Toxic isolectins from the mushroom *Boletus venenatus*

Masashi Horibe [a], Yuka Kobayashi[a,b,*], Hideo Dohra[c], Tatsuya Morita[a], Takeomi Murata[a], Taichi Usui[a], Sachiko Nakamura-Tsuruta[d], Masugu Kamei[b], Jun Hirabayashi[d], Masanori Matsuura[e], Mina Yamada[e], Yoko Saikawa[e], Kimiko Hashimoto[f], Masaya Nakata[e], and Hirokazu Kawagishi[a,g,*]

[a] *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[b] *J-Oil mills, INC, 11, Kagetoricho, Totsuka-ku, Yokohama, Kanagawa 245-0064, Japan*
[c] *Institute for Genetic Research and Biotechnology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*
[d] *Research Center for Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 2, Tsukuba, Ibaraki 305-8566, Japan*
[e] *Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522, Japan*
[f] *Kyoto Pharmaceutical University, 1 Shichono-cho, Misasagi, Yamashina-ku, Kyoto 607-8412, Japan*
[g] *Graduate School of Science and Technology, Shizuoka University, 836 Ohya, Suruga-ku, Shizuoka 422-8529, Japan*

* Corresponding authors. Tel: +81-45-852-4001 Fax: +81-45-852-6357 *E-mail address:* yuka.kobayashi@j-oil.com (Yuka Kobayashi). Tel and Fax: +81-54-238-4885 *E-mail address:* achkawa@agr.shizuoka.ac.jp (Hirokazu Kawagishi)

ABSTRACT

Ingestion of the toxic mushroom *Boletus venenatus* causes a severe gastrointestinal syndrome, such as nausea, repetitive vomiting, diarrhea, and stomachache. A family of isolectins (*Boletus venenatus* lectins, BVLs) was isolated as the toxic principles from the mushroom by successive 80% ammonium sulfate-precipitation, Super Q anion exchange chromatography, and TSK-gel G3000SW gel filtration. Although BVLs showed a single band on SDS-PAGE, they were further divided into eight isolectins (BVL-1 to -8) by BioAssist Q anion exchange chromatography. All the isolectins showed lectin activity and had very similar molecular weights as detected by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF-MS) analysis. Among them, BVL-1 and BVL-3 were further characterized and their complete amino acid sequences of 99 amino acids were determined and found to be identical to each other. In the hemagglutination inhibition assay, both proteins failed to bind to any mono- or oligo-saccharides tested and showed the same sugar binding specificity to glycoproteins. Among the glycoproteins examined, asialofetuin was the strongest inhibitor. The sugar binding specificity of each isolectin was also analyzed by using frontal affinity chromatography and surface plasmon resonance analysis, indicating that they recognized N-linked sugar chains, especially Galβ1→4GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc (Type II) residues in *N*-linked sugar chains. BVLs showed the fetal toxicity in mice upon intraperitoneal administration and caused diarrhea upon oral administration in rats.

Key words: Boletus venenatus; Boletaceae; mushroom; purification; lectin; lethal toxicity; diarrhea.

1. Introduction

People eat various kinds of wild mushrooms and a lot of them get poisoned by eating toxic mushrooms accidentally. Some of the toxic substances produced by the mushrooms have been isolated and characterized; low molecular toxins, illudin S and ustalic acid, have been obtained from *Lampteromyces japonicus* and *Tricholoma ustale*, respectively, and a metallo-protein has been reported as a toxin from *Rhodophylllus rhodopolius* (Nakanishi et al., 1963; McMorris et al., 1963; Matsumoto et al., 1965; Suzuki et al., 1987, 1988, 1990; Sano et al., 2002). However, many active principles of toxic mushrooms remain unknown.

The mushroom *Boletus venenatus* (Dokuyamadori or Tahei-iguchi in Japanese) has been proved to be toxic. Ingestion of the mushroom causes a severe gastrointestinal syndrome, such as nausea, repetitive vomiting, diarrhea, and stomachache. Among the symptoms, the major one is diarrhea. Recently, a protein showing lethal toxicity against mice, bolevenine, was isolated from the mushroom (Matsuura et al., 2007). However, it has been unclear whether the protein causes diarrhea in humans or not. In this study, we obtained a family of isolectins (BVLs) showing lethal toxicity to mice and giving a single band on SDS-PAGE from the mushroom, and further divided them into eight isolectins. Furthermore, we found that BVLs showed lectin activity and caused diarrhea in rats, and one of the isolectin was bolevenine.

Lectins are carbohydrate-binding proteins present in a wide variety of animals, plants and microorganisms. Mushroom lectins have been studied for biochemical reagents with valuable carbohydrate binding specificity, however, there is no report about lectins as diarrheal toxins (Kawagishi, 1995; Wang et al., 1998).

Here we describe the purification, and biochemical and molecular characterization of the isolectins from the mushroom.

2. Results

2.1. Purification of BVLs

Since the extract of *Boletus venenatu*s showed lectin activity and lethal toxicity to mice, the fractionation was guided by the two biological activities. The purification procedure is summarized in Table 1. After precipitation of the PBS-extract of the mushroom with ammonium sulfate, the precipitates further purified by anion-exchange chromatography and gel filtration in a two-step process. The toxicity and lectin activity cofractionated at all the steps of the isolation (data not shown) and the active fraction (*Boletus venenatus* lectins, BVLs) showed a single band on SDS-PAGE with an approximate mass of 11 kDa on SDS-PAGE regardless of the presence (lane 1) or absence (lane 2) of 2-mercaptoethanol (Fig. 1). HPLC gel filtration of BVLs also gave a single symmetrical peak at an elution volume corresponding to a molecular mass of 33 kDa (Fig. 3). The same result was obtained by FPLC gel filtration of the fraction (data not shown). The results of SDS-PAGE and gel filtration indicated that BVLs were homotrimers of identical 11 kDa-subunits with no disulfide linkage. However, the possibility that they were homotetramers cannot be excluded. Although BVLs appeared as a single band on SDS-PAGE (Fig. 1) and showed a symmetrical peak in HPLC gel filtration (Fig. 2A), isoelectric focusing of BVLs showed a very wide range of bands (Fig. 3A, lane 1). Therefore, those were further separated by HPLC anion-exchange chromatography, giving eight fractions (Table 1). Each fraction showed lectin activity and gave different bands from each other on isoelectric focusing (Fig. 3A, lanes 2 to 9). The isolated isolectins were named BVL-1 to -8, respectively.

2.2 Molecular properties of BVL-1 and BVL-3

The isoelectric focusing bands of BVLs converged to fewer bands upon treating with

PNGase F (Fig. 3B). MALDI-TOF-MS of each isolectin gave very similar molecular ions to each other (from *m/z* 10,947 to 10,995: BVL-1, *m/z* 10955; BVL-2, *m/z* 10947; BVL-3, *m/z* 10948; BVL-4, *m/z* 10953; BVL-5, *m/z* 10949; BVL-6, *m/z* 10954; BVL-7, *m/z* 10948; BVL-8, *m/z* 10950). Since BVL-1 and -3 had completely different pIs from each other, they were further characterized.

Amino acid composition analysis of BVL-1 found a high content of Asx, Thr, Glx and Gly, and a low content of Met, His and Cys (Table 2). *N*-Terminal amino acid sequence analysis of intact BVL-1 gave a sequence of 45 amino acids from the terminal. The protein was digested by *Achromobacter* protease I (Lys-C), *Clostridium histolyticum* protease (Arg-C) or *Staphylococcus aureus* V8 protease (Glu-C), and the resulting peptides were isolated by reversed-phase HPLC. Each of the purified peptides' sequences was determined by *N*-terminal amino acid sequence analysis and MALDI-TOF mass spectrometry. As a result, the complete amino acid sequence of BVL-1 was determined as shown in Fig. 4 (lane 1). The result of homology search by FASTA program is shown in Fig. 4. BVL-1 exhibited 75% similarity with a toxic lectin, bolesatine (length of compared sequence with BVL-1; 20 amino acids), from the mushroom *Boletus satanas*, 36% with hemagglutinin I from *Physarum polycephalum* (HA1) (over 56 amino acids), 31% with acetohydroxy acid isomeroreductase from *Kineococcus radiotolerans* (AAIK) (over 68 amino acids), 30% with acetohydroxy acid isomeroreductase from *Tharmobifida fusca* (AAIT) (over 70 amino acids), and 28% with acetohydroxy acid isomeroreductase from *Nocardioidea* sp. (AAIN) (over 70 amino acids).

