Purification and characterization of a lectin from the mushroom Hypsizigus marmoreus

Note

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Abstract

HML (*Hypsizigus marmoreus* lectin) was isolated from the mushroom *Hypsizigus marmoreus* using CM cation exchange, bovine submaxillary gland mucin affinity column and TSK-GEL G3000SW gel filtration chromatography. The results of SDS-PAGE, MALDI-TOF MS and gel filtration analysis of HML indicated that the lectin was a dimer with each subunit of 9.5 kDa. The partial amino acid sequences of HML were determined by *N*-terminal sequencing of peptides obtained by trypsin or Glu-C endopeptidase digest of the lectin. In the hemagglutination inhibition assay, HML did not bind to any mono- or oligo-saccharides tested. Among the glycoproteins examined, asialo-fetuin was the strongest inhibitor.

Keywords: Carbohydrate-binding specificity; *Lyophyllaceae* family; Partial amino acid sequences

Lectins are proteins or glycoproteins that are capable of specific recognition and binding to carbohydrate moieties of glycoproteins or glycolipids (Goldstein et al. 1980). Thus, lectins are used in studies of cell surface carbohydrate structures and oligosaccharides structures of glycoconjugates (Sharon and Lis 2004; Sharon 2007). Recently, new technologies such as lectin microarray have been in focus, therefore lectins with ever-unknown specific sugar binding properties are useful as a molecule that can be spotted on the array (Hirabayashi 2004; Kuno et al. 2005; Meany et al. 2011). Among lectins, mushroom lectins represent a class of intensely studied biomolecules, and more than 50 mushroom lectins have been purified to date and have attracted wide attention due to their biological activities (Kawagishi 1995; Kobayashi et al. 2004a,b; Kobayashi et al. 2005; Horibe et al. 2010; Kobayashi et al. 2012; Suzuki et al. 2012; Xu et al. 2014).

Hypsizigus marmoreus (bunashimeji in Japanese) is as popular as *Lentinula edodes* (shiitake) and *Flammulina velutipes* (enokitake) as an edible mushroom in Japan and readily available at supermarkets nationwide. Besides its good taste, many biological activities have been discovered in *H. marmoreus*: antioxidant (Matsuzawa et al. 1998; Lee et al. 2007), antiproliferative (Lam and Ng 2001), ribosome inactivating (Lam and Ng 2001; Wong et al. 2008), antiviral (Akihisa et al. 2005; Suzuki et al. 2011), anticancer (Chang et al. 2004; Chang et al. 2009) and ribonuclease (Guan et al. 2007) activities. However, there are no reported lectin or hemagglutinin from this mushroom.

In the course of our continuing screening for new mushroom lectins, we found lectin activity in the extract of *H. marmoreus*. Here, we describe the isolation and characterization of the lectin from this mushroom.

Purification of the lectin

The purification procedure is summarized in Table 1. In the present study, bovine

submaxillary gland mucin (BSM)-Toyopearl was chosen as the affinity support of choice as the hemagglutination activity of the crude extract from the mushroom was inhibited by BSM (Kobayashi 2004b). Initially, lyophilized fruiting bodies of *H. marmoreus* (JA Zennou) were homogenized and extracted with 50 mM acetate buffer, pH 5.0, overnight. The homogenate was centrifuged at 8,500*g* for 25 min and the resulting supernatant was applied to a column (5 × 13 cm) of CM-Toyopearl 650M (Tosoh, Tokyo, Japan) equilibrated with the buffer. After unbound materials were washed with the buffer, the bound fraction was eluted with 0.5 M NaCl in the buffer. The eluates were concentrated and equilibrated with 50 mM acetate buffer, pH 5.0, by ultrafiltration, and applied to a BSM-Toyopearl column (2.5 × 20 cm) equilibrated in the same buffer. After all unbound substances were removed by washing the column with the buffer, the bound fraction was then eluted by 0.5 M acetic acid. The lectin-containing fraction was dialyzed against 10 mM phosphate-buffered saline, pH 7.4 (PBS), containing 0.1% (w/v) sodium dodecyl sulfate (SDS), and applied to a TSK G3000SW column (7.8 × 300 mm; Tosoh, Tokyo, Japan) equilibrated in the same buffer. The eluant was dialyzed extensively against distilled water and ultra-filtered to give *H. marmoreus* lectin (HML).

