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# *Agrocybe cylindracea* lectin is a member of the galectin family

Fumio Yagi*, Hidenori Hiroyama and Shinichi Kodama

*Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto Kagoshima 890-0065, Japan*

**The complete amino acid sequence of Agrocybe cylindracea lectin was determined from the peptides obtained by chemical cleavages and enzymatic hydrolyses. The sequence shows 19.1% and 36.8% identity with those of human galectin-1 and Coprinus lectin-1, a fungal galectin, respectively. Seven residues, which are commonly found in carbohydrate recognizing domain (CRD) of galectins, were conserved. However, several insertions in the sequence, compared with those of human galectin-1 and Coprinus lectin-1, suggest that $\beta$-strands S2, F3, and S4 and the loop structures between $\beta$-strands F2 & S3 and F5 & S2 are different from those of galectins reported so far.**

***Keywords: Agrocybe cylindracea*, galectin, amino acid sequence, fungus**

## Introduction

In the previous paper [1], we reported the carbohydrate- binding specificity of the lectin from fruit bodies of *Agrocybe cylindracea*. The specificity of the lectin toward sialoconjugates was different from those of the lectins reported so far. The lectin can recognize glycoproteins with sugar chains containing NeuAc$\alpha$2-3Gal$\beta$1-3GlcNAc/GalNAc-. Except for this lectin, two lectins of fungal origin have been reported to have the affinity toward sialic acid itself or sialyltrisaccharides with $\alpha$2,6-linkage [2,3]. On the other hand, primary structures of several fungal lectins have been determined [4–9], but they don't show any similarity to animal and plant lectins. For elucidating structure and function relationships, the information on the structure is necessary. Therefore, we determined the primary structure of *Agrocybe cylindracea* lectin in this study.

## Materials and methods

*Materials*. Fruit bodies of *Agrocybe cylindracea* were obtained on our campus. The lectin was purified according to the method of Yagi et al. [1].

*Chemical cleavage of lectin*. The lectin was cleaved with 150 molar excess CNBr in 70% HCOOH under nitrogen gas at room temperature for 24 h according to the method of Gross and Witkop [10]. *o*-Iodosobenzoate was used for cleavage at tryptophan residue according to the method of Mahoney et al. [11]. Cleavage of Asn-Gly bond was done with 2 M hyroxylamine in 6 M guanidium- chloride at pH 9.0 and 45°C for 4 h by the method of Bornstein and Balian [12].

Tricine-sodium docecyl sulfate PAGE [13] was used to obtain the peptides after the chemical cleavages of the lectin. After electrophoresis, peptides were electroblotted to polyvinylidenedifluoride (PVDF) membranes according to the method of Matsudaira [14]. The peptides bands were visualized on PVDF membranes by staining with Coomassie Briliant Blue R250 and cut off for N-terminal sequencing.

Two N-terminal peptides were obtained by gel filtration on a column of Biogel P-30 (2 × 150 cm) in 0.1 M sodium phosphate buffer, pH 7.0 containing 6 M guanidium-chloride, measuring the absorption at 230 nm. After CNBr treatment, the reaction mixture was diluted 10-fold with water and dried *in vacuo*. Peptides were dissolved in in 0.1 M sodium phosphate buffer, pH 7.0 containing 6 M guanidium-chloride and applied onto the column of Biogel P-30. Peptides obtained by Asn-Gly cleavage was directly applied onto the column. The two N-terminal peptides separated by gel filtration were desalted on a column of Sephadex G25 (1.5 × 30 cm) with 5% acetic acid as a solvent and lyophilysed for enzyme digestion.