The sugar components in BVL-1 and -3 were identified as Glc: Gal: Man: L-Fuc: Xyl: GlcN in a 5.1: 1.9: 5.8: 6.2: 1.0: 1.0 and a 2.5: 2.4: 8.2: 9.3: 1.2; 1.0 molar ratio, respectively. Both proteins did not contain NeuAc and NeuGc.

2.3. Properties of BVL-1 as a lectin

BVL-1 agglutinated intact, Pronase-, trypsin-, or neuraminidase-treated human erythrocytes (Table 3).

The lectin activity was stable between pH 2.0 and 9.5 and below 80 ºC (data not shown). Since the lectin was not deactivated completely even at 100 ºC for 30 min (although the titer decreased from 2^8 to 2^2), the thermo-stability of the lectin at 100 °C was examined. The activity was completely retained even when treated for 20 min, but rapidly deactivated for 30 min, and completely deactivated for 60 min (data not shown). EDTA treatment of the lectin did not affect the activity. Addition of metal cations to the lectin also did not affect its activity at all.

Table 4 shows the inhibition of the hemagglutination activity of BVL-1 by various monosaccharides, oligosaccharides, and glycopeptides. None of the mono- and oligosaccharides used bound to the lectin. Asialo-fetuin exhibited the strongest inhibitory activity among the glycoproteins used, and thyroglobulin, fetuin, and α_1-acid glycoprotein also showed strong inhibition.

The sugar-binding specificity of BVL-1 was also investigated by surface plasmon resonance (SPR) analysis. BVL-1 was immobilized on the sensor chip CM-5 by amine coupling. Eight glycoproteins, fetuin, asialo-fetuin, asialo- bovine submaxillary mucin (BSM), BSM, porcine stomach mucin (PSM), thyroglobulin, α_1-acid glycoprotein, and transferrin, were used as analytes. Among them, asialo-fetuin, fetuin, α_1-acid glycoprotein and thyroglobulin, which inhibited the BVL-1-mediated hemagglutination, bound to the sensor chip. The binding of all the four glycoproteins to the immobilized lectin fitted best the 1:1 binding model among various models in the evaluating software and showed similar kinetic parameters to each other (Fig. 5, Table 5). Asialo-BSM, BSM, PSM, and transferrin, which showed weaker inhibitory activity or were not inhibitory in the hemagglutination assay, did not bind to the chip (data not shown).

The sugar-binding specificity of BVL-1 was also elucidated by frontal affinity chromatography (FAC) analysis. The amount of immobilized BVL-1 was determined to be 10 μg/ml. Among 114 kinds of pyridylaminated (PA)-glycans used (Fig. 6A), only eight glycans bound to the lectin (Fig. 6B). The strength of affinity of each PA-glycans for the immobilized

lectin was shown as V-V_0 value (μl).
Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc (PA-30, 19.1 μl) showed the strongest affinity to the immobilized lectin.
Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc (PA-38, 16.0 μl) and Galβ1→4GlcNAcβ1→2(Galβ1→4GlcNAcβ1→6)Manα1→6[Galβ1→4(Fucα1→3)GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc (PA-40, 14.3 μl) bound the lectin strongly.
Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc (PA-28, 10.8 μl),
Fucα1→6Galβ1→4GlcNAcβ1→2Manα1→6[Fucα1→6Galβ1→4GlcNAcβ1→4(Fucα1→6Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc (PA-44, 10.7 μl).
Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc (PA-37, 8.1 μl),
Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→3GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc (PA-29, 5.8 μl), and
Galβ1→4GlcNAcβ1→2Manα1→6[Galβ1→4(Fucα1→3)GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc (PA-39, 5.8 μl) also exhibited affinity for the lectin.

The results of the sequencing, the hemagglutination test, the hemagglutination inhibition test, the SPR experiment, and the FAC analysis of BVL-3 were completely the same as those of BVL-1 (data not shown).

2.4. Toxicity of BVLs

BVLs were injected intraperitoneally to mice at a dose of 0.5, 1.0, or 1.5 mg/mouse. Mice died within a day after the injection for all the concentrations. BVLs were orally force-fed to rats at a dose of 40 mg/kg body. The rats did not die but suffered from diarrhea after about 4 hours of the administration. On the other hand, before oral administration of BVLs to rats, an anti-diarrheal agent, loperamide, was orally injected. The pretreatment of the agent prevented the rats from suffering from diarrhea or showing any other abnormal symptoms.

3. Discussion

A family of isolectins, BVLs, was obtained from the toxic mushroom *B. venenatus* by successive chromatography. BVLs showed a single band on SDS-PAGE and gave a single symmetrical peak by HPLC and FPLC analyses (Figs. 1 and 2). However, the isoelectric focusing of BVLs showed a very wide range of bands. Therefore, they were further separated by HPLC anion-exchange chromatography, giving eight fractions (Table 1). Each fraction showed lectin activity, gave different bands from each other on isoelectric focusing (Fig. 3A), and was named BVL-1 to -8. A protein showing lethal toxicity against mice, bolevenine, has been isolated from this mushroom (Matsuura et al., 2007). In the report, only one fraction among several toxic ones, whose pI was 6.55, was purified, and its sequence of 18 amino acids from the *N*-terminal was determined. Judging from the pI value, one of the fractions purified in this study, BVL-1, is bolevenine.

BVL-1 to -8 had various pIs (Figs. 3a) and very similar molecular weights to each other (from *m/z* 10,947 to 10,995). Among them, BVL-1 and -3 showed completely different pI bands from each other. Therefore, the two isolectins were further characterized. The only difference between them was their neutral sugar compositions, although, both the proteins did not contain NeuAc and NeuGc. An explanation for this elimination of sugar chains from glycoproteins might be accounted due to MALDI-TOF mass measurement technique. The treatment of BVLs

with PNGase F gave fewer bands in isoelectric focusing than intact BVLs (Fig. 3B). These results and the slight differences of molecular mass among BVLs allowed us to elucidated that the difference of pIs among BVLs was due to the differences of their sugar chains and/or one or a few substitutions of amino acids in their sequences. However, the following possibility cannot be excluded; the isolectins contain some novel, covalently bound *N*- or *O*-glycans, or they contain some tightly, but non-covalently bound novel fungal glycan ligands, despite the extensive purification procedure.

The complete primary structure of BVL-1 was determined (Fig. 4). BVL-1 was composed of 99 amino acid residues and its calculated molecular mass was 10,943 Da. This molecular mass was in good agreement with the value (*m/z* 10,955) of the molecular ion peak obtained by MALDI-TOF mass spectrometry. FASTA search revealed that BVL-1 has a sequence homology with the partial sequence (20 *N*-terminal amino acids) of a toxic lectin, bolesatine, from the mushroom *Boletus satanas*. However, the complete amino acid sequence of bolesatine has not been determined yet (Kretz et al, 1992a).

