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A LECTIN FROM MYCELIA OF THE FUNGUS *GANODERMA LUCIDUM*

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Key Word Index—*Ganoderma lucidum*; Ganodermataceae; mycelium; fruiting body; lectin.

Abstract—A lectin (GLL-M) was isolated from mycelia of *Ganoderma lucidum* using affinity chromatography on BSM–Toyopearl. GLL-M is a monomer in its native form with a M_r of 18 000. Another lectin was also purified from fruiting bodies of the same fungus. The two lectins were partially compared with each other. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Many lectins have been isolated from the fruiting bodies of higher fungi (mushrooms) and characterized [1–25]. However, there are only a few reports which describe lectins in mycelia of higher fungi and no report which describes that lectin was isolated and its properties examined [4, 26]. We report herein the isolation and characterization of a lectin from mycelia of the fungus, *Ganoderma lucidum*, whose fruiting bodies are used in Chinese medicine; partial comparison of it with a lectin isolated from the fruiting bodies of the same fungus is made.

RESULTS AND DISCUSSION

The purification procedure for GLL-M is summarized in Table 1. Since both the culture broth and crude saline extract of the mycelia of *G. lucidum* showed haemagglutinating activity and preliminary experiments indicated that both lectins from the broth and the mycelia extract were identical, the lectin was isolated from the combined extracts from broth and mycelia. The crude lectin exhibited no remarkable binding specificity to any simple sugar and showed specificity against asialo-bovine submaxillary mucin (BSM), BSM, asialofetuin and fetuin in the haemagglutination-inhibition assay. Therefore, BSM–Toyopearl was chosen as the affinity support. All the lectin activity was adsorbed on the affinity column and eluted with 0.5 M acetic acid, giving the purified lectin, GLL-M. On the other hand, a lectin (GLL-F) was isolated from the fruiting bodies of the same fungus according to the method used for the mycelial lectin isolation (Table 2). The specific agglutination activity of GLL-F was much weaker than that of GLL-M. The low recovery of activity may be the result of deactivation with 0.5 M acetic acid in the affinity chromatography or irreversible adsorption of the lectins to the column.

Each purified lectin appeared as a single band on

Table 1. Purification of GLL-M (from 2 l of culture)

Fraction	Total protein (mg)	Total agglutination activity (titre)*	Specific agglutination activity (titre mg^{-1} protein)	Recovery of activity (%)†
80% Ammonium sulphate precipitate	1176	91 136	77.5	100
Anion-exchange chromatography	201.4	92 160	457.6	101
Affinity chromatography	2.2	12 288	5600	13.5

*Titre defined as reciprocal of end-point dilution exhibiting haemagglutination with pronase-treated type A erythrocytes in PBS.

†Based on initial ammonium sulphate precipitate.

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Table 2. Purification of GLL-F (from 500 g of fruiting bodies)

Fraction	Total protein (mg)	Total agglutination activity (titre)*	Specific agglutination activity (titre mg^{-1} protein)	Recovery of activity (%)†
80% Ammonium sulphate precipitate	1090	34 000	31	100
Anion-exchange chromatography	82.0	35 000	427	103
Affinity chromatography	4.1	2099	512	6.2

*,†Footnotes as Table 1.

SDS–PAGE (Fig. 1). GLL-M showed a single band corresponding to a M_r of 18 000 regardless of the presence or absence of 2-mercaptoethanol. The M_r of the native lectin was also 18 000 on the basis of the result of gel filtration on Superose 12 in (PBS) 10 mM phosphate-buffered saline (pH 7.4) suggesting that GLL-M is a monomer of M_r 18 000. On the other hand, GLL-F showed a band of M_r 16 000 on SDS–PAGE and the M_r of the native form by gel filtration on Superose 12 was estimated to be 19 000. These results indicate that GLL-F exists as a monomeric molecule in its native state or as a dimer which interacted with Superose 12 so that the estimated M_r is smaller than the true one.

