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Characterization and cloning of GNA-like lectin from the mushroom *Marasmius oreades*

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Abstract A new mannose-recognizing lectin (MOL) was purified on an asialofetuin-column from fruiting bodies of *Marasmius oreades* grown in Japan. The lectin (MOA) from the fruiting bodies of the same fungi is well known to be a ribosome-inactivating type lectin that recognizes blood-group B sugar. However, in our preliminary investigation, MOA was not found in Japanese fruiting bodies of *M. oreades*, and instead, MOL was isolated. Gel filtration showed MOL is a homodimer noncovalently associated with two subunits of 13 kDa. The N-terminal sequence of MOL was blocked. The sequence of MOL was determined by cloning from cDNA and by protein sequencing of enzyme-digested peptides. The sequence shows mannose-binding motifs of bulb-type mannose-binding lectins from plants, and similarity to the sequences. Analyses of sugar-binding specificity by hemagglutination inhibition revealed the preference of MOL toward mannose and thyroglobulin, but asialofetuin was the strongest inhibitor of glycoproteins tested. Furthermore, glycan-array analysis showed that the specificity pattern of MOL was different from those of typical mannose-specific lectins. MOL preferred complex–type N-glycans rather than high-mannose N-glycans.

Keyword Fungus · GNA-like · Lectin · Mannose-binding · *Marasmius oreades*

Abbreviations

GNA	*Galanthus nivalis* agglutinin
LDL	*Lyophyllum descades* lectin
MOA	*Marasmius oreades* agglutinin
MOL	*Marasmius oreades* lectin
RVL	*Remusatia vivipara* lectin
TxcL1	*Tulipa* hybrid lectin 1 with complex specificity

Introduction

Lectins are carbohydrate-binding proteins of nonimmune origin, which agglutinate cells and/or precipitate glycoconjugates by interacting with sugar chains [1]. The wide distribution of lectins in all biological species, from viruses and bacteria to animals and plants, suggests that lectins have important physiological functions. Many biological functions of these lectins have been elucidated, and they have been used in biological and biomedical applications. Many lectins from mushrooms have also been isolated, and several potential uses have been discovered, including mitogenic, antiproliferative, antitumor, antiviral, and immunestimulating functions [2].

Goldstein and Winter [3] reported that mushroom lectins have at least 6 families on the basis of their sequence homology and characteristics: fungal immunomodulatory protein, galectin family, mushroom lectin (CXL) family, ricin family, β-propeller-fold lectins and Jacalin-related lectin. However,

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the presence of more lectin families is assumed based on the sequences until now. One is the family monocot mannose-binding lectin and other is *Lyophyllum descades* lectin (LDL) [4]-type lectin. The sequence with the motif of monocot mannose-binding lectin was reported for several fungi including *Aspergillus oryzae* [5], and xylose-binding lectin from Ascomycota [6] was also deduced to be this type from the sequences of peptide fragments. On the other hand, LDL showed no sequence homology to lectins reported so far.

Kruger *et al.* [7] isolated and characterized *M. oreades* agglutinin (MOA) from fairy ring mushrooms. The mushroom is edible and popularly found in Europe and US. MOA strongly precipitated blood type B substance and presented a high affinity for Galα1-3Gal. Yagi *et al.* [8] reported that the diversity of mushroom lectin in a single species might be correlated with geographical location. Our preliminary finding for the extract from Japanese *M. oreades* indicated that the hemagglutination activity was not due to MOA but to a different lectin.

In this study, we describe the isolation, characterization, and heterologous expression of a new lectin MOL as GNA-like lectin.

Materials and methods

Materials

The fruit bodies of *M. oreades* were collected in June in Jigenji Park in Kagoshima, Japan. They were stored at −80 °C until use. CNBr-activated Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). Trypsin, chymotrypsin and *Staphylococcus aureus* V8 protease were purchased from Wako Chemical Industries (Osaka, Japan). Sugars and glycoproteins were purchased from Sigma (St. Louis, Mo). All other reagents were commercially available. Asialofetuin-Sepharose 4B was prepared by coupling asialofetuin to CNBr-activated Sepharose 4B.

Isolation of the lectin from fruiting bodies of M. oreades (MOL)

All steps of purification were done at 4 °C. The fruiting bodies of *M. oreades* (100 g) were homogenized with 10 volumes of phosphate buffered saline (PBS), pH 7.5. After the extraction overnight, the extract was filtered and centrifuged at 10,000*g* for 20 min. The clear supernatant was concentrated against ammonium sulfate. The precipitate was collected by centrifugation at 15,000*g* for 20 min and then dissolved in PBS. The clear, crude extract obtained was loaded onto an asialofetuin-Sepharose 4B column (1.5×12 cm) equilibrated with PBS. Unbound protein was washed with PBS, and the adsorbed lectin was then eluted with 0.2 M glycine-HCl containing 0.5 M NaCl, pH 2.5. Eluted fractions were neutralized with 2 M Tris–HCl, pH 8.0, immediately. Hemagglutination activity was measured throughout all purification with trypsinized rabbit erythrocytes.