The sugar binding specificity of BVL-1 was analyzed by the hemagglutination inhibition test, the SPR experiment, and the FAC analysis (Tables 4 and 5, Figs. 5 and 6). In the hemagglutination inhibition test and the SPR experiment, asialo-fetuin, thyroglobulin, fetuin, and α_1-acid glycoprotein showed potent affinity for the lectin. In the FAC analysis, only eight glycans (PA-30, V- V_0 = 19.1 μl; PA-38, 15.0; PA-40, 14.3; PA-28, 10.8; PA-44, 10.7; PA-37, 8.1; PA-29, 5.8; PA-39, 5.8) among 114 kinds of PA-glycans used bound to the lectin (Fig. 5). The common structure of seven sugars except for PA-29 (Galβ1→3GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc, Type I) among the eight ones is Galβ1→4GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc (Type II). Comparison of the structure of the strongest haptenic sugar, PA-30, with those of PA-38, PA-40 and PA-44 indicates that the attached NeuAc or L-Fuc to the common sugar chain weakened the affinity for the lectin. The best three of the haptenic sugars, PA-30, PA-38 and PA-40, have four Gal®1□4GlcNAc residues in their molecules. In contrast, LacNAc (Galβ1→4GlcNAc) and a synthetic LacNAc polymer, poly(LacNAc-pAP/Gln-co-Gln), did not bind to the lectin even at 1.0 M and 1 mg/ml in the hemagglutination test, respectively (Table 4). In addition, all the seven glycoproteins that inhibited the BVL-1-mediated hemagglutination were digested with Pronase severely and the resulting reaction mixtures showed much weaker inhibitory activity toward the lectin-mediated hemagglutination than intact glycoproteins at the same concentrations. These results allowed us to conclude that this lectin mainly recognized *N*-linked sugar chains, especially Galβ1→4GlcNAcβ1→4Manβ1→4GlcNAcβ1→4GlcNAc (Type II) residues in the sugar chains, and plural sugar chains that were close to each other strengthened the binding of the lectin to the sugar chains.

BVLs were orally force-fed to rats at a dose of 40 mg/kg body. The rats did not die but suffered from diarrhea after the administration. Human diarrhea occurs by various causes. In general, absorption of water from the intestines is suppressed by inhibition of intestinal Na^+,K^+-ATPase, resulting in diarrhea. It was deduced that ustalic acid and its derivatives from the toxic mushroom *Tricholoma ustale* inhibited the enzyme (Sano et al., 2002). However, BVLs did not inhibit the enzyme even at 1 mM (data not shown). On the other hand, an anti-diarrheal agent, loperamide, prevented the BVLs-administrated rats from suffering from diarrhea. One of the causes of diarrhea is hyper-contraction of intestinal smooth muscles followed by abnormal increase of intestinal motility. The agent suppresses the symptom. BVLs might have acted as enterokinetic substances in the intestines.

Some toxic compounds have been isolated from diarrhea-causing mushrooms (Nakanishi et al., 1963; McMorris et al., 1963; Matsumoto et al., 1965; Suzuki et al., 1987, 1988, 1990; Kretz et al., 1989; Sano et al., 2002). Although those compounds exhibited lethal toxicity to mice and/or rats, there is no experimental evidence that those are the true "diarrhea-causing principles" in the toxic mushrooms. Bolesatine, which was isolated from the same genus to the BVLs-producing mushroom, showed inhibition of protein synthesis (Kretz et al; 1989, 1992a, b; Basset et al, 1995), agglutination property (Gachet et a., 1996; Ennamany et al., 1998), lipid

peroxidation property (Ennamany et al., 1995), and resistance to proteolysis (Kretz et al, 1991, 1992a). However, the relationship between those biological activities of bolesatine and diarrhea has not been clarified yet. To the best of our knowledge, this is the first report that the active principles, which were proved to cause diarrhea in the animal experiment, were purified from a diarrhea-causing mushroom.

4. Experimental

4.1. Materials

Toyopearl SuperQ, TSK-gel G3000SW, TSK-gel G3000SWXL, and TSK-gel BioAssist Q columns were products of Tosoh. MALDI-TOF mass spectra were acquired on an AutoFlex (Bruker Daltonics). Ultrafiltration membrane, YM-100, was a product of Millipore. Lysyl endopeptidase, endopeptidase Glu-C, and Arg-C were products of Wako Pure Chemicals, Sigma, or Takara Bio Inc.. ABEE reagent (ethyl *p*-aminobenzoate) and Wakosil-II column were obtained from Wako Pure Chemicals. Poly(LacNAc-pAP/Gln-co-Gln) was synthesized as described previously (Totani et al., 2003; Zeng et al., 2000). All the other sugars and glycoproteins for the hemagglutinating inhibition tests and the SPR analyses were purchased from Nacalai Tesque, Wako Pure Chemicals, Calbiochem, or Sigma. Loperamide hydrochloride was a product of Wako Pure Chemicals. BIAcore 2000 was a product of GE Healthcare Bio-Sciences Corp. PA-oligosaccharides for FAC analysis were purchased from Takara Bio Inc.. HiTrap NHS-activated Sepharose (activated agarose gel) were purchased from GE Healthcare Bio-Sciences Corp.. Stainless steel empty miniature column (inner diameter, 2 mm; length, 10 mm; bed volume, 31.4 μl) were obtained from Shimadzu Co..

4.2. Fungus materials

Mature fruiting bodies of *Boletus venenatus* Nagasawa were collected at Narusawa village, Yamanashi Prefecture, Japan and identified by one of the authors (H. K.). A voucher specimen of the organism (BV-09-03) has been deposited in Faculty of Agriculture, Shizuoka University, Japan. The fruiting bodies of *B. venenatus* were frozen upon collection and stored at –20 ºC.

4.3. Purification of BVLs

All of the procedures were carried out at 4 °C. After defrosting, the fruiting bodies of *B. venenatus* were homogenized and extracted with 10 mM phosphate-buffered saline, pH 7.4 (PBS) overnight. The homogenate was centrifuged at 8,500 x g for 15 min, and solid ammonium sulfate was added to the resulting supernatant to obtain 80% saturation. After standing overnight, the precipitates were collected by centrifugation and dialyzed extensively against distilled water and lyophilized. The lyophilized dialyzate was redissolved in 50 mM Tris-HCl buffer, pH 8.5, and applied to a column of Toyopearl SuperQ (5.0 x 20 cm) equilibrated with the buffer. After unbound materials were washed with the buffer, the bound fraction was desorbed with 50 mM NaCl in the buffer. The eluates were concentrated and equilibrated with PBS by ultrafiltration, and further separated by gel filtration on a TSK-gel G3000SW column (2.15 x 60 cm) equilibrated with the buffer. The lectin-containing fraction was dialyzed against 50 mM Tris-HCl buffer, pH 8.5, and divided into eight fractions by anion-exchange chromatography using a BioAssist Q column (1.0 x 10 cm) with a linear gradient elution of NaCl (0-1 M) in this buffer. Each fraction was dialyzed against distilled water and lyophilized, giving BVL-1 to BVL-8.

4.4. Hemagglutination and Inhibition Assay

Intact, Pronase-treated, trypsin-treated, and neuraminidase-treated human erythrocytes were prepared as described previously (Kawagishi et al., 1994a,b, 2000; Kobayashi et al., 2004). The hemagglutinating activity of the lectin was determined by a two-fold serial dilution procedure using intact, Pronase-treated, trypsin-treated, and neuraminidase-treated human erythrocytes. The hemagglutination titer was defined as the 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 using Pronase-treated human O erythrocytes.

4.5. SDS-PAGE and Isoelectric focusing

SDS-PAGE was done by the method of Laemmli (Laemmli, 1970). Samples were heated in the presence or absence of 2-mercaptoethanol for 10 min at 100 °C. Gels were stained with Coomassie Brilliant Blue. The molecular mass standards (GE Healthcare Bio-Sciences Corp.) used were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-macroglobulin (14.4 kDa).

Isoelectric focusing on a gel (PhastGel IEF, pH 3 - 9) was done in a Phastsystem (GE Healthcare Bio-Sciences Corp.). The pI standards (GE Healthcare Bio-Sciences Corp.) used were trypsinogen (pI 9.30), lentil lectin basic band (8.65), lentil lectin middle band (8.45), lentil lectin acidic band (8.15), myoglobin basic band (7.35), myoglobin acidic band (6.85), human carbonic anhydrase B (6.55), bovine carbonic anhydrase B (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), and amyloglucosidase (3.50).

4.6. Gel filtration for estimation of molecular mass

Gel filtration by FPLC was carried out on a Sephacryl S-300HR column (2.6 × 60 cm) operating at 4 ºC in PBS at a flow rate of 1 ml/min. Fractions were collected by monitoring absorbance at 280 nm. Gel filtration by HPLC was carried out on a TSK-gel G3000SWXL 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).