Molecular properties of HML

HML gave a single band with an apparent mass of 10 kDa on SDS-PAGE (Laemmli 1970) in the presence of 2-mercaptoethanol (Fig. 1). Gel filtration for determination of its molecular mass by HPLC was carried out on a TSK-gel G3000SW$_{XL}$ column (7.8 × 300 mm) operating at room temperature in PBS at a flow rate of 0.5 ml/min. The molecular mass was calibrated with the standard proteins (Sigma, MO, USA). HML gave a single symmetrical peak at an elution volume corresponding to a molecular mass of about 18 kDa (Fig. 2). Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry

(MALDI−TOF MS) analysis was performed on AutoFlex (Bruker Daltonics, MA, USA), using 10 mg/ml super 2-hydroxy-5-methoxybenzoic acid (Bruker Daltonics), as a matrix. The MALDI-TOF mass spectrum of the lectin gave a main peak at *m/z* 9,576 and a small peak at *m/z* 19,251 (Fig. 3). The results of SDS-PAGE, gel filtration, and MALDI-TOF MS analysis indicated that the molecular mass of HML was 19 kDa and it was composed of two identical 9.5 kDa-subunits.

N-terminal amino acid sequence analysis of intact HML suggested that the amino terminus of HML was blocked. Thus, HML was digested with sequencing grade modified trypsin (Promega, WI, USA) or Endoproteinase Glu-C from *Staphylococcus aureus* strain V8 (Sigma), and the resulting peptides were isolated by reverse-phase HPLC. Each purified peptide sequence was analyzed on a PPSQ-21A protein sequencer (Shimadzu, Kyoto, Japan). As a result, three partial amino acid sequences of HML were determined as shown in Table 2. The partial amino acid sequences of these three peptides obtained by protein sequencing were very short, but covered about 40% of HML judging from its molecular mass. These sequences show similarity to those of many proteins by BLASTP search against non-redundant (nr) protein sequences and Swiss-Prot database. However, no known lectins were found by the homology search, indicating that HML might be a new type of lectin. Further amino acid sequencing analysis or molecular cloning of HML is needed to conclude it.

Properties of HML as a lectin

The hemagglutinating activity of the lectin was determined by a two-fold serial dilution procedure using Pronase-treated rabbit erythrocytes. The hemagglutination titer was defined as a reciprocal of the highest dilution exhibiting hemagglutination. Inhibition was expressed as the minimum concentration of each sugar or glycoprotein required for inhibition of

hemagglutination of titer 4 of the lectin. Table 3 shows the inhibition of hemagglutination activity of HML by various monosaccharides, oligosaccharides, and glycopeptides. HML did not bind to any mono- or oligo-saccharides tested. Among the glycoproteins examined, the most potent inhibitor was asialo-fetuin (minimum inhibitory concentration (MIC), 7.81 μg/ml). Thyroglobulin, fetuin, BSM and α_1-acid glycoprotein were also inhibitory at higher concentrations, 15.6, 15.6, 31.3 and 125 μg/ml, respectively, than asialo-fetuin. Asialo-fetuin that carries both *N* and *O* linked oligosaccharides was the most potent inhibitor of the hemagglutination activity. Fetuin has a triantennary structure with terminal Galβ1-4GlcNAc and three *O*-linked structure (Townsend et al. 1986). Thyroglobulin contains mainly diantennary Galβ1-4GlcNAc *N*-glycans (Takeya et al. 2007). Mucin contains several Galβ1-3GalNAcα1 *O*-glycans. HML showed high affinity for fetuin and thyroglobulin. The affinity of HML towards fetuin and thyroglobulin suggested that the Galβ1-4GlcNAc *N*-glycans structure was important for the interaction with HML. In basidiomycetes, lectins from *Volvariella volvacea* (VVL) (She et al. 1998), *Mycoleptodonoids aitchisonii* (MAL) (Kawagishi et al. 2001) and *Ganoderma lucidum* (Thakur et al. 2007) have been reported with hemagglutination activity inhibited only by glycoproteins. Hemagglutinating activity of VVL was inhibited by thyroglobulin and MAL exhibited strong sugar binding specificity towards asialo BSM. In the case of *G. lucidum* lectin, asialo-fetuin was most potent inhibitor. The results of the hemagglutination inhibition test of HML suggest that the carbohydrate-binding specificity of HML was most similar to that of *G. lucidum* lectin. At present, we cannot explain the reason why the hemagglutination mediated by HML was inhibited by BSM, but asialo-BSM, PSM (porcine stomach mucin) and asialo-PSM. More detailed analysis of its binding specificity is necessary.