Molecular mass determination of MOL

The molecular mass of MOL was measured by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS, Bruker Daltonics, Bremen, Germany). Tricine SDS-PAGE was performed in 4 % stacking gel and 16.5 % resolving gel as described by Schagger and Jagow [9]. The gel was stained with Coomassie Brilliant Blue R-250. The molecular mass of MOL was measured by gel filtration on a TSKgel G2000SW (7.5×300 mm) column.

Amino acid sequencing of MOL

MOL (4.0 nmol each) was digested with trypsin (enzyme: substrate=1:50, mol/mol) or chymotrypsin (1:50) in 100 μL of 50 mM sodium bicarbonate (pH 8.0) at 37 °C for 24 h. MOL (4.0 nmol each) was digested with *S. aureus* V8 protease (enzyme: substrate=1:50, mol/mol) in 100 μl of 50 mM phosphate buffer (pH 7.8) at 37 °C for 24 h. Each digest was separated by reverse-phase HPLC using a COSMOSIL 5 C_{18} MS-II column (4.6×250 mm; Nacalai, Kyoto, Japan) with a linear gradient of acetonitrile (0–48 % in 60 min) in 0.1 % (v/v) trifluoroacetic acid. The N-terminal amino acid sequences of MOL and its proteolytic peptide fragments were analyzed with an Applied Biosystems Procise 492 protein sequencer [10].

Genomic DNA sequence analysis

Genomic DNA of *M. oreades* was isolated using a DNeasy plant mini kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Templates (70 ng/μL) were amplified using two sets of degenerate primers designed from partial amino acid sequence of MOL MQTDGNCV (residues 63–70) and AVWHTATN (78–85). PCR was performed with TAKARA LA Taq (Takara Bio, Kyoto, Japan) for 25 cycles of repeating denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1 min to obtain the gene-specific sequence of the genomic DNA encoding MOL.

cDNA sequencing of MOL

Total RNA was purified from 100 mg of *M. oreades* using an RNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized using ReverTra Ace -α-® (Toyobo, Osaka, Japan) according to the supplier's protocols. Two μg of total RNA was used as a template, and transcription was carried out at 42 °C for 20 min. Synthesized cDNA was used as a template for PCR. 3′-RACE was

performed using a 3′-full RACE Core set (Takara Bio) following the manufacturer's protocols. The template cDNA was synthesized using 2.5 μM of an oligo-d(T) primer containing an adapter sequence. The primer MOL-F1 (ATGCAGACAGATGGTAATTGCGTCGGTTAC) was synthesized on the basis of the partial sequence of the genomic DNA. The reverse primer corresponded to the adapter sequence of the transcript primer. 3′-RACE was performed with 5 ng of template cDNA, 5 μM of primer MOL-F1, and 3 μM of adapter primer. The procedure consisted of 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C 2 min. 5′-RACE was performed using a 5′-full RACE Core set (Takara Bio) following the manufacturer's protocols. cDNA was synthesized with 200 pmol/ml of the 5′-terminal phosphorylated transcript primer. Digestion of RNA generated a single strand cDNA, which was then ligated to form a concatemer or circular template. 5′-RACE was performed using TAKARA LA Taq containing 1 μl of the template with 5 μM of primers S1 (GGACTGGGATTTGAACGGAGCGAATTCGAC) and A1 (CAACGGTCTCAGCAATTTAGTGGACCACGG). The amplification consisted of 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min followed by a denaturation step at 94 °C for 3 min using the product. Nested PCR was done under the same conditions with the internal primer S2 (GGAACGTTTCGTTATGACCAGTTGTGTAC) and A2 (CCGCCTTTGGCATAGACAACCAAGTTTCCG). The resulting product was analyzed as described above.

DNA sequence analysis

The PCR products were subcloned into the plasmid pGEM-T (Promega, Madison, WI) after purification by agarose gel electrophoresis. DNA sequencing was performed by cycle sequencing on an ABI DNA Sequencer (Model 310; Applied BioSystems, Foster City, CA) using the T7promoter and SP6 promoter primers and BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems). Sequence data were analyzed using GENETIX-MAC (ver. 15). Homologous sequences were searched using the BLAST and FASTA programs. Sequence alignments were made using the CLUSTAL W program [11].