4.7. MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectra were acquired on an AutoFlex (Bruker Daltonics). The spectra were measured in linear mode using 20 kV ion acceleration without post acceleration. ⟨-Cyano-4-hydroxycinnamic acid was used as the matrix. The spectra were recorded at a detector voltage of 1.65 kV and were the averaged results of at least 300 laser shots. Each sample was dissolved in 0.1% trifluoroacetic acid (TFA)-CH_3CN (2:1 v/v) and mixed with the matrix solution (1:1 or 1:4 v/v). The mixture (1 ⌈l) was put on a stainless target and crystallized at room temperature. A mass calibration procedure was employed prior to the analysis of a sample using protein calibration standards (Bruker Daltonics).

4.8. Amino acid composition analysis and N-*Terminal Sequence Analysis*

Each sample was hydrolyzed with 6 M HCl at 110 °C for 24 h in a sealed evacuated tube and analyzed on a Hitachi L-8900 amino acid analyzer. The cysteine content was determined by carboxymethylation of the protein with iodoacetic acid followed by hydrolysis under the same

conditions as that of the intact protein (Moore, 1963). The content of tryptophan was estimated by the spectrometric method of Edelhoch (Edelhoch, 1967).

The *N*-terminal amino acid of the intact protein was analyzed on a PPSQ-21A protein peptide sequencer (Shimadzu).

4.9. N-*Glycanase Digestion*

BVLs (50 μg) were dissolved in PBS (0.1 ml), heated to 100 °C for 10 min, and then cooled to room temperature. To the solution, Nonidet P-40 was added at a final concentration of 0.5% (w/v), and further incubated for 18 h at 37 °C in the presence or absence of 5 unit of *N*-glycanase F (Roche). After the treatment, samples were dialyzed against distilled water and analyzed on isoelectric focusing.

4.10. Proteinase Digestion and Peptide Sequence Analysis

Each sample (0.5 mg) was reduced with dithiothreitol (0.5 mg) at room temperature for 5 h, *S*-carboxymethylated with ICH_2COOH (1.25 mg) at room temperature for 30 min, dialyzed against 0.1% TFA in H_2O, and digested with a lysyl endopeptidase, Achromobacter proteinase I (Enzyme(E)/Substrate(S)=1:100 (w/w)), in 0.1 M Tris-HCl buffer (pH 9.0) for 2 h at 37 °C. The *S*-carboxymethylated lectin was also digested with an endoproteinase Arg-C from *Clostridium histolyticum* (E/S=1:50 (w/w)) in 50 mM sodium phosphate buffer (pH 8.0) at 37 °C for 12 h, or an endoproteinase Glu-C from Staphylococcus aureus V8 (E/S=1:50 (w/w)) in 50 mM ammonium bicarbonate buffer (pH 7.8) for 2-12 h at 37°C. The resulting peptides were separated by reversed-phase HPLC using a TSK-gel Superoctyl column (4.6 x 100 mm) with a linear gradient of 0-80% acetonitrile/0.1% TFA in H_2O at a flow rate of 0.5 ml/min. The effluent was monitored at 215 nm. After the isolation, each peptide was analyzed by an AutoFlex MALDI-TOF Mass Spectrometer (Bruker Daltonics). Homology of the sequences with other proteins was searched by FASTA service.

4.11. Neutral sugar content estimation and sugar composition analysis

The sugar content was measured by the phenol-sulfuric acid method with reference to Glc. Neutral and amino sugar compositions were determined as described previously (Kobayashi et al., 2004; Kawagishi et al., 2000; Yasuno et al., 1997). Briefly, the purified protein (0.2 mg) was dissolved in 20 μl distilled water in a test tube to which 4 M TFA (20 μl) was added. The test tube was incubated at 100 °C in a hot block bath. After 4 h, the tube was cooled to room temperature and the solvent was removed by using a centrifugal concentrator at 35 °C. The dried sample was derivatized with *p*-aminobenzoic ethyl ester (ABEE) in the presence of borane-pyridine complex at 80 °C. After 1 h, the reaction mixture was cooled to room temperature. The distilled water (0.2 ml) and an equal volume of chloroform were added to the reaction mixture. After vigorous vortexing, the sample was centrifuged (6,000 x g, 1 min). The upper aqueous layer was analyzed by reversed-phase HPLC under the following conditions: column, Wakosil-II 5C18HG (4.6 × 150 mm); solvent, A 0.02% TFA/CH_3CN (90/10), B 0.02% TFA/CH_3CN (50/50); program, 0-45 min (B conc. 0%), 45-55 min (B conc. 100%), 55-70 min (B conc. 0%); flow rate, 1 ml/min; temperature, 45 °C; detection, fluorescence at 305 nm (excitation) and 360 nm (emission). The monosaccharide and amino monosaccharide standards used were GlcNAc, GalNAc, Glc, Gal, Man, Xyl, and L-Fuc.

Sialic acid composition was determined according to the method of Hara *et al*. (Hara et al., 1986, 1989). Briefly, the protein (10 μg) was dissolved in 10 μl distilled water in a test tube to which 25 mM HCl (400 μl) was added. The test tube was incubated at 80 °C in a hot block bath. After 1 h, the tube was cooled to room temperature and the solvent was removed by using a centrifugal concentrator at 35 °C. The dried sample was derivatized with 1,2-diamino-4,5-methylenedioxybenzene at 65 °C. After 2.5 h, the reaction mixture was cooled

to room temperature. The sample was analyzed by reversed-phase HPLC under the following conditions: column, Wakosil-II 5C18HG (4.6 × 150 mm); solvent, A MeOH/CH_3CN/H_2O (3/1/10 v/v/v), B MeOH/CH_3CN/H_2O (1/1/1 v/v/v); program, 0-35 min (B conc. 0%), 35-45 min (B conc. 100%), 45-60 min (B conc. 0%); flow rate, 1 ml/min; temperature, 35 °C; detection, fluorescence at 373 nm (excitation) and 448 nm (emission). The sialic acid standards used were NeuAc and NeuGc.

4.12. Thermostability, pH stability and metal cation requirements

The thermostability and pH stability of the lectin were examined as described previously (Kawagishi et al, 1994). Briefly, samples in PBS were heated for 30 min at the temperatures indicated, cooled on ice, and titrated. In another experiment, samples in PBS were heated for 70 min at 100 °C, cooled on ice, and titrated. The pH stability of the lectin was measured by incubating the samples in the following buffers for 12 h at 4 °C, dialyzing against PBS, and titrating in PBS: 50 mM glycine-HCl buffer (pH 2.0-3.0), 50 mM sodium acetate buffer (pH 4.0-5.5), 50 mM sodium phosphate buffer (pH 6.0-7.5), 50 mM Tris-HCl buffer (pH 8.0-8.5), and 50 mM glycine-NaOH buffer (pH 9.0-11.0). To examine metal cation requirements of the hemagglutination by the lectin, the sample (0.1 mg/ml) was incubated in 10 mM EDTA for 1 h at room temperature, dialyzed against PBS, and titrated. To the demetalized lectin, 0.1 M metal cation ($CaCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$) was added, and the solution was incubated for 1 h at room temperature and titrated.

