 mg/L) at 37 °C, under 5% CO_2 and 95% air. Actively growing cells were transiently transfected using DEAE-dextran (*9*) (10 μg/1 × 10^6 cells) and the pBIIXLUC reporter plasmid (1 μg/1 × 10^6 cells) containing two binding sites for NF-kappa B. Transfection solution containing THP-1 cells was incubated for 7 min in a 37 °C water bath. The transfected cells were then resuspended in RPMI 1640 medium containing 10% FBS and plated out in 96-well plates at a cell density of 2 × 10^5 cells per well. After 24 h, *M. esculenta* extracts, fractions, and purified galactomannan polysaccharide were added to transfected cells. At 4 h after addition of samples, cells were harvested and luciferase activity was measured. Cells were harvested using Packard filter plates and lysed using 30 μL of luciferase mix (1:1, LucLite luciferase/1 × PBS, 1 mM Ca, and Mg). Luciferase light emission was measured using a Packard microplate scintillation counter in single photon mode. Activation is reported as a percentage relative to maximal activation of NF-kappa B by 10 μg/mL LPS.

RESULTS AND DISCUSSION

The immunostimulatory properties of *M. esculenta* were evaluated and characterized using a luciferase reporter gene based bioassay in which luciferase expression is driven by the binding of NF-kappa B. The activation of transcription factor NF-kappa B controls the expression of multiple genes in activated monocytes and macrophages (*10*). Target genes regulated by NF-kappa B include proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, receptors, and inhibitors of apoptosis (*11*).

The most active crude extract resulted from extraction of the lyophilized *M. esculenta* material with 95% EtOH/H$_2$O. At 75 μg/mL this extract increased NF-kappa B directed luciferase expression to levels equivalent to those achieved by the maximally activating concentration of LPS (10 μg/mL). Fractionation of the 95% EtOH/H$_2$O extract used both reversed-phase vacuum liquid chromatography and Sephadex LH-20. Final purification was accomplished using HPLC−SEC where the active polysaccharide eluted as the earliest peak which was isolated through repeated injection to obtain sufficient quantity for structural characterization and immunostimulatory evaluation. Using this isolation procedure, the recovery of the active galactomannan was calculated to be about 2.0% of the freeze-dried *M. esculenta* material.

Figure 1 presents a dose−response for both LPS and the purified galactomannan polysaccharide isolated from *M. esculenta*. The EC$_{50}$ (50% of maximal LPS induction) value for NF-kappa B directed luciferase expression for three different preparations of this galactomannan is about 3.0 μg/mL. Compared with bacterial LPS (EC$_{50}$ value of 0.5 μg/mL), this galactomannan polysaccharide is about one-sixth as potent.

It is possible that the observed NF-kappa B activation by this galactomannan is due to an endotoxin-mediated response. To
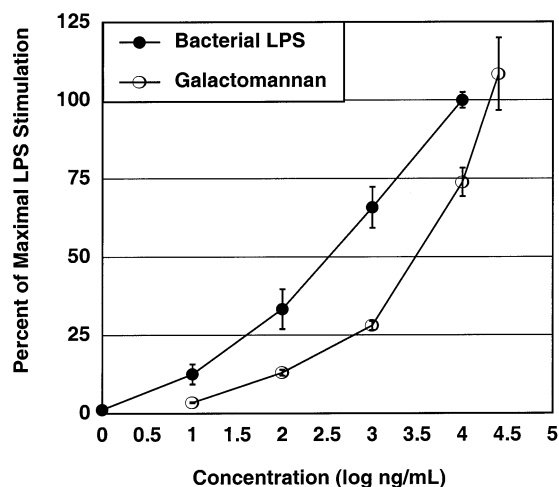

Figure 1. Dose response for galactomannan polysaccharide isolated from *Morchella esculenta* and bacterial LPS for activation of NF-kappa B in THP-1 human monocytes at 4 h. Samples were run in quadruplicate. Results are reported as means ± standard deviation.