*Enzymatic digestion*. Digestion of intact lectin with $\alpha$-chymotrypsin (EC 3.4.21.1, Wako Pure Chemical Ltd., Osaka, Japan) was done at an enzyme/substrate ratio of 1:100 (w/w) in 0.1 M Tris HCl buffer, pH 8.0, at 37°C for 2 h. *Staphylococcus*

*To whom correspondence should be addressed: Fumio Yagi, Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University, 1-21-24 Korimoto Kagoshima 890-0065, Japan. Fax and Tel.: +81-99-285-3441; E-mail: fyagi@chem.agri.kagoshima-u.ac.jp

*aureus* V8 protease (EC 3.4.21.19, Wako Pure Chemical Ltd.) digestion of the intact protein was done at an enzyme/substrate ratio of 1:50 w/w in 0.1 M $NaHCO_3$ at 37°C for 24 h.

The N-terminal peptide obtained by Asn-Gly cleavage was hydrolyzed with endoproteinase Asp-N (Takara Shuzo Co. Ltd., Kyoto, Japan) at an enzyme/substrate ratio of 1:50 w/w in 50 mM sodium phosphate buffer, pH 8.0 in the presence of 2 M urea at 37°C for 18 h. N-terminal peptide obtained by CNBr treatment was hydrolyzed with thermolysin (EC 3.4.24.4, Wako Pure Chemical Ltd.) at an enzyme/substrate ratio of 1:40 w/w in 50 mM Tris HCl buffer, pH 8.0 in the presence of 2 mM $CaCl_2$ and 8 urea at 37°C for 2 h.

After enzymatic hydrolyses, soluble peptides were separated with a column of YMC Cel C4 300S-5 (4.6 × 250 mm) in the gradient of 0–60% acetonitrile in 0.1% TFA) at 0.8 ml/min. Neutral solvent system (0–60% acetonitrile in 5 mM potassium phosphate buffer, pH 6.0) was used for rechromatography of chymotryptic peptides after separation by the acidic solvent system.

N-terminal peptide C1 which was obtained after separation of chymotryptic peptide was hydrolyzed with acylamino acid releasing enzyme (EC 3.4.19.1, Takara Shuzo Co. Ltd.) at an enzyme/substrate ratio of 1:50 w/w in 50 mM sodium phosphate buffer, pH 7.2 at 37°C for 2 h.

*Amino terminal peptide sequencing*. Peptide sequencing was done with a model 492 protein sequencer fitted with a model 140 C PTH analyzer (Perkin Elmer Applied Biosystems Division Japan, Tokyo).

*Carboxy terminal sequencing*. C-terminal amino acid sequencing was performed with a Procise C494 protein sequencer (Perkin Elmer Applied Biosystems Division) according to the method of Boyd et al. [15].

*Amino acid analysis*. Peptides were hydrolyzed with constant boiling HCl at 110°C for 24 h. Amino acids were analyzed with a Waters Pico-Tag system after derivatization with phenylisothiocyanate according to the method of Heinrikson and Meredith [16].

*Mass spectrometry*. Molecular masses were determined for intact protein and N-terminal peptides with sinapic acid and $\alpha$-cyano-4-hydroxycinnamic acid as matrices, respectively, by a MALDI-TOF mass spectrometer (Voyager DE, Perseptive Biosystems Inc., Tokyo, Japan).