4.13. FAC analysis

The lectin was dissolved in 0.2 M $NaHCO_3$ containing 0.5 M NaCl (pH 8.3) and coupled to HiTrap NHS-activated Sepharose by following the manufacturer's instructions. After washing and deactivation of excess active groups by 0.5 M Tris-HCl buffer containing 0.5 M NaCl (pH 8.3), the lectin-immobilized Sepharose beads were suspended in 10 mM Tris-HCl buffer, pH 7.4, containing 0.8% NaCl (TBS) and the slurry was packed into a stainless steel column (2.0 × 10 mm) and connected to the FAC-1 machine, which had been specially designed and manufactured by Shimadzu Co.. The amount of immobilized protein was determined by measuring the amount of uncoupled protein in the washing solutions by the method of Bradford (Bradford , 1976). The flow rate and the column temperature were kept at 125 μl/min and 25 °C, respectively. After equilibration with TBS, an excess volume (0.5 - 0.8 ml) of PA-glycans (2.5 or 5.0 nM) was successively injected into the columns by an auto-sampling system. Elution of each PA-glycan was monitored by measuring fluorescence (excitation and emission wave lengths, 310 and 380 nm, respectively). The elution front relative to that of a standard oligosaccharide (PA-01), i.e., V-V_0, was then determined. V is elution volume of each PA sugar. For the determination of V_0, PA-01, which has no affinity to the lectin, was used (Hirabayashi et al., 1998, 2000; Arata et al., 2001)

4.14. SPR analysis

Real time detection of the lectin binding to glycoproteins was recorded by using a BIAcore 2000 (Kobayashi et al., 2004, 2005; Kawagishi et al., 2001). Intact lectin was immobilized covalently via its primary amines to carboxyl groups within a dextran layer on the sensor chip CM-5 according to the manufacture's specifications. After chip activation with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 10 mM *N*-hydroxysuccinimide, the sample (in 10 mM sodium acetate buffer, pH 5.0) at a concentration of 10 μg/ml was passed through the flow cell at a rate of 5 μl/min. After immobilization, the chip was capped by exposure to 1 M ethanolamine.

All the sample analyses were performed at a flow rate of 20 μl/min. Before loading of analytes, the chip was equilibrated with 10 mM Hepes containing 0.15 M NaCl, 3 mM EDTA

and 0.005% surfactant P20, pH 7.4 (HBS-EP). Each analyte at various concentrations in the same buffer was injected over the immobilized ligand. After injection of the analyte, HBS-EP was introduced onto the sensor surface to start dissociation.

The experimental sensorgrams were fitted to various kinetic models in BIAevaluation 3.2 software (GE Healthcare Bio-Sciences Corp.). Association and dissociation rate constants (*ka* and *kd*) were calculated by using BIAevaluation 3.2 software. The affinity constant (K_D) was calculated from the *ka* and *kd*. For the calculation of rate constants, samples were appropriately diluted in HBS-EP at various concentrations.

4.15. Animal experiments

Eight-week-old male mice of the ddy strain weighing 20-25 g and five-week-old male rats of the Wistar strain weighing 90-100 g were obtained from Japan SLC. The animals were housed in hanging stainless steel wire-cages and kept in an isolated room at a controlled temperature (23-25 °C) and ambient humidity (50 - 60%). Lights were maintained on a 12-hour light-dark cycle. Animals were acclimated to the facility for 4 or 5 days. After the acclimation, BVLs were injected intraperitoneally at a dose of 0.5, 1.0, or 1.5 mg/mouse (one group, 3 mice) or orally force-fed to rats at a dose of 40 mg/kg body weight by using a catheter (one group, 3 rats). As the control, rats were treated with saline. In another experiment, loperamide (30 mg in 5 ml of 0.3 % carboxymethylcellulose) was orally administrated to rats (30 mg/kg body weight). After 30 min of the administration, BVLs were orally force-fed to the rats at a dose of 40 mg/kg body weight by using a catheter (one group, 3 rats). The experimental design was approved by the Laboratory Animal Care Committee of the Faculty of Agriculture, Shizuoka University.

Acknowledgements

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Abbreviations: ABEE, *p*-aminobenzoic ethyl ester; BSM, bovine submaxillary mucin; FAC, frontal affinity chromatography; HBS-EP, 10 mM Hepes containing 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20, pH 7.4; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PA, pyridylaminated; PBS, 10 mM phosphate-buffered saline, pH 7.4; PSM, porcine stomach mucin; SPR, surface plasmon resonance; TBS, 10 mM Tris-HCl buffer containing 0. 15 M NaCl,, pH 7.4; TFA, trifluoroacetic acid; All sugars were of D-configuration unless otherwise stated.

References

Arata, Y., Hirabayashi, J., Kasai, K. 2001. Sugar binding properties of the two lectin domains of the tandem repeat-type galectin LEC-1 (N32) of *Caenorhabditis elegans*. J. Biol. Chem. 276, 3068 - 3077.

Basset, L., Ennamany, R., Portail, J. P., Kretz, O., Deffieux, G., Badoc, A., Guillemain, B., Creppy, E. E. 1995. Effects of bolesatine on a cell line from the SP2/O thymic lymphosarcoma. Toxicology 103, 121-125.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram

quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Edelhoch, H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. Biochemistry 6, 1948-1954.

Ennamany, R., Marzetto, S., Saboureau, D., Creppy, E. E. 1995. Lipid peroxidation induced by bolesatine, a toxin of *Boletus satanas*: implication in m5dC variation in Vero cells related to inhibition of cell growth. Cell Biol. Toxicol 11, 347-354.

Ennamany, R., Bingen, A., Creppy, E. E., Kretz, O., Gut, J. P., Dubuisson, L., Balabaud, C., Bioulac Sage, P., Kirn, A. 1998. Aspirin and heparin prevent hepatic blood stasis and thrombosis induced by the toxic glycoprotein Bolesatine in mice. Hum. Exp. Toxicol. 17, 620-624.

Gachet, C., Ennamany, R., Kretz, O., Ohlmann, P., Krause, C., Creppy, E. E., Dirheimer, G., Cazenave, J. P. 1996. Bolesatine induces agglutination of rat platelets and human erythrocytes and platelets in vitro. Hum. Exp. Toxicol. 15, 26-29.

Hara, S., Yamaguchi, M., Takemori, Y., Nakamura, M., Ohkura, Y. 1986. Highly sensitive determination of *N*-acetyl- and *N*-glycolylneuraminic acids in human serum and urine and rat serum by reversed-phase liquid chromatography with fluorescence detection. J. Chromatogr. 377, 111-119.

Hara, S., Yamaguchi, M., Takemori, Y., Furuhata, K., Ogura, H., Nakamura, M. 1989. Determination of mono-*O*-acetylated *N*-acetylneuraminic acids in human and rat sera by fluorometric high-performance liquid chromatography. Anal. Biochem. 179, 162-166.

Hirabayashi, J., Dutta, S. K., Kasai, K. 1998. Novel galactose-binding proteins in Annelida. Characterization of 29-kDa tandem repeat-type lectins from the earthworm *Lumbricus terrestris*. J. Biol. Chem. 273, 14450 - 14460

Hirabayashi, J., Arata, Y., Kasai, K. 2000. Reinforcement of frontal affinity chromatography for effective analysis of lectin–oligosaccharide interactions. J. Chromatogr. A 890, 261-271.

Kawagishi H. 1995. Mushroom lectins. Food Rev. Int., 11, 63-68.

Kawagishi, H., Yamawaki, M., Isobe, S., Usui, T., Kimura, A., Chiba, S. 1994a. Two lectins from the marine sponge *Halichondria okadai*; an *N*-acetyl-sugar-specific lectin (HOL-I) and an *N*-acetyllactosamine-specific lectin (HOL-II). J. Biol. Chem. 269, 1375-1379.

Kawagishi, H., Mori, H., Uno, A., Kimura, A., Chiba, S. 1994b. A sialic acid-binding lectin from the mushroom *Hericium erinaceum*. FEBS Lett. 340, 56-58.

Kawagishi, H., Suzuki, H., Watanabe, H., Nakamura, H., Sekiguchi, T., Murata, T., Usui, T., Sugiyama, K., Suganuma, H., Inakuma, T., Ito, K., Hashimoto, Y., Ohnishi-Kameyama, M., Nagata, T. 2000. A lectin from an edible mushroom *Pleurotus ostreatus* as a food intake-suppressing substance. Biochim. Biophys. Acta 1474, 299-308.

Kawagishi, H., Takagi, J., Taira, T., Murata, T., Usui, T. 2001. Purification and characterization of a lectin from the mushroom *Mycoleptodonoides aitchisonii*. Phytochemistry 56, 53-58.

Kobayashi, Y., Kobayashi, K., Umehara, K., Dohra, H., Murata, T., Usui, T., Kawagishi, H. 2004. Purification, characterization and sugar-binding specificity of an *N*-glycolylneuraminic acid-specific lectin from the mushroom *Chlorophyllum molybdites*. J. Biol. Chem. 279, 53048-53055.