Expression of the recombinant MOL (rMOL)

A full-length coding sequence PCR product was digested with *Nde*I and *Bam*HI and cloned into an isopropyl-1-thio-α-D-galactopyranoside-inducible pET3a expression vector (Novagen, Darmstadt, Germany). rMOL was expressed in a BL21 (DE3) strain of *E. coli*. Induced bacteria were collected by centrifugation and then resuspended in PBS. After the cell suspension was sonicated, the insoluble fraction was removed by centrifugation (10,000× *g*, 20 min). rMOL was purified by affinity chromatography with a column of asialofetuin-Sepharose 4B in a similar manner for MOL.

Protein determination

Protein amounts were determined by the method of Lowry *et al.* [12] with bovine serum albumin as the standard.

Hemagglutination and inhibition assay

Hemagglutination assays were performed in 96-well microtiter plates, in a final volume of 70 μl containing 50 μl of serially diluted lectin solution and 20 μl of a 4 % (v/v) suspension of trypsinized rabbit erythrocytes. After incubation for 1 h at room temperature, the agglutination titer was recorded for the maximum dilution giving positive agglutination. Carbohydrate-binding specificity was determined by the inhibition of agglutination of trypsinized rabbit erythrocytes. Stock solutions of the inhibitors (25 μl) were diluted twofold in 96-well microtiter plates, followed by the addition of 25 μl of lectin solutions with a hemagglutination titer of 8. After 1 h of incubation, 20 μl of 4 % trypsinized rabbit erythrocyte suspension was added to the mixture, and agglutination was measured after standing for 1 h at room temperature. The results were expressed as the minimum concentration of inhibitor required for the complete inhibition of hemagglutination.

Glycan array

Sugar-binding profiling of MOL was determined as described by Tateno *et al.* [13].

Glycoproteins and glycoside-polyacrylamide used for glycan-array are shown in Table 1.

Thermal stability

Thermal stability profile was obtained by incubating aliquots of MOL at 25–100 °C in a water bath for 30 min. After the thermal treatment, the lectin solutions were chilled on ice, and the remaining activity was determined. Activity at each temperature was expressed as the relative activity compared with the control at 4 °C.

Results and discussion

Purification of MOL

Table 2 shows the summary of purification of MOL from 100 g of fruiting bodies by asialofetuin-Sepharose 4B. Its yield was 75 %, and 16.2 mg of MOL was obtained.

