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A new α-galactosyl-binding protein from the mushroom *Lyophyllum decastes* ☆

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Abstract

A new α-galactosyl binding lectin was isolated from the fruiting bodies of the mushroom *Lyopyllum decastes*. It is a homodimer composed of noncovalently-associated monomers of molecular mass 10,276 Da. The lectin's amino acid sequence was determined by cloning from a cDNA library using partial sequences determined by automated Edman sequencing and by mass spectrometry of enzyme-derived peptides. The sequence shows no significant homology to any known protein sequence. Analysis of carbohydrate binding specificity by a variety of approaches including precipitation with glycoconjugates and microcalorimetric titration reveals specificity towards galabiose (Gal α1,4Gal), a relatively rare disaccharide in humans. The lectin shares carbohydrate binding preference with the Shiga-like toxin, also known as verocytoxin, present in the bacteria *Shigella dysenteriae* and *Escherichia. coli* 0157:H7, both of which are causes of outbreaks of sometimes fatal food-borne illnesses.

Keywords: Alpha-galactosyl; Galabiose; Mushroom lectin; *Lyophyllum decastes*; P antigens; Verocytoxin; Shiga-like toxin

Alpha galactosyl-binding proteins are valuable reagents in the glycobiologists' armentarium. However, relatively few have been described and characterized. They include the GS I-B_4[1] isolectin from the seeds of *Griffonia simplicifolia*, which is highly specific for terminal nonreducing α-galactosyl groups but exhibits only slight preference for the nature and linkage to the subterminal sugar [1–3], and the lectin present in the mushroom *Marasmius oreades*, which is highly specific for the Gal α1,3Gal disaccharide with enhanced binding activity to the linear and branched human blood group type B trisaccharides [4,5].

The GS I-B_4 isolectin has found multiple biomedical applications. It agglutinates Ehrlich ascites tumor cells [6] and 3T3 cells [7]. It has been employed for the isolation of murine laminin [8] and a family of alpha-galactosyl-terminated glycoproteins from Ehrlich ascites tumor cells [9]; and for the detection of α-galactosyl-terminated glycoproteins in the basement membranes in a variety of tissues of the rat, mouse, and rabbit [10].

Because of its pronounced specificity for the human blood type B epitope and related structures, the *M. oreades* lectin is capable of discerning the sequence of three sugar residues (Gal α1,3Gal β1,4Glc/GlcNAc) in glycoproteins and glycolipids [5]. The fluorescein-labeled lectin reacts strongly with α-galactosyl-terminated glycans of porcine organs and tissues, the so-called xenotransplantation antigen [5]. When injected into mice the lectin binds specifically

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[1] *Abbreviations used:* GS I-B_4, B_4 isoform of *Griffonia simplicifolia* lectin I; LDL, *Lyophyllum decastes* lectin; MOA, *Marasmius oreades* agglutinin; PBS/E, phosphate-buffered saline with EDTA (10 mM sodium phosphate; pH 7.2, 0.15 M NaCl, 1.25 mM EDTA, 0.04% NaN_3).

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to the glomeruli resulting in hematuria, proteinuria and fibrin thrombi, and has thus been implicated as a model for hemolytic–uremic syndrome [11]. The fluorescein-labeled lectin may be visualized in the glomeruli [11]. Immobilized MOA binds murine laminin, bovine thyroglobulin, and a fraction of α_2-macroglobulin in human type B serum containing the blood group B epitopes [12].

In this communication, we describe the isolation and characterization of a new α-galactosyl-binding lectin from the mushroom *Lyophyllum decastes*. We also compare the carbohydrate binding properties of the three specific α-galactosyl-binding lectins: those from *M. oreades*, *Griffonia simplicifolis* isolectin GS I-B4 and *L. decastes*.

Methods

Extraction and purification of LDL

Lyophyllum decastes (Fr.) Singer 1951 (syn: *L. aggregatum* (Schaeff.) Kühner 1938) was collected as clusters of fruiting bodies from a grassy area in the vicinity of Ann Arbor, MI in late spring. A voucher specimen was deposited in the University of Michigan herbarium. Subsequent collections were made at the same site at various times during the summer, and in the following year.

Mushrooms were washed, diced and extracted (40 g fresh weight) in 200 ml PBS/E containing ascorbic acid (1 g/L) and thiourea (10 mM), 200 μL of protease inhibitor cocktail (Sigma P8215, formulation for fungal tissue), and 5 g insoluble polyvinylpolypyrrolidone, by blending in a Waring Blendor to a smooth consistency and stirring 2 h. The extract was filterered through four layers of cheesecloth, brought to 20% saturation of $(NH_4)_2SO_4$ (114 g/L) and centrifuged. The clarified crude extract was taken to 80% saturation of $(NH_4)_2SO_4$ (424 g/L of 20% supernatant), and the precipitated protein recovered by centrifugation was redissolved in approx 10% of the original volume of PBS/E, dialyzed and centrifuged. The clarified 80% fraction was applied to a column (2.5×12 cm) of melibiose–Sepharose, which was then washed until the washings were essentially free of 280 nm-absorbing material, and the bound lectin activity eluted with a linear gradient (300 ml total volume) from 0 to 0.2 M lactose in PBS/E. Peak fractions absorbing at 280 nm were pooled separately, the late-eluting partially resolved doublet being pooled into early and late halves. The latest eluting (frct. IIb) was the fraction used for subsequent studies, unless noted otherwise.