Kobayashi, Y., Nakamura, H., Sekiguchi, T., Takanami, R., Murata, T., Usui, T., Kawagishi, H. 2005. Analysis of the carbohydrate binding specificity of the mushroom *Pleurotus ostreatus* lectin by surface plasmon resonance. Anal. Biochem. 336, 87-93.

Kretz, O., Creppy, E. E., Boulanger, Y., Dirheimer, G. 1989. Purification and some properties of bolesatine, a protein inhibiting in vitro protein synthesis, from the mushroom Boletus satanas Lenz (Boletaceae). Arch. Toxicol. Suppl. 13, 422-427.

Kretz, O., Creppy, E. E., Dirheimer, G. 1991. Disposition of the toxic protein, bolesatine, in rats: its resistance to proteolytic enzymes. Xenobiotica 21, 65-73.

Kretz, O., Reinbolt, J., Creppy, E. E., Dirheimer, G. 1992a. Properties of bolesatine, a translational inhibitor from Boletus satanas Lenz. Amino-terminal sequence determination and inhibition of rat mitochondrial protein synthesis. Toxicol. Lett. 64-65 Spec No, 763-766.

Kretz, O., Barbieri, L., Creppy, E. E., Dirheimer, G. 1992b. Inhibition of protein synthesis in liver

and kidney of mice by bolesatine: mechanistic approaches to the mode of action at the molecular level. Toxicology 73, 297-304.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Matsumoto, T., Shirahama, H., Ichihara, A., Fukuoka, Y., Takahashi, Y., Mori, Y., Watanabe, M. 1965. Structure of lampterol (illudin S). Tetrahedron 21, 2671-2676.

Matsuura, M., Yamada, M., Saikawa, Y., Miyairi, K., Okuno, T., Konno, K., Uenishi, J., Hashimoto, K., Nakata, M. 2007. Bolevenine, a toxic protein from the Japanese toadstool *Boletus venenatus*. Phytochemistry 68, 893–898.

McMorris, T. C., Anchel, M. 1963. The structures of Basidiomycete metabolites illudin S and illudin M. J. Am. Chem. Soc. 85, 831-832.

Moore, S. 1963. On the determination of cystine as cysteic acid. J. Biol. Chem. 238, 235-237.

Nakanishi, K., Tada, M., Yamada, Y., Ohashi, M., Komatsu, N., Terakawa, H. 1963. Isolation of lampterol, an antitumor substance from *Lampteromyces japonicus*. Nature 197, 292-292.

Nakanishi, K., Ohashi, M., Tada, M., Yamada, Y. 1965. Illudin S (lampterol). Tetrahedron 21, 1231-1246.

Sano, Y., Sayama, K., Arimoto, Y., Inakuma, T., Kobayashi, K., Koshino, H., Kawagishi, H. 2002. Ustalic acid as a toxin and related compounds from the mushroom *Tricholoma ustale*. Chem. Commun., 1384 -1385.

Suzuki, K., Une, T., Fujimoto, H., Yamazaki, M. 1987. Studies on the toxic components of *Rhodophylllus rhodopolius*. I. The biological activities and screening of the toxic principles. Yakugaku Zasshi 107, 971-977.

Suzuki, K., Une, T., Fujimoto, H., Yamazaki, M. 1988. Studies on the toxic components of *Rhodophylllus rhodopolius*. II. Partial purification and properties of the hemolysin from *Rhodophylllus rhodopolius*: examination on the condition of the hemolysis. Yakugaku Zasshi 108, 221-225.

Suzuki, K., Une, T., Yamazaki, M., Takeda, T. 1990. Purification and some properties of a hemolysin from the poisonous mushroom *Rhodophylllus rhodopolius*. Toxicon 28, 1019-1028.

Totani, K., Kubota, T., Kuroda, T., Murata, T., Hidari, K., Suzuki, T., Suzuki, Y., Kobayashi, K., Ashida, H., Yamamoto, K., Usui, T. 2003. Chemo-enzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses. Glycobiology 13, 315-316.

Wang H., Ng T. B. and Ooi V. E. C. 1998. Lectins from mushrooms. Mycol. Res., 102, 8, 897-906.

Yasuno, S., Murata, T., Kokubo, K., Kamei, M. 1997. Two-mode analysis by high-performance liquid chromatography of *p*-aminobenzoic ethyl ester-derivatized monosaccharides. Biosci. Biotech. Biochem. 61, 1944-1946.

Zeng, X., Nakaaki, Y., Murata, T., Usui, T. 2000. Chemoenzymatic synthesis of glycopolypeptides carrying α-Neu5Ac-(2→3)-β-D-Gal-(1→3)- α-D-GalNAc, β-D-Gal-(1→3)-α-D-GalNAc, and related compounds and analysis of their specific interactions with lectins. Arch. Biochem. Biophys. 383, 28-37.

Fig. legends

Fig. 1. SDS-PAGE of BVLs
Lane M, marker proteins; Lane 1, BVL non-reduced; Lane 2, BVLs reduced with 2-mercaptoethanol.

Fig. 2. HPLC profile of BVLs. A. Elution profile of BVLs. Column, TSK-gel G3000SWXL (7.8 × 300 mm); temperature, room temperature; solvent, PBS; flow rate , 0.5 ml/min; detection, 280 nm. B. Estimation of molecular weight. 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. Isoelectric focusing of BVL-1 to -8
A. Lane M, marker proteins; Lane 1, BVLs; Lanes 2 to 9, BVL-1 to -8. B. Lane M, marker proteins; Lane 1, BVLs; Lane 2, *N*-glycanase F-treated BVLs.

Fig. 4. Sequence comparison of BVL-1 and other proteins
Residues in Lanes 1 to 6 describe the amino acid sequences of BVL-1, bolesatine, HA1, AAIK, AAIT, and AAIN respectively. The identical residues with BVL-1 are displayed in black shading.

Fig. 5. Sensorgrams showing the interaction between immobilized BVL-1 and glycoproteins
A, analyte, fetuin; B, asialo-fetuin; C, $\langle_1$-acid glycoprotein; D, thyroglobulin.

Fig. 6. FAC analysis of binding of PA-oligosaccharides immobilized BVL-1
A, structures of PA-oligosaccharides tested; B, retardation volume of each PA-sugar in BVL-1-immobilized column (V-V_0 in ⌈l, y-axis).

Figure(s)

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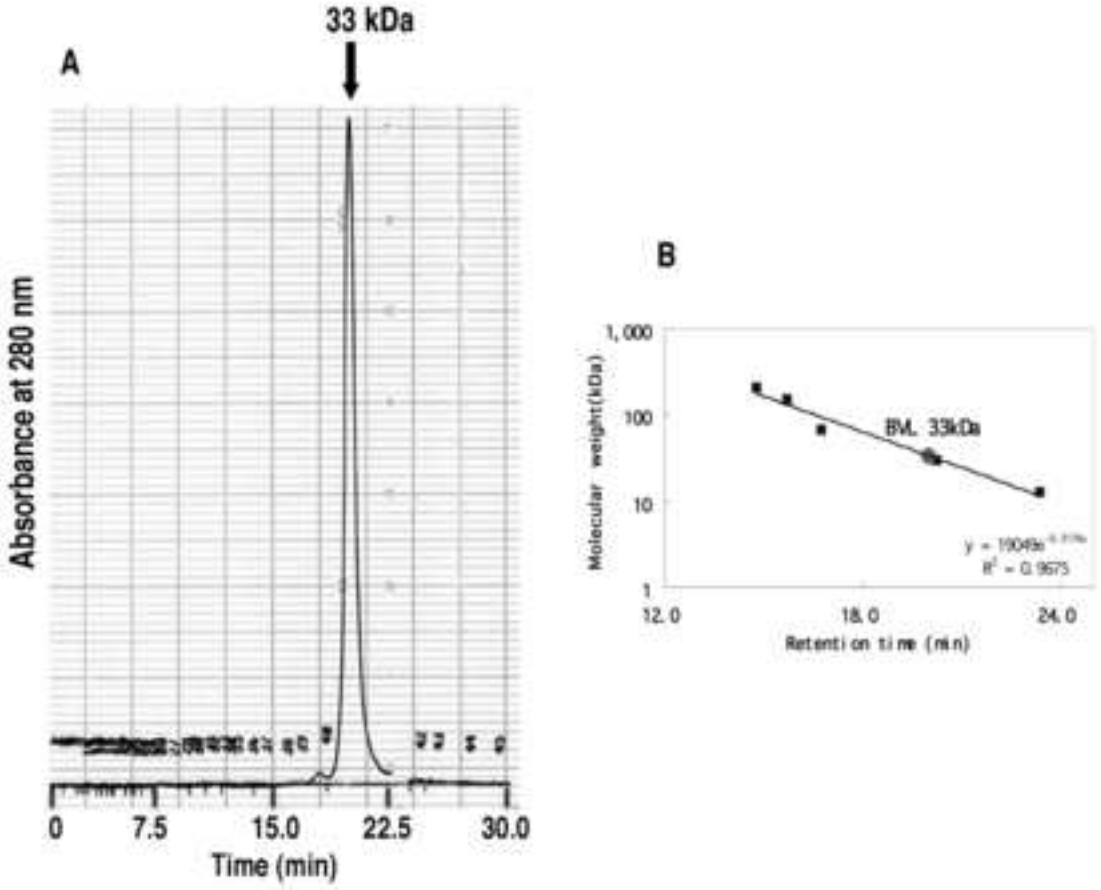