Some lectins can specifically bind to fucose, galactose/*N*-acetylgalactosamine, mannose/glucose, *N*-acetylglucosamine, sialic acid or complex oligosaccharides

(Guzman-Partida et al. 2004). Therefore, such lectins have become invaluable tools for chemical and biological research of carbohydrate-containing compounds as well as for clinical uses (Van Damme et al. 1997; Loris et al. 1998; Lam and Ng 2011). The result of the hemagglutination inhibition test of HML suggested that the lectin belongs to the group termed "complex" with specificity towards *N*-glycans. FVA-L from *Flammulina velutipes* (Ng et al. 2006), lectin IV from *Griffonia simplicifolia* (Delbaere et al. 1993), PHA-L from *Phaseolus vulgaris* (Loris et al. 1998), PF2 from *Olneya tesota* (Guzman-Partida et al. 2004) and VL1 from *Acacia constricta* (Guzman-Partida et al. 2004) also belong to this group. To our knowledge, this is the first report of isolation and characterization of the lectin from *Hypsizigus marmoreus.*

Disclosure

The authors declare no conflict of interest. All the experiments undertaken in this study comply with the current laws of Japan.

Acknowledgments

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Figure legends

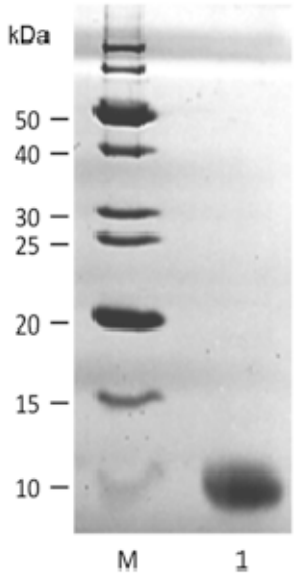

Fig. 1 – SDS-PAGE of HML. Lane M, marker proteins; lane 1, HML reduced with 2-mercaptoethanol. Samples were heated in the presence of 2-mercaptoethanol for 10 min at 100°C and SDS-PAGE (12% polyacrylamide gel) was done by the Laemmli method (Laemmli 1970), with Coomassie Brilliant Blue staining. The molecular mass on SDS-PAGE was estimated using recombinant ladder markers (10 to 100 kDa; XL-Ladder Low; APRO Life Science Institute, Tokushima, Japan).

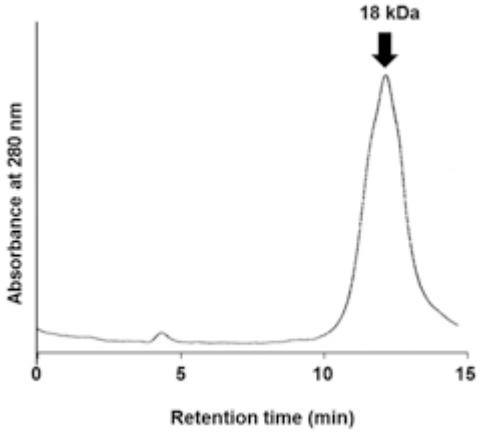

Fig. 2 – HPLC profile of HML. Gel filtration by HPLC was carried out on a TSK-gel G3000SW$_{XL}$ column (7.8 × 300 mm) operating at room temperature in PBS at a flow rate of 0.5 mL/min. Fractions were collected by monitoring absorbance at 280 nm. The molecular mass was calibrated with the following standard proteins (Sigma); bovine thyroglobulin (669 kDa), horse spleen apoferritin (443 kDa), sweet potato β-amylase (200 kDa), yeast alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa).