Table 1 Glycans used for glycan array

Number	Trivial name	Presentation	Glycans
1	aFuc	PAA	Fucα1-PAA
2	Fucα2Gal	PAA	Fucα1-2Galβ1-PAA
3	Fucα3GlcNAc	PAA	Fucα1-3GlcNAcβ1-PAA
4	Fucα4GlcNAc	PAA	Fucα1-4GlcNAcβ1-PAA
5	H type1	PAA	Fucα1-2Galβ1-3GlcNAcβ1-PAA
6	H type2	PAA	Fucα1-2Galβ1-4GlcNAcβ1-PAA
7	H type3	PAA	Fucα1-2Galβ1-3GalNAcα1-PAA
8	A	PAA	GalNAcα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-PAA
9	B	PAA	Galα1-3(Fucα1-2)Galβ1-4GlcNAcβ1-PAA
10	Le^a	PAA	Galβ1-3(Fucα1-4)GlcNAcβ1-PAA
11	[3S]Le^a	PAA	$(3OSO_3)$Galβ1-3(Fucα1-4)GlcNAcβ1-PAA
12	Le^b	PAA	Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-PAA
13	Le^x	PAA	Galβ1-4(Fucα1-3)GlcNAcβ1-PAA
14	Le^y	PAA	Fucα1-2Galβ1-4(Fucα1-3)GlcNAcβ1-PAA
15	aNeu5Ac	PAA	Neu5Acα2-PAA
16	aNeu5Gc	PAA	Neu5Gcα2-PAA
17	Siα2	PAA	Neu5Acα2-8Neu5Acα2-PAA
18	Siα3	PAA	Neu5Acα2-8Neu5Acα2-8Neu5Acα2-PAA
19	3′SiaLe^c	PAA	Neu5Acα2-3Galβ1-3GlcNAcβ1-PAA
20	3′SL	PAA	Neu5Acα2-3Galβ1-4Glcβ1-PAA
21	3′SLN	PAA	Neu5Acα2-3Galβ1-4GlcNAcβ1-PAA
22	sLe^a	PAA	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAcβ1-PAA
23	sLe^x	PAA	Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAcβ1-PAA
24	6′SL	PAA	Neu5Acα2-6Galβ1-4Glcβ1-PAA
25	FET	Glycoprotein	Fetuin (Complex-type N-glycans and O-glycans)
26	AGP	Glycoprotein	α1-acid glycoprotein (Complex-type N-glycans)
27	TF	Glycoprotein	Transferrin (Complex-type N-glycans)
28	TG	Glycoprotein	Porcine thyroglobulin (Complex and high-mannnose-type N-glycans)
29	bGal	PAA	Galβ1-PAA
30	[3S]bGal	PAA	$(3OSO_3)$Galβ1-PAA
31	A-di	PAA	GalNAcα1-3Galβ1-PAA
32	Lac	PAA	Galβ1-4Glcβ1-PAA
33	Le^c	PAA	Galβ1-3GlcNAcβ1-PAA
34	[3′S]Le^c	PAA	$(3OSO_3)$Galβ1-3GlcNAcβ1-PAA
35	LN	PAA	Galβ1-4GlcNAcβ1-PAA
36	[3′S]LN	PAA	$(3OSO_3)$Galβ1-4GlcNAcβ1-PAA
37	[6S]LN	PAA	Galβ1-4$(6OSO_3)$GlcNAcβ1-PAA
38	[6′S]LN	PAA	$(6OSO_3)$Galβ1-4GlcNAcβ1-PAA
39	bGalNAc	PAA	GalNAcβ1-PAA
40	di-GalNAcβ	PAA	GalNAcβ1-3GalNAcβ1-PAA
41	LDN	PAA	GalNAcβ1-4GlcNAcβ1-PAA
42	GA2	PAA	GalNAcβ1-4Galβ1-4Glcβ1-PAA
43	Asialo-FET	Glycoprotein	Asialo fetuin (Desialylated complex-type N- and O-glycans)
44	Asialo-AGP	Glycoprotein	Asialo α1-acid glycoprotein (Desialylated complex-type N-glycans)
45	Asialo-TF	Glycoprotein	Asialo transferrin (Desialylated complex-type N-glycans)
46	Asialo-TG	Glycoprotein	Asialo porcine thyroglobulin (Desialylated complex-type N-glycans, high-mannose-type N-glycans)
47	βGlcNAc	PAA	GlcNAcβ1-PAA
48	[6 S]bGlcNAc	PAA	$(6OSO_3)$GlcNAcβ1-PAA
49	Agalacto-Fet	Glycoprotein	Agalacto fetuin (Agalactosylated complex-type N- and O-glycans)
50	Agalacto-AGP	Glycoprotein	Agalacto α1-acid glycoprotein (Agalactosylated complex-type N-glycans)

Table 1 (continued)

Number	Trivial name	Presentation	Glycans
51	Agalacto-TF	Glycoprotein	Agalacto transferrin (Agalactosylated complex-type N-glycans, high-mannose-type N-glycans)
52	OVM	Glycoprotein	Ovomucoid (Complex-type N-glycans)
53	OVA	Glycoprotein	Ovoalbumin (Hybrid-type N-glycans)
54	aMan	PAA	Manα1-PAA
55	bMan	PAA	Manβ1-PAA
56	[6P]Man	PAA	($6OPO_4$)Manα1-PAA
57	INV	Glycoprotein	Yeast invertase (High mannose-type N-glycans)
58	Tn	PAA	GalNAcα1-PAA
59	Core1	PAA	Galβ1-3GalNAcα1-PAA
60	Core2	PAA	Galβ1-3(GlcNAcβ1-6)GalNAcα1-PAA
61	Core3	PAA	GlcNAcβ1-3GalNAcα1-PAA
62	Core4	PAA	GlcNAcβ1-3(GlcNAcβ1-6)GalNAcα1-PAA
63	Forssman	PAA	GalNAcα1-3GalNAcβ1-PAA
64	Core6	PAA	GlcNAcβ1-6GalNAcα1-PAA
65	Core8	PAA	Galα1-3GalNAcα1-PAA
66	[3′S]Core1	PAA	($3OSO_3$)Galβ1-3GalNAcα1-PAA
67	Galb-Core3	PAA	Galβ1-4GlcNAcβ1-3GalNAcα1-PAA
68	Asialo-BSM	Glycoprotein	Asialo bovine submaxillary mucin (Tn)
69	Asialo-GP	Glycoprotein	Asialo human glycophorin MN (T)
70	STn	PAA	Neu5Acα2-6GalNAcα1-PAA
71	STn (Gc)	PAA	Neu5Gcα2-6GalNAcα1-PAA
72	ST	PAA	Neu5Acα2-3Galβ1-3GalNAcα1-PAA
73	Siaα2-6Core 1	PAA	Galβ1-3(Neu5Acα2-6)GalNAcα1-PAA
74	BSM	Glycoprotein	Bovine submaxillary mucin (Sialyl Tn)
75	GP	Glycoprotein	Human glycophorin (Disialyl T and sialyl Tn)
76	aGal	PAA	Galα1-PAA
77	Galα1-2Gal	PAA	Galα1-2Galβ1-PAA
78	Galα1-3Gal	PAA	Galα1-3Galβ1-PAA
79	Galα1-3Lac	PAA	Galα1-3Galβ1-4Glcβ1-PAA
80	Galα1-3LN	PAA	Galα1-3Galβ1-4GlcNAcβ1-PAA
81	Galα1-4LN	PAA	Galα1-4Galβ1-4GlcNAcβ1-PAA
82	Melibiose	PAA	Galα1-6Glcβ1-PAA
83	aGlc	PAA	Glcα1-PAA
84	bGlc	PAA	Glcβ1-PAA
85	Maltose	PAA	Glcα1-4Glcβ1-PAA
86	HA	BSA	Hyaluronic acid-BSA
87	CSA	BSA	Chondoroitin Sulfate A-BSA
88	CSB	BSA	Chondoroitin Sulfate B-BSA
89	HS	BSA	Heparan Sulfate-BSA
90	HP	BSA	Heparin-BSA
91	KS	BSA	Keratan Sulfate-BSA
92	aRha	PAA	Rhamnoseα1-PAA
93	Mannan (SC)	Glycoprotein	*S. cerevisiae* mannan
94	Mannan (CA)	Glycoprotein	*C.albicans* mannan
95	Zymosan	Glycoprotein	Zymosan
96	Chitobiose	PAA	GlcNAcβ1-4GlcNAcβ1-PAA
97	BSA	BSA	-
98	Negative PAA	PAA	-
99	Marker		
100	BG		