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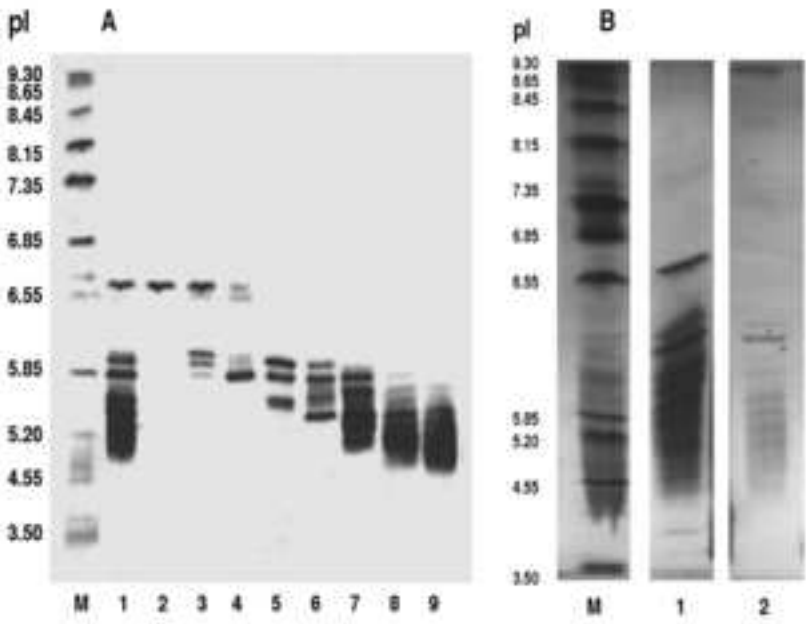

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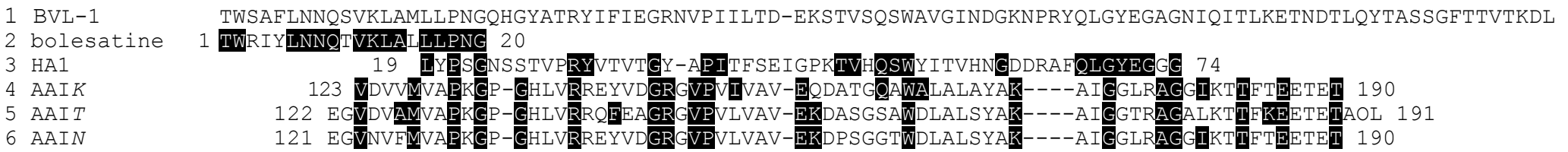

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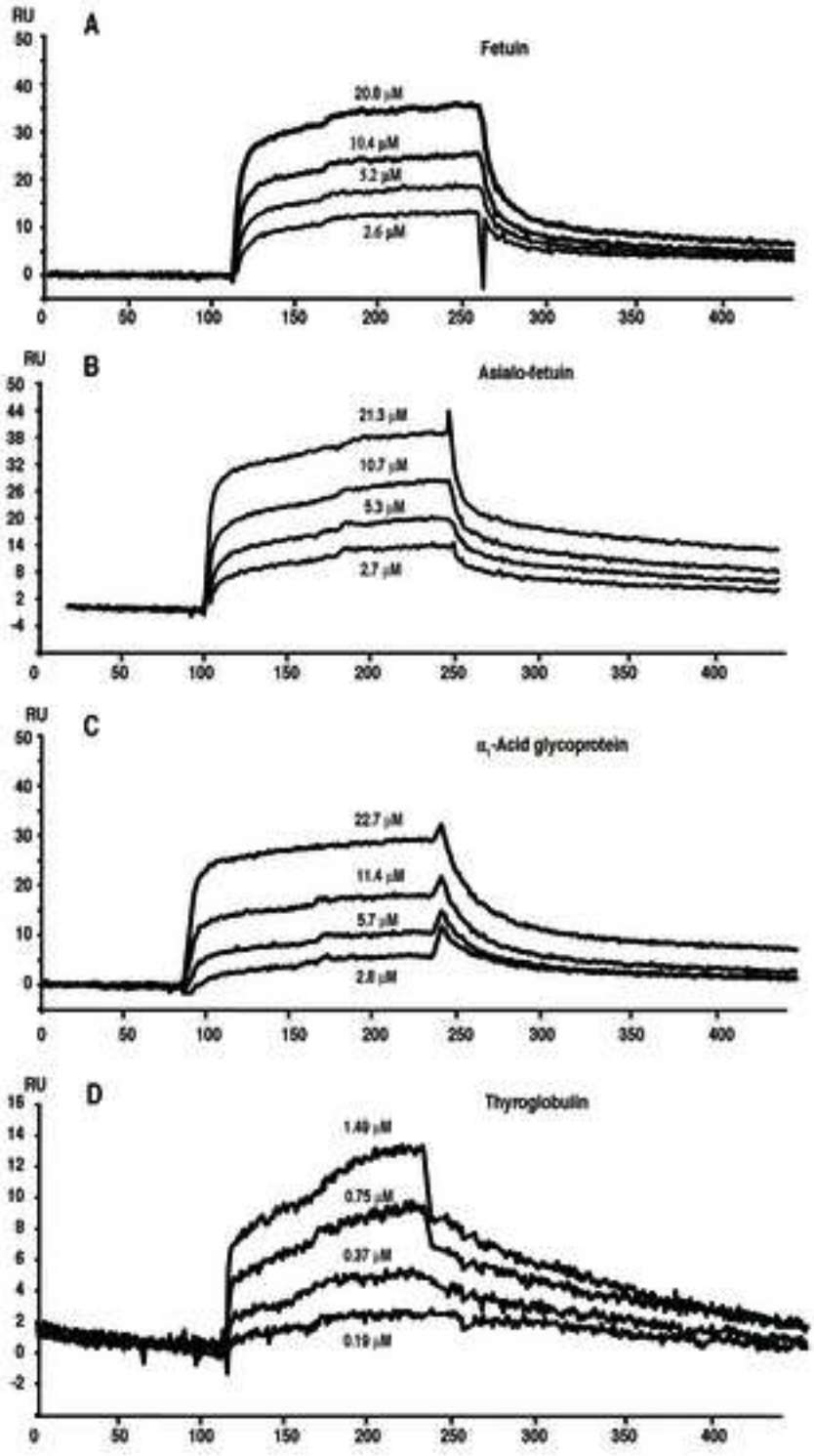