Amino acid analysis of GLL-M indicated a high content of Gly, Ala, Asx and Thr, and a low content of Arg, Lys, Met and His (Table 3). Although 2 nmol of the protein was applied to the sequence, the *N*-terminal amino acid could not be detected. This suggests that the *N*-terminus of the protein is blocked.

After isoelectric focusing of GLL-M, the lectin gave a band at pH 4.5 (Fig. 2), suggesting that there are no isolectins due to the charge-difference of the protein. The carbohydrate content of the lectin amounted to 4.0%. Haemagglutination was not affected by demetallization and addition of $CaCl_2$, $MgCl_2$, $ZnCl_2$ or $MnCl_2$ to the demetallized lectins did not cause any change of activity. The lectin did not agglutinate any type of native human erythrocyte, but pronase-treated blood cells caused agglutination by the lectin; the titre of GLL-M (1 mg ml^{-1}) was 5600 to all of the cell-types.

Table 3. Amino acid composition of GLL-M

Amino acid	Mol%	Amino acid	Mol%
Asx	11.0	Ile	4.6
Thr	10.4	Leu	7.1
Ser	8.9	Tyr	2.6
Glx	8.0	Phe	4.3
Gly	12.0	Lys	1.2
Ala	11.0	His	1.3
Val	8.0	Arg	1.1
Cys	ND*	Pro	7.3
Met	1.2	Trp	ND

*Not determined.

Similarly, GLL-F was also inactive to intact human erythrocytes and caused haemagglutination to the enzyme-treated cells; the titre of GLL-M (1 mg ml^{-1}) was 512 to the cells regardless of blood type.

The results of haemagglutination inhibition assays of GLL-M and GLL-F are shown in Table 4. GLL-M-induced haemagglutination was not inhibited by any mono- or oligosaccharide tested, but some glycoproteins inhibited haemagglutination. Asialo-BSM was the best inhibitor, and BSM, asialofetuin and fetuin were also inhibitory. Haemagglutionation by GLL-F was inhibited by high concentrations of glucosamine and galactosamine, although mannosamine, *N*-acetylglucosamine, *N*-acetylgalactosamine and *N*-acetylmannosamine were inactive. Among the glycoproteins,

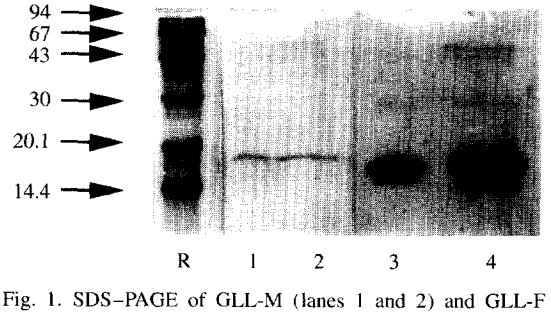

Fig. 1. SDS–PAGE of GLL-M (lanes 1 and 2) and GLL-F (lanes 3 and 4). Lanes 1 and 3, in the absence of 2-mercaptoethanol; lanes 2 and 4, in the presence of the reagent. Lane R contains marker proteins (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin in kDa).

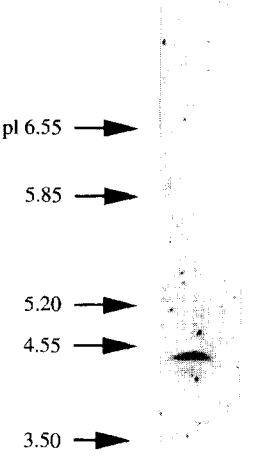

Fig. 2. Isoelectric focusing of GLL-M.