Table 2 Purification of MOL

Purification step	Total activity (titer)	Total protein (mg)	Specific activity (titer/mg)	Purification (fold)	Yield (%)
Crude extract	32,000	2,500	12.8	1	100
Asialofetuin-Sepharose 4B	24,000	16.2	1,480	115	75.0

Amino acid sequence of MOL

The peptide sequences after enzymatic digestion and Edman sequencing were assigned to the amino acid sequence deduced from the DNA (Fig. 1). cDNA sequencing of MOL was done by PCR and 5′- and 3′-RACE. The cDNA comprised 561 nucleotides with an open reading frame of 360 nucleotides encoding 120 amino acids (GenBankTM accession number AB 649027). The results agreed with the peptide sequence, but 3 residues (107–109) were not obtained from the peptide sequences. Two putative N-glycosylation sites were found in the sequence, but MOL was not glycosylated.

Expression and characterization of MOL

The MOL open reading frame was cloned into a pET-3a expression vector. The protein was produced in *E. coli* BL21 (DE3) and purified as described above. rMOL had an electrophoretic mobility in SDS-PAGE identical to that of native protein at 13 kDa (Fig. 2) and eluted as a dimer at the same elution volume as MOL from a TSKgel G2000SW column.

The molecular mass of MOL is calculated to be 13,103 from 120 amino acids. The MOL and rMOL were also subjected to MALDI-TOF mass spectrometry. MOL showed a molecular mass of 13,012.8±3.0 Da whereas rMOL had a slightly lower mass of 12,968.5±3.0 Da. The N-terminus of MOL was blocked but chymotryptic peptides of MOL showed SYVHPYGSTLPENG. The N-terminal sequence was also determined to be SYVHPYGSTL from rMOL. The initial methionine residue was removed.

Hemagglutination activity of MOL

MOL agglutinated native and trypsinized rabbit erythrocytes at a minimum concentration of 0.7 μg/ml. However, MOL did not agglutinate any human erythrocytes, irrespective of trypsin treatment. MOL retained full hemagglutinating activity after the heat treatment at 60 °C for 30 min. Treatment at 70 °C for 30 min inactivated MOL completely.

Sugars and glycoproteins specificity of MOL

The inhibition of the hemagglutinating activity of MOL and rMOL by various saccharides and glycoproteins is summarized in Table 3. For MOL, mannose was weakly inhibitory, other mono- and oligosaccharides were not inhibitory, and