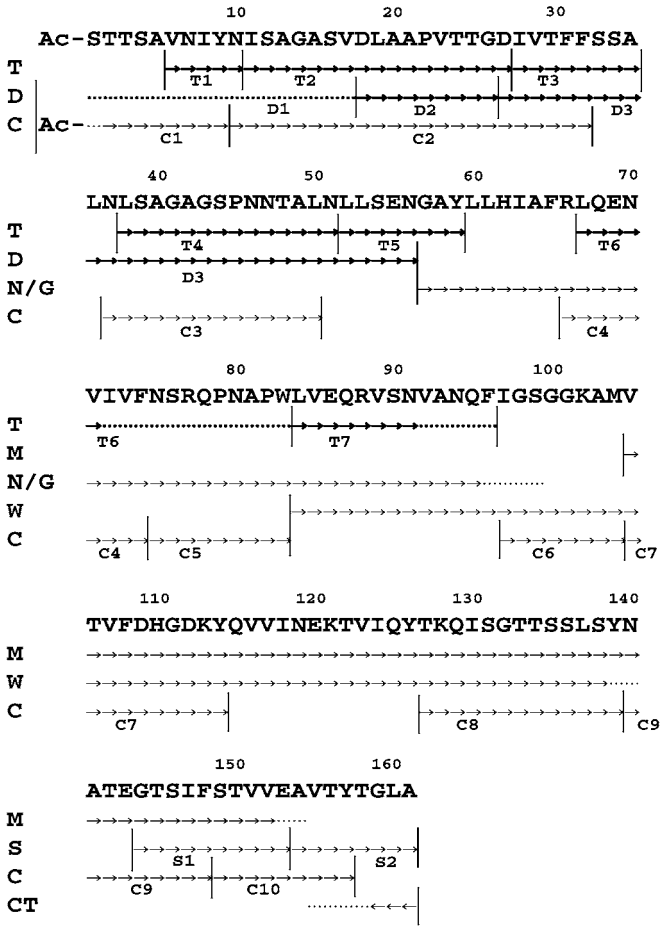


**Figure 1.** Amino acid sequence of *Agrocybe cylindracea* lectin. The arrows indicate the residues determined by N-terminal and C-terminal sequencing. The vertical lines are N-termini and C-termini of the peptides. Dotted lines show that the sequences continue. Bold lines show the peptides derived from N-terminal fragments by Asn-Gly cleavage and CNBr treatment. M, peptide obtained by CNBr cleavage; C1-10, chymotryptic peptides; N/G, C-terminal peptide by hydroxylamine cleavage; D1-3, peptides obtained after proteinase Asp-N digestion of N-terminal fragment by Asn-Gly cleavage; W, by *o*-iodosobenzoate cleavage; S1 & 2, peptides obtained by V8 protease; T1-7, peptides obtained after thermolysin digestion of N-terminal fragment by CNBr cleavage; CT, by C-terminal sequencing.

## Results and discussion

The sequence of Agrocybe lectin was determined from peptides obtained by chemical cleavages and enzymatic digestions (Figure 1). Approximately 91% of amino acids in the structure have been identified at least twice by sequencing different peptides. The recoveries of some chymotryptic peptides from the whole protein were low, but recoveries of peptides obtained by thermolysin and proteinase Asp-N digestion of N-terminal sequences were relatively high (Table 1). Recoveries of three C-terminal peptides were 38% for CNBr cleavage, 49% for *o*-iodosobenzoate cleavage, and 30% for Asp-Gly cleavage.

C-Terminal sequence of the lectin after residue G57 was easily determined. Sequence (G57–Q95) of the C-terminal peptide obtained by Asn-Gly cleavage, was linked to peptide M(V105 up to T150) obtained by CNBr treatment with peptide W (L84 up to S138) by *o*-iodosobenzoate treatment. C-terminal sequence of the lectin was determined to be -GLA by C-terminal sequencer. Peptide M was linked to the C-terminal sequence–GLA with chymotryptic peptide C10 (S149-Y157) and peptide S2 (A154-A161) by V8 protease.

N-terminus of the lectin was blocked as reported previously [1]. Three peptides D1-3 were obtaind after proteinase Asp-N hydrolysis of the N-terminal peptide. The N-terminus of peptide

**Table 1.** Yield of each peptide after enzymatic hydrolysis. Yields were calculated by amino acid analysis

| *Peptide* | *Residue number* | *Yield (%)* | *Peptide* | *Residue number* | *Yield (%)* |
|---|---|---|---|---|---|
| C1 | 1–9 | 30 | T1 | 6–10 | 60 |
| C2 | 10–32 | 11 | T2 | 11–27 | 43 |
| C3 | 37–50 | 28 | T3 | 28–35 | 56 |
| C4 | 66–74 | 37 | T4 | 38–51 | 47 |
| C5 | 75–83 | 40 | T5 | 52–59 | 35 |
| C6 | 97–104 | 12 | T6 | 67–83 | 53 |
| C7 | 105–114 | 12 | T7 | 84–96 | 37 |
| C8 | 127–139 | 23 | | | |
| C9 | 140–148 | 16 | D1 | 1–17 | 60 |
| C10 | 149–157 | 52 | D2 | 18–26 | 51 |
| | | | D3 | 27–56 | 46 |
| S1 | 144–153 | 29 | | | |
| S2 | 154–161 | 56 | | | |

The yields of peptides T1-7 and D1-3 were calculated based on the respective N-terminal peptides. Peptide nomenclatures are shown in Figure 1.

