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Toxic isolectins from the mushroom *Boletus venenatus*

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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 (*B. 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 Bio-Assist 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 mass spectrometry (MALDI-TOF-MS) analysis. Among them, BVL-1 and -3 were further characterized with their complete amino acid sequences of 99 amino acids 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, asialo-fetuin 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 ingestion resulted in fatal toxicity in mice upon intraperitoneal administration and caused diarrhea upon oral administration in rats.

1. Introduction

People eat various kinds of wild mushrooms and many get poisoned by accidentally eating toxic mushrooms. Some of the toxic substances produced by mushrooms have been isolated and characterized; for example, 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 *Rhodophyllus rhodopolius* (Nakanishi et al., 1963, 1965; McMorris and Anchel, 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. These gave a single band on SDS–PAGE from the mushroom, and could be divided them into eight isolectins. Furthermore, we found

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-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; 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.

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

Step	Total protein (mg)	Total agglutination (titer)[a]	Specific agglutination activity (titer/mg)	Recovery of activity (%)
80%$(NH_4)_2SO_4$ precipitate	1420	45,44,000	3200	100
SuperQ	84.6	21,65,760	25,600	47.7
Gel filtration (BVLs)	48.1	12,31,360	25,600	27.1
BioAssist Q				
BVL-1	3.9	99,840	25,600	2.2
BVL-2	2.0	51,200	25,600	1.1
BVL-3	1.1	28,160	25,600	0.6
BVL-4	2.1	53,760	25,600	1.2
BVL-5	7.6	1,94,560	25,600	4.3
BVL-6	2.1	53,760	25,600	1.2
BVL-7	2.3	58,880	25,600	1.3
BVL-8	0.7	17,920	25,600	3.9

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

that BVLs showed lectin activity and caused diarrhea in rats, and one of the isolectins 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).

Herein, we describe the purification, and biochemical and molecular characterization of the isolectins from this mushroom species.

2. Results

2.1. Purification of BVLs

Since the extract of *B. venenatus* showed lectin activity and lethal toxicity to mice, its fractionation was guided by 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 were further purified by anion-exchange chromatography and gel filtration in a two-step process. The toxicity and lectin activity cofractionated in all the steps of the isolation (data not shown) and the active fraction (*B. 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. 2). 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 by HPLC gel filtration (Fig. 2A), isoelectric focusing of BVLs gave 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 2–9). The isolated isolectins were named BVL-1 to -8, respectively.

2.2. Molecular properties of BVL-1 and -3

The isoelectric focusing bands of BVLs converged to fewer bands upon treating with PNGase F (Fig. 3B). MALDI-TOF-MS of each

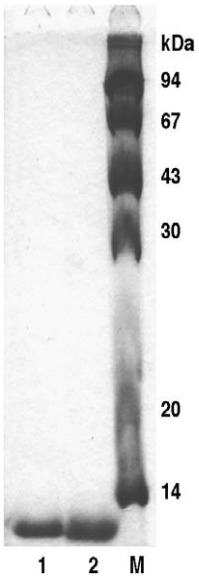

Fig. 1. SDS–PAGE of BVLs: (Lane M) marker proteins. (Lane 1) BVLs non-reduced. (Lane 2) BVLs reduced with 2-mercaptoethanol.

isolectin gave very similar molecular ions to each other (from *m/z* 10,947 to 10,955: BVL-1, *m/z* 10,955; BVL-2, *m/z* 10,947; BVL-3, *m/z* 10,948; BVL-4, *m/z* 10,953; BVL-5, *m/z* 10,949; BVL-6, *m/z* 10,954; BVL-7, *m/z* 10,948; BVL-8, *m/z* 10,950). Since BVL-1 and -3 had completely different p*I*s from each other, they were further characterized.

The amino acid composition analysis of BVL-1 established 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 also 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 peptide sequences was determined by *N*-terminal amino acid sequence analysis and MALDI-TOF-MS. 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: GlcNAc 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 M 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).

Lectin activity was stable between pH 2.0 and 9.5 and below 80 °C (data not shown). Since the lectin was not deactivated completely even at 100 °C 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