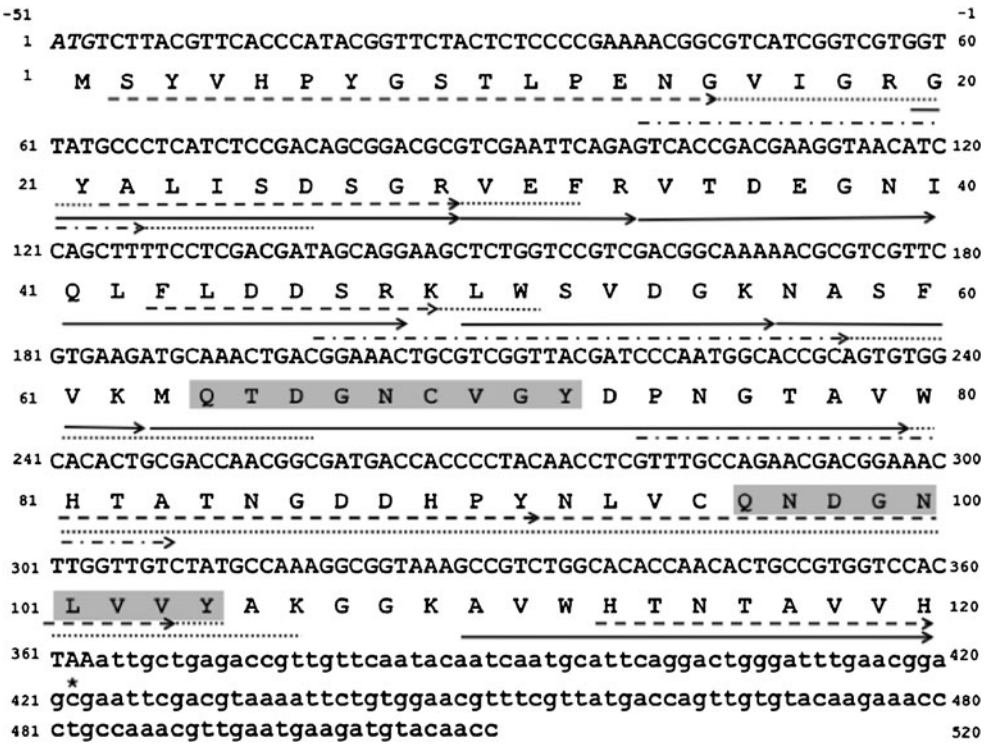

Fig. 1 Nucleotide sequence and deduced amino sequence of MOL. The numbers on the left and right margins indicate the order of amino acid residues and nucleotides, respectively. Amino acid residues of mannose-binding motif are shaded in gray. The arrows represent amino acid residues determined by Edman degradation. Dashed line is chymotryptic peptides, continuous line is tryptic peptides, long dashed dotted line is *S. aureus* V8-digested peptides, and dotted line is putative site of a digestion. Initiation and stop codons are indicated in italics and by an asterisk, respectively

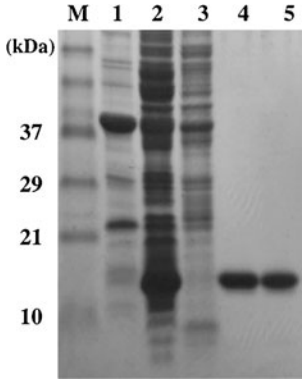

Fig. 2 Tricine SDS-PAGE analysis of MOL and recombinant MOL. Lane M, protein molecular weight marker; Lane 1, precipitation of bacterial lysate; Lane 2, supernatant of bacterial lysate; Lane 3, flow-through fraction; Lane 4, purified recombinant MOL by affinity chromatography on asialofetuin-Sepharose 4B column; Lane 5, purified MOL by affinity chromatography on asialofetuin-Sepharose 4B column

asialofetuin was the most potent inhibitor. Quail ovomucoid and bovine thyroglobulin with trimannosyl core and high mannose-N-glycans respectively weakly inhibited hemagglutination, but quail albumin and bovine pancreatic RNase B with only high mannose-N-glycans did not. The specific activity and hemagglutination inhibition of rMOL were almost the same as the native lectin.

The result of glycan array analysis of rMOL is shown in Fig. 3. MOL did not react to invertase and ovalbumin, proteins with high mannose N-glycans, and yeast mannans, but preferred asialo- and agalacto-derivatives of α_1-acid glycoprotein, fetuin, and transferin. Tateno *et al.* [13] showed the sugar binding-pattern of concanavalin A, a typical mannose-binding lectin with the same method. The pattern of Con A is quite different from that in Fig. 3. Apparently, sugar-binding specificity of MOL is different from those of typical mannose-binding lectins, and close to that of TxcL1 [14].