For protein sequence analysis, mass spectrometric analysis, or glycan array binding, samples (1–2 mg) of the purified fraction IIb were dialyzed against distilled water, lyophilized, and reconstituted in the appropriate solvent and concentration immediately before the analysis. The remainder of the purified preparations were stored at concentrations of approx. 1–2 mg/ml in PBS/E at 4 °C. Automated Edman sequencing and MALDI-TOF mass spectrometry were performed by the Protein Structure Facility at the University of Michigan. Detailed specificity of the lectin was determined by the Consortium for Functional Glycomics using a glycan array.

Other analytical methods

Protein estimation, SDS–PAGE, hemagglutination assays, and precipitin assays were performed by previously described methods [4]. Ultraviolet absorption spectra were recording using a Shimadzu UV160U spectrophotometer. Size estimation was performed by molecular sieve chromatography using a Beckman System Gold HPLC (Beckman–Coulter, Fullerton, CA) with a TOSOH GSK2000SW_{XL} column (7.8×300 mm; Supelco, Inc., Bellefonte, PA) with UV absorbance detection. The column was calibrated with bovine serum albumin (67 kDa), hen ovalbumin (44 kDa), soybean trypsin inhibitor (22 kDa), and cytochrome c (13.5 kDa), with blue dextran 2000 and adenosine as markers for V_0 and V_1, respectively. Microcalorimetric titrations were performed either in a CSC-4200 isothermal titration calorimeter as previously described [4], or in a VP-ITC (MicroCal, Northhampton, MA, USA) at 25 °C. The lectin in PBS was titrated with ligand in the same buffer. Oligosaccharide ligands were available in this laboratory from previous studies, or were purchased from Sigma Chemical Co., St. Louis, or from V-Labs, Inc., Covington, LA. Concentrations and titration volumes were adjusted so that the titration proceeded to at least a 5-fold excess of ligand at the expected stoichiometry. Data were analyzed using Origin ver. 7 software supplied with the instrument.

RNA isolation and cDNA synthesis

Mushrooms collected as above were quick-frozen in liquid nitrogen and stored at −80 °C. Frozen tissue was ground to a fine powder with a mortal and pestle on dry ice and RNA was isolated from the frozen powder using the Trizol (Invitrogen) recommended protocol. Briefly, the tissue was homogenized with a glass homogenizer in 0.4 ml of Trizol per 100 μg of tissue, followed by chloroform extraction. mRNA was then isolated using Oligotex mRNA Mini Kit (Qiagen).

RT-PCR was performed with the mRNA, using oligonucleotide primers based on the peptide sequences determined by automated Edman sequencing that allowed for the lowest amount of degeneracy. Various combinations of primers were employed; however, the forward primer (5′-G CNTGYTGGAARGCNAAYWSNTG-3′) and reverse primer (5′-CCR TANGGNGGYTTRTARTTRTA-3′) generated from the underlined octapeptides in the protein sequence ACWKANSCPGSAFESKDRLRR FALLYCRYNYKPPYGQF proved most suitable. RT-PCR was performed using the Superscript First Strand Synthesis system for RT-PCR (Invitrogen) and Platinium Pfx (Invitrogen). The PCR product obtained, of approximately 170 bps, was isolated and cloned into Zero Blunt TOPO PCR cloning kit (Invitrogen) for sequencing by the University of Michigan Sequencing Core.

The cDNA so obtained was extended by 3′-RACE using First Choice RLM-RACE kit (Ambion) with the primer GCCTTTGGCTATGC ATCCGCGG. The product, approximately 280 bps, was cloned into Zero Blunt TOPO PCR cloning kit and sequenced as above. A full length cDNA was cloned using Zero Blunt PCR cloning Kit (Invitrogen) with the primers CTAAAGCTCCTCGCAGTTACC as the reverse primer and GCGTGCT GGAAGGCGAATAGC as the forward primer, then sequenced.

Results

Identification and purification of LDL

A crude extract of 40 gm of fresh tissue of the mushroom *L. decastes* in 200 ml of extraction buffer agglutinated formaldehyde-stabilized rabbit erythrocytes at approx. 8-fold dilution, whereas human A, B, or O-type erythrocytes were not agglutinated by the undiluted extract. Upon concentrating the extracted protein approx. 8-fold by precipitation with $(NH_4)_2SO_4$, slight agglutination of all types of human cells was observed. The agglutination of rabbit cells was inhibited by melibiose at 1 mM, but required 6 mM MeαGal, or 25 mM lactose or LacNAc for inhibition. GalNAc and methyl β-fructopyranoside exhibited partial inhibition at 25 mM, but mannose, glucose, GlcNAc, xylose, L-fucose, and *N*-acetylneuraminic acid were non-inhibitory. Based on these observations, a column of melibiose–Sepharose was used for affinity purification of the lectin. The column retained all agglutination activity from the applied concentrated crude extract. A sharp peak of protein was eluted with 0.1 M lactose,