Table 4. Inhibition of haemagglutination activity by GLL-M and GLL-F

Inhibitor*	Minimum inhibitory concentration†	
	GLL-M	GLL-F
	(mM)	
Glucosamine	NI‡	200
Galactosamine	NI	200
	(μg ml^{-1})	
Asialo-BSM	16	31
Asialofetuin	31	63
BSM	31	31
Fetuin	125	31

*D, L-Arabinose, D, L-fucose, ribose, 2-deoxyribose, fructose, glucose, *N*-acetylglucosamine, galactose, *N*-acetylgalactosamine, mannose, mannosamine, *N*-acetylmannosamine, *N*-acetylneuraminic acid, xylose, lactose, lactobionic acid, lactulose, melibiose, *N*-acetylchitobiose and chitobiose exhibited no inhibition at concentrations up to 0.2 M. Galβ1 → 4GlcNAc, Galβ1 → 6GlcNAc, Galβ1 → 4GalNAc, GlcNAcβ1 → 4Man and Manβ1 → 4Man exhibited no inhibition at concentrations up to 50 mM. Human transferrin and α_1-acid glycoprotein exhibited no inhibition at concentrations up to 1 mg ml^{-1}.

†Minimum concentrations required for inhibition of four haemagglutinating doses of the lectins.

‡Not inhibited at concentrations up to 0.2 M.

asialo-BSM, BSM and fetuin were the most potent inhibitors.

Many lectins from the fruiting bodies of fungi are known [1–25]. However, little is known about lectins from mycelia of higher fungi. To our knowledge, only a few examples have been reported; Guillot *et al.* [4] isolated a lectin from the fruiting bodies of *Lactarius deliciosus* and detected lectin activity in mycelia. However, the lectin from the mycelia has not been isolated [4]. Lz-8, an immunomodulatory protein, has been isolated from the mycelia of *G. lucidum* and its complete amino acid sequence has been determined [26, 27]. The protein showed haemagglutinating activity but it has not been characterized as a lectin. Furthermore, its amino acid sequence was completely different from GLL-M whose complete amino acid sequence is being determined now and the partial sequence of the lectin has been determined (data not shown). GLL-M and GLL-F are apparently different proteins, judging by their molecular size and sugar-binding properties. Detailed properties of GLL-F will be published elsewhere.

EXPERIMENTAL

Materials. Sepharose 4B, Superose 12 HR 10/30 and ProRPC HR5/10 columns were products of Pharmacia. Amino-Toyopearl and Toyopearl 55S were purchased from Tosoh (Japan). Spectra/Por 1 was a product of Spectrum Medical Industries (U.S.A.). All glycoproteins and lactulose for haemagglutinating inhibition tests were products of Sigma. Galβ1 → 4GlcNAc, Galβ1 → 6GlcNAc and Galβ1 → 4GalNAc were synthesized by enzymatic methods [28, 29]. All the other sugars for the tests were obtained from Nacalai Tesque (Japan); they are of the D-configuration, unless stated otherwise. All other chemicals were reagent grade.

Cultivation of mycelia. Ganoderma lucidum was cultivated in a medium containing 5% glucose, 1% malt extract, 0.5% KH_2PO_4, 0.25% $MgSO_4$, 0.2% peptone and 0.2% yeast extract with shaking for *ca* 1 month at 30°.

Isolation of GLL-M. All procedures were carried out at 4°. The culture was centrifuged (10 000 *g*, 15 min) and divided into supernatant (culture broth) and residue (mycelia). Mycelia were homogenized in saline with a blender and extracted for *ca* 2 hr with stirring. The resulting suspension was centrifuged (10 000 *g*, 15 min). These extraction procedures were repeated ×3. Solid $(NH_4)_2SO_4$ was added to the combined supernatant to give 80% satn. The ppt. collected by centrifugation (10 000 *g*, 15 min) was dialysed, first against tap H_2O and then dist. H_2O, and lyophilized. Solid $(NH_4)_2SO_4$ was also added to the culture broth to give 80% satn. The ppt. was collected by centrifugation (10 000 *g*, 15 min), dialysed and lyophilized. Lyophilized material from culture broth and mycelia were combined, redissolved in 10 mM Pi buffer (pH 6.0) and applied to a ZetaPrep 60 DEAE disc equilibrated with the buffer. After exhaustive washing with the buffer, the lectin-containing fr. was eluted with PBS. The eluates were applied directly to a BSM–Toyopearl column equilibrated with PBS. After the column was washed with PBS and then dist. H_2O, the lectin was desorbed with 0.5 M HOAc. Eluates were immediately neutralized with 0.5 M NH_4OH, dialysed with Spectra/Por 1 against dist. H_2O and lyophilized, providing the purified lectin.