Table 3 Inhibition by sugars and glycoproteins of hemagglutinating activity of MOL and rMOL Minimum concentration required for the complete inhibition of titer 4 hemagglutinating activity

Inhibitor	Minimum inhibitory concentration	
	MOL	rMOL
	(mM)	
D-Mannose	200	200
	(mg/mL)	
BSM	0.25	0.25
AsialoBSM	0.125	0.125
Asialofetuin	0.008	0.004
Ovomucoid (quail)	0.25	0.125
Thyroglobulin (bovine)	0.125	0.125

Arabinose, L-fucose, galactose, glucose, rhamnose, and xylose were not inhibitory at 200 mM, and lactose and laminaribiose were not at 100 mM. AsialoPSM, bovine pancreatic RNase B, fetuin, ovalbumin, quail ovalbumin, PSM, and yeast mannan were not inhibitory at 1.0 mg/mL, and porcine thyroglobulin was not at 0.5 mg/mL

Sequence homology

BLAST searches showed the highest similarity of the MOL sequence to *Galanthus nivalis* agglutinin (GNA)-like protein from liverwort *Marchantia polymorpha* [15], mannose-binding lectin from *Zea mays* [16], mannose specific lectin from *Allium ampeloprasum* [17], antifungal protein from *Gastrodia elata* (NCBI accession number AAG53445), mannose-binding lectin from *Picea abies* (NCBI accession number AAZ30388), and D-mannose binding lectin from *Signiliparus rugosus* (NCBI accession number ZP_07963966). All of these proteins were bulb-type mannose-specific lectins that have the mannose-binding motif QXDXNXVXY (Fig. 4) [18]. The sequence of the first bulb-type mannose-specific lectin was reported from *G. nivalis* [19], and subsequently, many lectins have been isolated from monocots and liverworts. And the genes were also found in bacteria *Signiliparus*, conifers, and some fungi [5, 18]. Furthermore, one lectin of Ascomycota is deduced to be this family of lectin from the partial sequences [6]. However, the Ascomycota lectin showed completely different sugar-binding specificity, showing the preference to xylose and inulin. MOL also recognized complex N-glycans rather than high mannose N-glycans, though the two mannose-binding motifs were found. The sugar binding specificity of MOL is closer to those of GNAmaize [16] and RVL [20], which recognized not only high mannose N-glycans but also complex N-glycans. MOL showed sequence homology to mannose-binding lectins from *Z. may* and *M. polymorpha*, with sequence identities of 38 %. MOL included two tandemly repeated homologous domains (residues 64–84 and 96–116) with 62 % internal homology.

Conclusion

This is the first report on Basidiomycota mannose-binding GNA-like lectin with the mannose-binding motif found in many monocots. The finding shows that the bulb-type lectin is common in plant and fungi as similarly known for the ricin type lectins and the Jacalin-related lectins. However, the sugar binding specificities of MOL were close to that of GNAmaize and RVL, and was different from that of Ascomycota lectin. These GNA-like lectins are found not to be highly mannose-specific. Japanese *M. oreades* may contain