D1 was blocked. Peptide D2(D18-G26) and D3(D27-N56) were linked each other with chymotryptic peptide C2(N10-F32) and thermolyic peptide T2(I10-D27). Peptide D3 was linked to the C-terminal peptide (G57-) obtained by Asn-Gly cleavage with thermolytic peptide T5(L52-Y59). Peptide C1 was assigned to the N-terminus of this lectin, because the N-terminus of C1 was blocked. After digestion of C1 with acylamino acid releasing enzyme, the sequence T2-Y9 was determined. Furthermore, amino acid composition of peptide C1 and peptide D1 were compatible with the sequences S1-Y9 and S1-V17, respectively. Molecular mass of C1 was determined to be 997.9 by mass spectrometer in good agreement with the calculated value of 998.3, assuming that the N-terminus was acetylated. Thus, we obtained the sequence of a total of 161 residues. The calculated molecular mass of the lectin including N-terminal acetyl group was 17,088.0, coinciding with the value 17,111.8 obtained by mass spectrometer.

However, several amino acid variants were found in the peptides. At residue 100 of peptide W, aspartic acid was also found and the ratio of Asp to Gly was 2:3. At residues 133–141 in peptide M, major sequence was TTSSLSYNA and minor one was PTTSLSYLV. At residues 133, 135, 140 and 141, the ratio of major amino acid to minor one was 3:2. Peptides corresponding to the minor sequence were also obtained by chymotryptic digestion. Generally, cells of fungi are known to be heterokaryons. Therefore, in most cases, heterogeneity of proteins from fungi come from multigenes. It has been reported that two Coprinus cinereus lectins are derived from different genes [8].

Two possible N-glycosylation sites (N10-S12 and N37-S39) were found, though the lectin was not glycosylated.