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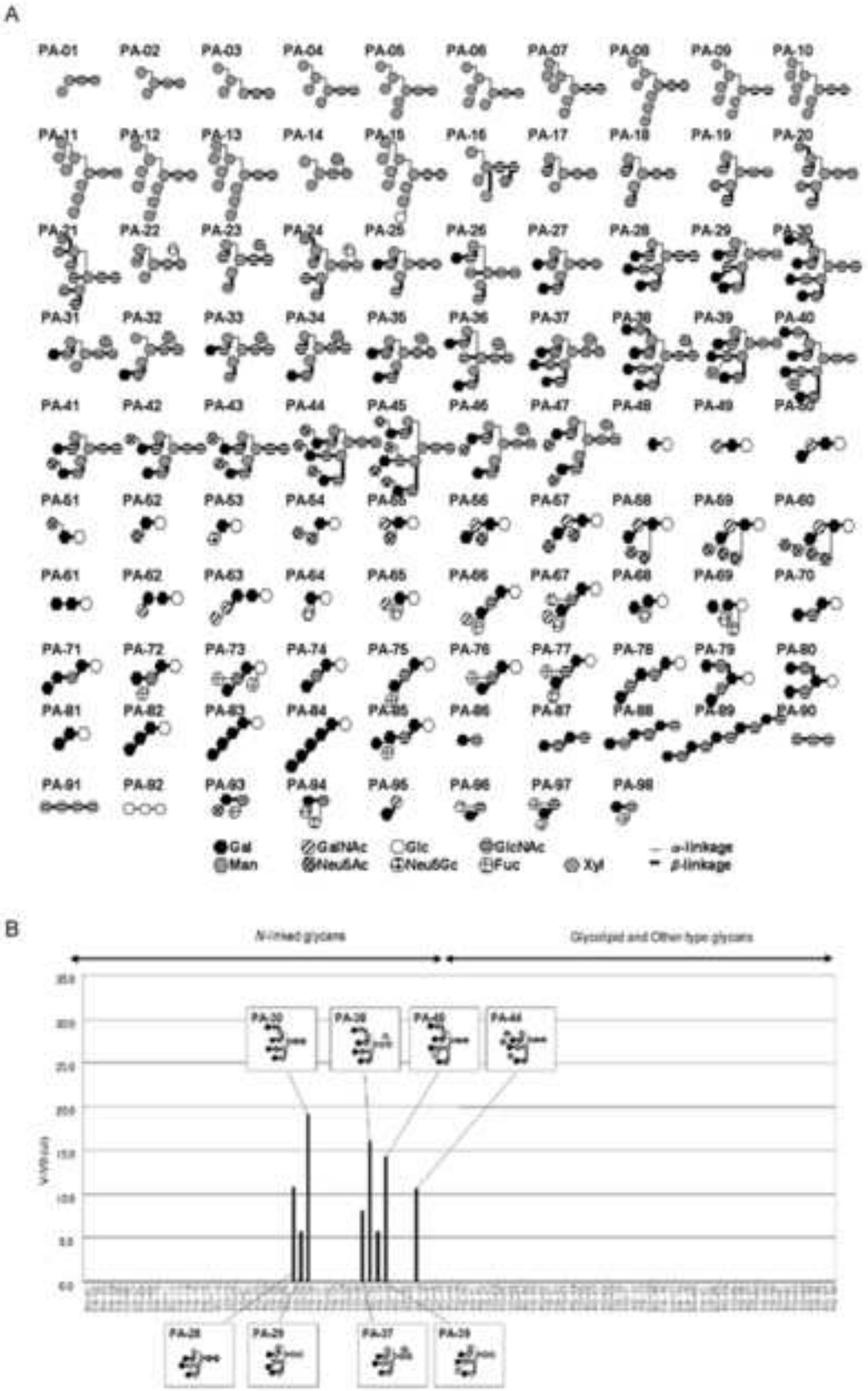

Horibe et al., Fig. 6

Table 1

Purification of BVL-1 to BVL-8 from 100 g of the fresh fruiting bodies of *Boletus venenatus*

Step	Total protein (mg)	Total agglutination activity (titer)[a]	Specific agglutination activity (titer/mg)	Recovery of activity (%)
80%$(NH_4)_2SO_4$ Precipitate	1420	4544000	3200	100
SuperQ	84.6	2165760	25600	47.7
Gel filtration (BVLs)	48.1	1231360	25600	27.1
Bio Assist Q				
BVL-1	3.9	99840	25600	2.2
BVL-2	2.0	51200	25600	1.1
BVL-3	1.1	28160	25600	0.6
BVL-4	2.1	53760	25600	1.2
BVL-5	7.6	194560	25600	4.3
BVL-6	2.1	53760	25600	1.2
BVL-7	2.3	58880	25600	1.3
BVL-8	0.7	17920	25600	3.9

[a] Titer was defined as the reciprocal of the end-point dilution exhibiting the hemagglutination.

Table 2
Amino acid composition of BVL-1

Amino acid	mol%	Amino acid	mol%
Asx	12.0	Met	0.2
Thr	11.1	Ile	5.3
Ser	6.7	Leu	8.8
Glx	10.4	Tyr	3.6
Pro	5.8	Phe	2.7
Gly	10.0	Lys	4.9
Ala	7.1	His	0.9
Cys	0	Trp	3.6
Val	4.9	Arg	2.0

Table(s)

Table 3

Agglutination profiles of BVL-1

Group of erythrocytes	Log_2 of titer [a]			
	Untreated	Pronase treated[b]	Trypsin treated[c]	Neuraminidase treated[d]
Human A	2	13	8	7
Human B	2	13	8	7
Human O	2	13	8	7

[a] Titer was defined as the reciprocal of the highest dilution exhibiting hemagglutination.

[b] 10% suspension of erythrocytes in PBS (10 ml) was treated with Pronase (5.0 mg) for 30 min at 45°C.

[c] 10% suspension of erythrocytes in PBS (10 ml) was treated with trypsin (1.0 mg) for 180 min at 37°C.

[d] 10% suspension of erythrocytes in PBS (10 ml) was treated with neuraminidase (1 U/ml) for 60 min at 37°C.

Table 4
Inhibition of BVL-1-mediated hemagglutination by glycoproteins

Inhibitor[a]	MIC[b] (μg/ml)
Asialo-fetuin	0.49
Thyroglobulin	1.95
Fetuin	3.91
α_1-Acid glycoprotein	3.91
Asialo-BSM	15.6
BSM[c]	15.6
PSM[d]	15.6

[a] Glucose, galactose, mannose, fructose, fucose, L-fucose, arabinose, L-arabinose, ribose, glucosamine, galactosamine, mannosamine, raffinose, L-rhamnose, saccharose, lactulose, lactose, lactitol, GlcNAc, GalNAc, ManNAc, Me α-Glc, Me β-Glc, Me α-Gal, Me β-Gal, Me α-Man, Me α-GlcNAc, Me β-GlcNAc, Me β-GalNAc, melibiose, xylose, galactulonic acid, gluculonic acid, 2-deoxyglucose, 2-deoxyribose, 2'-fucosyllactose, 3-fucosyllactose, GlcNAc[(β1-4)GlcNAc]$_n$(n=1-4), Ph α-GalNAc, and Ph β-GalNAc did not inhibit at concentrations up to 0.4 M. LacNAc did not inhibit at concentrations up to1.0 M. *N*-Acetylneuraminic acid and *N*-glycolylneuramic acid did not inhibit at concentrations up to 40 mM. Transferrin, hyaluronan, albumin, mannan, and poly(LacNAc-pAP/Gln-co-Gln) did not inhibit at concentrations up to 1 mg/ml.
[b] Minimum inhibitor concentration required for inhibition of 4 hemagglutination dose of the lectin.
[c] BSM : bovine submaxillary gland mucin.
[d] PSM : porcine stomach mucin.

Table 5

Binding kinetics of interaction between immobilized BVL-1 and glycoproteins

Analyte	*ka* ($M^{-1}S^{-1}$)	*kd* (S^{-1})	*KD* (M)
Asialo-fetuin	9.72×10^{2}	2.75×10^{-3}	2.83×10^{-6}
Fetuin	1.66×10^{3}	3.90×10^{-3}	2.35×10^{-6}
α_1-Acid glycoprotein	3.27×10^{2}	4.40×10^{-3}	1.35×10^{-5}
Thyroglobulin	1.04×10^{4}	5.77×10^{-3}	5.56×10^{-7}