Isolation of GLL-F. Chipped fresh fruiting bodies were extracted overnight with stirring. The resulting suspension was centrifuged (10 000 *g*, 15 min). These extraction procedures were repeated ×2. Solid $(NH_4)_2SO_4$ was added to the combined supernatant to give 80% satn. The ppt. collected by centrifugation (10 000 *g*, 15 min) was dialysed, first against tap H_2O and then dist. H_2O, and lyophilized. The lyophilized materials were redissolved in 10 mM acetate buffer (pH 3.6) applied to a ZetaPrep 60 DEAE disc equilibrated with the buffer. After exhaustive washing with buffer, the lectin-containing fr. was eluted with 10mM Pi buffer (pH 6.1). The eluates were concd by ultrafiltration, dialysed against PBS and applied to a BSM–Toyopearl column equilibrated with PBS. After the column was washed with PBS and then dist. H_2O, the lectin was desorbed with 0.5 M HOAc. The eluates were immediately neutralized with 0.5 M NH_4OH, dialysed with Spectra/Por 1 against dist. H_2O and lyophilized, providing the purified lectin.

Erythrocytes. Human blood was collected in 3% Na citrate. The erythrocytes were washed ×3 with PBS and suspended at a concn of 3% in the buffer.

Enzyme treatment of erythrocytes. A 10% suspension of erythrocytes in PBS (10 ml) was treated with Actinase E (7 mg) for 30 min at 47°, then the erythrocytes were washed × 3 with the buffer and

suspended at a concn of 3% in the buffer.

Haemagglutination test. Agglutination of 3% erythrocytes and inhibition of the agglutination by sugars and glycoproteins were done in microtitre U-plates. The titre is defined as the reciprocal of the end-point dilution causing haemagglutination. Inhibition is expressed as the minimum concn of each sugar or glycoprotein required for inhibition of haemagglutination of titre 4 of the lectin.

SDS–PAGE. SDS–PAGE was carried out by the method of ref. [30]. Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100°. Gels were stained with Coomassie Brilliant Blue. The M_r standards (Pharmacia) used were phosphorylase B (M_r 94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and α-lactalbumin (14 400).

Gel filtration. Gel filtration for measuring the M_r of native lectin was carried out on a Superose 12 HR10/30 column with a FPLC system. The M_r standards (Pharmacia) used were thyroglobulin (M_r 669 000), ferritin (440 000), catalase (232 000), aldolase (158 000), BSA (67 000), ovalbumin (43 000), chymotrypsinogen A (25 000), and ribonuclease A (13 700).

Isoelectric focusing. Isoelectric focusing was performed on LKB Ampholine Pagplate, pH 3.5-9.5. The gel was stained with Coomassie Brilliant Blue.

Amino acid analysis. Protein was hydrolysed with 6 M HCl at 110° for 24 hr. The resulting amino acids were analysed with an amino acid analyser.

N-*Terminal amino acid analysis.* The *N*-terminal amino acid of GLL-M was analysed on a Pulsed Liquid Protein/Peptide Sequencer equipped with a HPLC system (Applied Biosystems).

Sugar content. Sugar was measured by the PhOH–H_2SO_4 method with ref. to glucose [31].

Effect of metal cations on lectin activity. To examine metal cation requirements of haemagglutination by the lectins, samples were demetallized by the method of ref. [32].