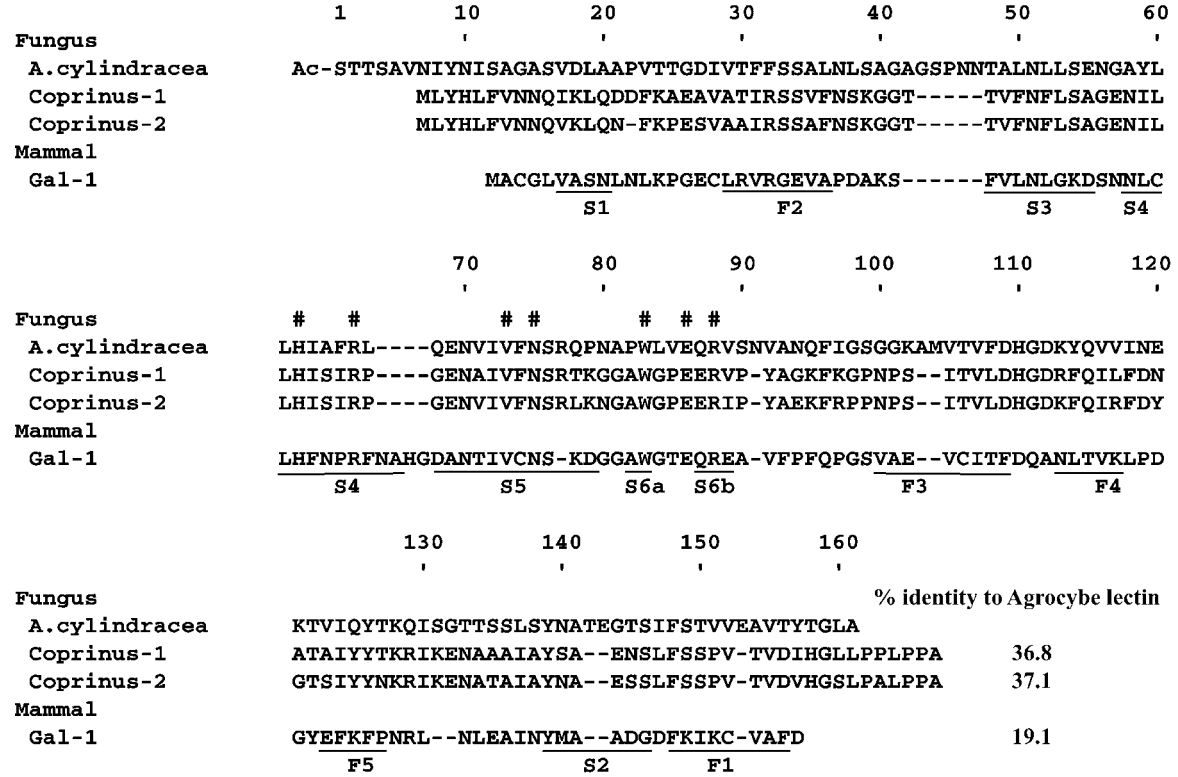


**Figure 2.** Comparison of the sequence of *Agrocybe* lectin with fungal lectins [8] and human galectin-1, Gal-1 [17]. Dashed lines indicate the gaps inserted for maximizing the match. # show the residues conserved in CRD of galectins [18,19]. Beta strands S1-6b and F1-5 of human galectin-1 with the underlines are deduced from those of bovine spleen galectin-1 [20].

The primary structure of Agrocybe lectin shows the similarity to those of fungal galectins. Figure 2 shows the comparison of the sequences of Agrocybe lectin and some galectins [8,17]. Seven residues that are known as carbohydrate binding residues in most galectins are conserved in Agrocybe lectin. However, sequence identity of Agrocybe lectin was only 19% to human galectin-1 and 37% to Coprinus galectins. N-terminus of Agrocybe lectin was acetylated, differing from those of Coprinus galectins [8]. No cysteine residue was found in fungal galectins. In comparison of fungal galectins with human galectin-1, deletion of 4 residues between residue 67 and 68 of Agrocybe lectin, and insertion of two residues (residues 131 and 132 of Agrocybe lectin) were found. In comparison of Agrocybe lectin with Coprinus galectins, five insertions (residues 43–47, 91, 103 & 104, 142 & 143, and 153) were found.

In a previous paper, we showed a characteristic carbohydrate binding specificity of Agrocybe lectin. This lectin can recognize lactose, N-acetyl lactosamine, Gal$\beta$1-3GlcNAc and Gal$\beta$1-3GalNAc. Most galectins can bind the former 3 disaccharides but only a few can bind Gal$\beta$1-3GalNAc [21–24]. Furthermore, NeuAc$\alpha$2-3lactose and other trisaccharides with NeuAc$\alpha$2-3Gal were potent inhibitors for hemagglutination by Agrocybe lectin. NeuAc$\alpha$2-3lactose was 100 times potent inhibitor than lactose. So far, no galectin with strong affinity toward sialoconjugates has been reported. The binding affinity of other galectins toward saccharides decrease with the substitution by N-acetyl neuraminic acid at 3′O of galactosyl moiety of lactose [21]. Thus, Agrocybe lectin showed a unique carbohydrate binding specificity, differing from galectins previously reported.

The CRD seem to be around the residues 48–90 in this lectin, and the sequence identity in this region to those of human galectin-1 and Coprinus galectin-1 are 35.7% and 48.8%, respectively. However, some $\beta$-strands of Agrocybe lectin may be different from those of other galectins. Apparently, strands S4, F3, and S2 are different from those of human galectin-1 in length. The loops between strands F2 & S3, and F5 & S2 are supposed to be different from other galectins. At present, the three-dimensional structure of the sugar-lectin complex is being analyzed. The three-dimensional structure will elucidate the characteristic carbohydrate binding specificity of Agrocybe lectin.

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