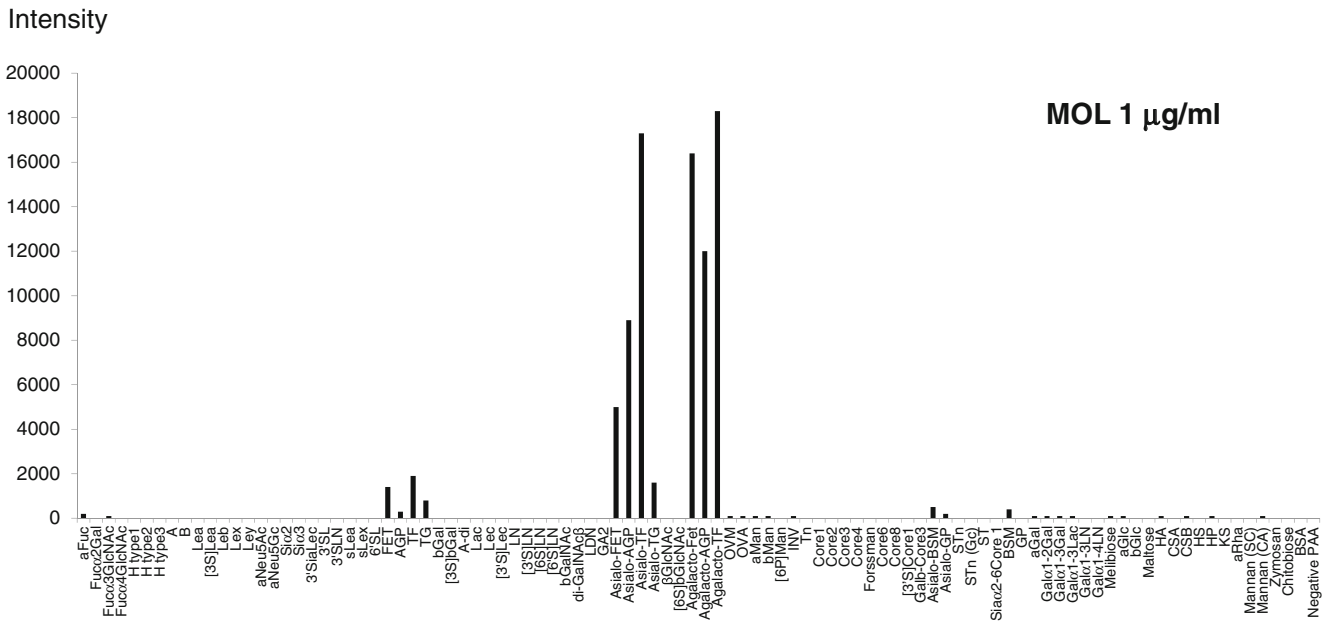

Fig. 3 Glycan-array of MOL. Cy3-labeled MOL was applied on the array and binding was detected by the scanner. Scan image of MOL was analyzed with the Array Pro analyzer ver. 4. 5

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P. abies          ----------------------MGYGTLDNGDWLMVGMSIFS----KDRSVELRMQDDGK 34
Z. mays           ----------------------MGYGTLDNGDWLMVGMSIFS----KDRSVELRMQDDGK 34
M. polymorpha     ----------------------MGYGTLDNGDWLMVGMSIFS----KDRSVELRMQDDGK 34
S. rugosus        -----------------------MATLQAGQSLAKGQSLSS----DNGVFTLTLQDDGN 32
MOL               -----------------MSYVHPYGSTLPENGVIGRGYALIS----DSGRVEFRVTDEGN 39
A. ampeloprasum   MGRTTPSPKLIMSITTVAAILTILASTCMARNLLTNGEGLYAGQSLDVEQYKFIMQDDCN 60
G. elata          --MAASASTAVILFFAVTTMMSLSAIPAFASDRLNSDHQLDTGGSLAQGGYLFIIQNDCN 58
R. vivipara       ---------------------------LGTNYLLSGQTLDTEGHLKNGDFDLVMQDDCN 32
                                              :  .  : :          : : :: :

P. abies          LAIYYNNRCAWQSTD-QQISNAKGAITQGDGNFCIYDKNGKATWHTNTAAPKGDNRTFVA 93
Z. mays           LAIYYNNRCAWQSTD-QQISNAKGAIMQGDGNFCIYDKNGKATWHTNTAAPKGDNKTFVA 93
M. polymorpha     LAIYYNDRCAWQSTD-QQISNAKGAIMQGDGNLCIYDKNGKATWHTNTAAPSGDNKTLLS 93
S. rugosus        LVLAEG-STPVWSTQ-TNGTGASRLEVQTDGNVVLYTDSNESKWATGTSG-----QVRLE 85
MOL               IQLFLDDSRKLWS---VDGKNASFVKMQTDGNCVGYDPNGTAVWHTATNGD--DHPYNLV 94
A. ampeloprasum   LVLYEYSTPIWASNTGVTGKNGCRAVMQKDGNFVVYDVNGRPVWATNSVRG--NGNYILV 118
G. elata          LVLYDNNRAVWASGTNGKASN-CFLKMQNDGNLVIYSGS-RAIWASNTNRQ--KGNYYLI 114
R. vivipara       LVLYNGN---WQSNTANNGRD-CKLTLTDYGELVIKNGDGSTVWKSGAQSV--KGNYAAV 86
                  : :         *       .         *:      .  . * : :

P. abies          VQDDGNLVLYRDGGATPIWSSKSNK---------------------------------- 118
Z. mays           VQDDGNLVLYRDGGATPIWSSKSNK---------------------------------- 118
M. polymorpha     VQDDGNLVLYKNGGATPIWSSKSNK---------------------------------- 118
S. rugosus        LQNDRNLVVYGADGS-ALWNSGTVTDKPIVPTPAAAEVVEEEPAAPEPQTYVVESGDTLS 144
MOL               CQNDGNLVVYAKGGK-AVWHTNTAVVH-------------------------------- 120
A. ampeloprasum   LQQDRNVVIY----GSDIWSTGTYRRS-------------------------AGGPVVM 148
G. elata          LQRDRNVVIYDNSNN-AIWATHTNVGN-----------------------------AEI 143
R. vivipara       VHPDGRLVVLGPS-------------------------------------------VF 101
                   : * .:*:
```

Fig. 4 Comparison of sequences of MOL with some bulb-type mannose-specific lectins. Sequence alignment of bulb-type mannose-specific lectins from *P. abies, Z. may*, *M. polymorpha*, *A. ampeloprasum*, *G. elata*, *S. rugosus*, *R. vivipara*, and MOL, performed using Clustal W. Identical residues are indicated by asterisks, and similar residues are indicated by dashes or colons. The amino acids that constitute the mannose-binding motifs are shaded in gray

a lectin differing from those of European and US *M. oreades* [21, 22].

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