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REFERENCES

1. Debray, H. and Montreuil, J. (1989) *Carbohydr. Res.* **185**, 15.
2. Eifler, R. and Ziska, P. (1980) *Experientia* **36**, 1285.
3. Guillot, J., Genaud, L., Gueugnot, J. and Damez, M. (1983) *Biochemistry* **22**, 5365.
4. Guillot, J., Giollant, M., Damez, M. and Dusser, M. (1991) *J. Biochem. (Tokyo)* **109**, 840.
5. Horejsi, V. and Kocourek, J. (1978) *Biochim. Biophys. Acta* **538**, 299.
6. Kawagishi, H. and Mizuno, T. (1988) *FEBS Letters* 227, 99.
7. Kawagishi, H., Nomura, A., Yumen, T. and Mizuno, T. (1988) *Carbohydr. Res.* **183**, 150.
8. Kawagishi, H., Nomura, A., Mizuno, T., Kimura, A. and Chiba, S. (1990) *Biochim. Biophys. Acta* **1034**, 247.
9. Kawagishi, H., Abe, Y., Nagata, T., Kimura, A. and Chiba, S. (1991) *Agric. Biol. Chem.* **55**, 2485.
10. Kawagishi, H., Mori, H., Uno, A., Kimura, A. and Chiba, S. (1994) *FEBS Letters* **340**, 56.
11. Kawagishi, H. (1995) *Food Rev. Int.* **11**, 63.
12. Kochibe, N. and Furukawa, K. (1980) *Biochemistry* **19**, 2841.
13. Kochibe, N. and Matta, K. L. (1989) *J. Biol. Chem.* **264**, 173.
14. Lin, J. and Chou, T. (1984) *J. Biochem. (Tokyo)* **96**, 35.
15. Oguri, S., Yoshida, M. and Nagata, Y. (1994) *Biosci. Biotech. Biochem.* **58**, 502.
16. Oguri, S. and Nagata, Y. (1994) *Biosci. Biotech. Biochem.* **58**, 507.
17. Presant, C. A. and Kornfeld, S. (1972) *J. Biol. Chem.* **247**, 6937.
18. Sage, H. J. and Vazquez, J. J. (1967) *J. Biol. Chem.* **242**, 120.
19. Sage, H. J. and Connett, S. L. (1969) *J. Biol. Chem.* **244**, 4713.
20. Sueyoshi, S., Tsuji, T. and Osawa, T. (1985) *Biol. Chem. Hoppe-Seyler* **366**, 213.
21. Synchrova, H., Ticya, M. and Kocourek, J. (1985) *Can. J. Biochem. Cell Biol.* **63**, 700.
22. Yagi, F. and Tadera, K. (1988) *Agric. Biol. Chem.* **52**, 2077.
23. Yatohgo, T., Nakata, M., Tsumura, Y., Hashimoto, Y. and Yamamoto, S. (1988) *Agric. Biol. Chem.* **52**, 1485.
24. Kawagishi, H., Wasa, T., Murata, T., Usui, T., Kimura, A. and Chiba, S. (1996) *Phytochemistry* **41**, 1013.
25. Kawagishi, H. and Mori, H. (1991) *Biochim. Biophys. Acta* **1076**, 179.
26. Kino, K., Yamashita, A., Yamaoka, K., Watanabe, J., Tanaka, S., Ko, K., Shimizu, K. and Tsunoo, H. (1989) *J. Biol. Chem.* **264**, 472.
27. Tanaka, S., Ko, K., Kino, K., Tsuchiya, K., Yamashita, A., Murasugi, A., Sakuma, S. and Tsunoo, H. (1989) *J. Biol. Chem.* **264**, 16372.
28. Sakai, K., Katsumi, K., Ohi, H., Usui, T. and Ishido, Y. (1992) *J. Carbohydr. Chem.* **11**, 553.
29. Usui, T., Suzuki, M., Sato, T., Kawagishi, H., Adachi, K. and Sano, H. (1994) *Glycoconjugate J.* **11**, 105.
30. Laemmli, U. K. (1970) *Nature* **227**, 680.
31. Dubois, M., Gilles, K. A., Hammilton, J. K., Rebers, P. A. and Smith, F. (1956) *Analyt. Chem.* **280**, 350.
32. Pandolfino, E. R. and Magnuson, J. A. (1980) *J. Biol. Chem.* **225**, 870.