Table 1. Glycosyl Composition and Glycosyl Linkage Data for Galactomannan Isolated from *Morchella esculenta;* Data Obtained from One Experiment

glycosyl residue	mol %	glycosyl linkage	% present
mannose	62.9	1,2-linked mannose	40.4
galactose	20.0	1,4-linked glucose	19.8
N-acetyl glucosamine	7.9	terminal 1-galactose	11.1
glucose	6.5	2,3-linked mannose	10.9
rhamnose	2.7	1,6-linked mannose	7.6
		terminal 1-mannose	7.0
		3,6-linked galactose	3.2

address this possibility two experiments were conducted. First, polymyxin B (10 μg/mL) was added in combination with three different preparations of the galactomannan (25 μg/mL) to observe whether there was any reduction in NF-kappa B activation. Polymyxin B is a polycationic antibiotic known to block many of the biological effects of LPS by binding to the lipid A portion of the molecule. All three galactomannan preparations were insensitive to polymyxin B addition (data not shown). In contrast, addition of polymyxin B to LPS (10 μg/mL) suppressed NF-kappa B activation by 82%. The second experiment examining possible endotoxin-mediated effects looked for the presence of 3-β-hydroxymyristate in the glycosyl composition analysis. No detectable levels of 3-β-hydroxy-myristate were detected, and thus, it is unlikely that the observed macrophage activation by this galactomannan is due to endo-toxin contamination.

Glycosyl composition and glycosyl linkage analysis for the galactomannan polysaccharide are summarized in **Table 1**. The major glycosyl components are mannose (62.9%) and galactose (20.0%). For linkage analysis, the major derivatives included 1,2-linked mannose (40.4%), 1,4-linked glucose (19.8%), terminal 1-galactose (11.1%), and 2,3-linked mannose (10.9%). No glycosyl linkages were observed for the minor sugar residues rhamnose and *N*-acetylglucosamine. Because of the many different glycosyl linkages of this polysaccharide (refer to **Table 1**), the anomeric configurations for each linkage have not yet been determined. The molecular weight was estimated to be about 1.0 million Da. Coomassie blue based protein determinations on three separate sample preparations indicate that this polysaccharide contains only about 2.4% protein.

Various polysaccharides isolated from *M. esculenta* have been reported in the literature. In the early work of Ito et al. (*12*) a galactomannan polysaccharide was isolated from *M. esculenta* and was reported to be ineffective against treatment of various mouse tumors. However, no structural data (e.g., molecular weight, percent composition of glycosyl units, and glycosyl linkages) were provided for that polysaccharide. Therefore, it is difficult to evaluate whether the galactomannan polysaccharide isolated in the current research is different from the galactomannan previously reported. Nevertheless, our research represents the first detailed structural characterization of a galactomannan polysaccharide from *M. esculenta* that is biologically active.

Additional polysaccharides have been isolated from the nutrient liquor resulting from fermentation of *M. esculenta*. These include two polysaccharide fractions, MEP-SP2 and MEP-SP3, that have molecular weights of 23,000 Da and 44,000 Da, respectively (*13*). The glycosyl composition of MEP-SP2 is mannose, glucose, arabinose, and galactose (mole ratio of 1.75:4.13:0.71:0.68). The glycosyl composition of MEP-SP3 is xylose, glucose, mannose, fructose, arabinose, and galactose (mole ratio of 3.58:14.9:3.85:1.77:51.3:0.53). Another polysaccharide (*14*) isolated from this material has a molecular weight of 11,500 Da and is composed of xylose, glucose, arabinose, and galactose (mole ratio of 0.29:0.24:0.61:0.39). Clearly, the galactomannan identified in the present study differs from these other polysaccharides in both molecular weight and composition.

Medicinal and edible fungi have had a long history of use in traditional medicine. It is well-known that many of these mushrooms contain bioactive polysaccharides that exhibit anticancer, antimicrobial, and immunomodulatory effects (*15*). A few of these polysaccharides (e.g., schizophyllan, lentinan, and krestin) have been developed as pharmaceuticals in Japan where they are clinically used as biological response modifiers for cancer treatment (*16*). The present study reports the structural characterization of a high-molecular-weight galactomannan that activates THP-1 monocytes that was isolated from wild-grown *M. esculenta*. Further research is needed to determine whether there are any in vivo benefits comparable to the in vitro effects reported here. Although it is unlikely that this polysaccharide would be absorbed after oral administration (because of its high molecular weight), it is possible that it could exert a therapeutic effect by direct interaction with the mucosal immune system of the GI tract. Investigation of this possibility could lead to the development of a *M. esculenta* polysaccharide preparation for use as a dietary supplement or pharmaceutical.

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