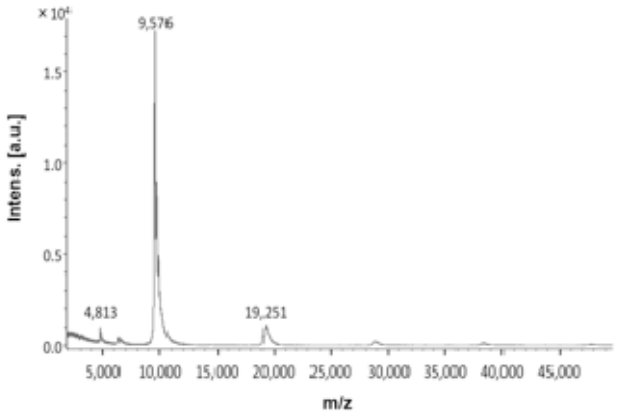

Fig. 3 – MALDI-TOF Mass Spectrometry of HML. The MALDI-TOF mass spectrum was acquired on an AutoFlex. Each sample was dissolved in 0.1% trifluoroacetic acid (TFA):acetonitrile (2:1 v/v) and mixed with the matrix solution (1:4 v/v). The mixture (1 µl) was put on a stainless steel target and crystallized at room temperature. The spectrum was measured in linear mode using 20-kV ion acceleration without post-acceleration. The spectrum was recorded at a detector voltage of 1.65 kV, and was expressed as the averaged results of at least 300 laser shots.

Table 1 – Purification of HML from 100 g of the fruiting bodies of *Hypsizigus marmoreus*.

Fraction	Total protein (mg)	Total agglutination (titer)[a]	Specific agglutination (titer/mg protein)	Recovery of activity (%)
Extract	1.1×10^3	7.0×10^4	64	100
CM cation-exchange chromatography	20	4.1×10^4	2.0×10^3	59
BSM-affinity chromatograhy	0.090	1.4×10^3	1.6×10^4	2.3
Gel filtration chromatography	0.045	8.8×10^2	2.2×10^4	1.2

[a] The hemagglutinating activity was determined by a 2-fold serial dilution procedure using rat erythrocytes. Titer was defined as the reciprocal of the end-point dilution exhibiting the hemagglutination.

Table 2 – Partial amino acid sequences of HML.

Peptide		Sequence
Trypsin-1	(*m/z* 2,425; calcd, 1,478.6)	LTDPTMIFDSQPN
Glu-1	(*m/z* 1,358; calcd, 1,283.3)	DLGSNCGGVFDAE
Glu-2	(*m/z* 1,331; calcd, 1,238.2)	AHMTVSFDSDE

Table 3 – Inhibition of hemagglutination activity by HML.

Inhibitors[a]	Minimum inhibitory concentration[b] (μg/ml)
Asialo-fetuin	7.81
Thyroglobulin	15.6
Fetuin	15.6
BSM[c]	31.3
α_1-acid glycoprotein	125

[a] Glucose, galactose, mannose, L-fucose, arabinose, L-arabinose, raffinose, saccharose, lactose, methyl α-glucoside, methyl β-glucoside, methyl β-galactoside, methyl α-mannoside, melibiose, 2-deoxyglucose and 2-deoxyribose did not inhibit at all at concentrations up to 0.4 M. Asialo-BSM, PSM[d] and asialo-PSM did not inhibit at concentrations up to 1 mg/ml.

[b] These are minimum inhibitory concentrations required for inhibition 4 hemagglutination doses of the lectin.

[c] BSM : Bovine submaxillary gland mucin.

[d] PSM : Porcine stomach mucin.