Note

Structural Analysis of N-Linked Oligosaccharide of Mitogenic Lectin-B from the Roots of Pokeweed (Phytolacca americana)†

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The structure of an asparagine (N-) linked oligosaccharide of pokeweed lectin-B (PL-B) and the amino acid sequences around two glycosylation sites were identified. The pyridylamin (PA) oligosaccharide prepared from PL-B was eluted as a single peak on both reversed-phase (RP-) HPLC and size-fractionation (SF-) HPLC, and its structure was estimated to be Manz1 → 6(Manz1 → 3)(Xylβ1 → 2) Manβ1 → 4GlcNAcβ1 → 4(Fucz1 → 3)GlcNAc by a combination of component analysis, successive exoglycosidase digestions, IS-MS analysis, and 500 MHz 1H-NMR. Two trypic glyc peptides were isolated from the reduced and S-pyridylethylated PL-B after gel filtration followed by RP-HPLC, indicating the presence of two glycosylation sites in PL-B. The amino acid sequences around the two glycosylation sites were determined to be Cys-Gly-Val-Asp-Phe-Gly-Asn(CHO)-Arg.

Key words: N-linked oligosaccharide; plant glycoprotein; IS-MS; mitogenic lectin; Phytolacca americana

Pokeweed lectin is a lectin specific for N-acetylglucosamine-containing saccharides and stimulates peripheral lymphocytes to undergo mitosis by binding to their cell surfaces. Previously we isolated three homologous lectins, designated PL-A, PL-B, and PL-C, with different molecular masses from the roots of pokeweed (Phytolacca americana) and characterized them.13 We analyzed their amino acid sequences and found that they are composed of several cysteine-rich chitin-binding domains like wheat germ agglutinin.2-3 Of these lectins, PL-B is a glycoprotein with molecular mass of 36 kDa composed of 7 domains,8 and has the most potent hemagglutinating and mitogenic activities.13 However, the structure of the N-linked oligosaccharide(s) of PL-B and the number of glycosylation site(s) remain to be discovered. In this paper, we describe the structural analysis of N-linked oligosaccharide of PL-B and amino acid sequences around the glycosylation sites.

Most analytical methods in this study are described in our previous papers. PL-B was prepared from pokeweed roots13 and pyridylaminated oligosaccharide from PL-B was prepared by coupling to 2-aminoptyridine after hydrazinolysis and N-acetylation.44 Separation of PA-oligosaccharides was done by reversed-phase (RP-) HPLC with a Cosmosil SC18-AR column (Nakarai Chemicals Co.) or size-fractionation HPLC with an Asahipak NH2P-50 column (Showa Denko Co.) using a JASCO 880-PU HPLC apparatus with a JASCO 821-FP Intelligent Spectrofluorometer.52 Sugar composition was analyzed by gas liquid chromato graphy as trimethylsilyl derivatives after methanolysis.53 Exoglycosidase digestions were done using about 100 pmol of PA-oligosaccharide. Reactions were stopped by boiling the digests for 2 min and a part of the digest was analyzed by size-fractionation HPLC. All authentic PA-sugar chains were prepared as described in our previous paper.61 Ion-spray mass (IS-MS) was done using a Perkin Elmer Sciex API-III. Mass spectrometer was operated in

Fig. 1. HPLC of PA-Oligosaccharide Derived from PL-B.

(i) RP-HPLC of PA-oligosaccharide derived from PL-B. PA-oligosaccharide was pooled as indicated by the bar. (ii) SF-HPLC of the PA-oligosaccharides derived by the exoglycosidases. A, PA-oligosaccharides pooled in I; B, the β-mannosidase digest of A; C, the β-xylosidase digest of B; D, the α-fucosidase digest of C; E, the β-mannosidase digest of C. M1-M5 indicates ManαGlcNAcβ-Glu-ManαGlcNAcβ-Glu-ManαGlcNAcβ-Glu-ManαGlcNAcβ-Glu-ManαGlcNAcβ-Glu.
the positive mode with 4200 V of the ion spray voltage. The collisionally activated dissociation (CAD) spectrum was measured with argon as collision gas (collision energy 120 eV). The scanning was done with a step size of 0.5 Da and the CAD daughter ion spectrum was recorded from m/z 100 to 1300. Nuclear magnetic resonance (NMR) spectroscopic analysis was done in D₂O using acetone (2.255 ppm) as internal standard at 21 C using a Varian VXR-500 Instrument.

Twenty mg of PL-B was hydrazinolized and the liberated oligosaccharide was N-acetylated and pyridylaminated. The PA-oligosaccharide thus obtained was eluted as a single peak at the elution position of M3FX on both RF-HPLC (Fig. 1-1) and SF-HPLC (Fig. 1-II-A), and its sugar composition was estimated to be Man₁₀₆, Fuc₁₀₈, Xyl₁₁₀₈, GlcNAc₁₀₂, and PA-GlcNAc₁₀₂, indicating that the N-linked oligosaccharide of PL-B is a complex type one.

The PA-oligosaccharide obtained in Fig. 1-1 (indicated by a bar) was digested successively with α-mannosidase (jack bean; Sigma Co.), β-xylosidase (Pomacea canaliculata), and β-fucosidase (Pomacea canaliculata) or β-mannosidase (Achatina fulica; Seikagaku Kogyo Co.) and the resulting PA-oligosaccharides were analyzed by SF-HPLC. As shown in Fig. 1-II, the peak of PA-oligosaccharide at the elution position of M3FX (A) moved successively to the elution positions of MFX (B), MF (C), M1 (D), or FGN2 (E), respectively, indicating that the PA-oligosaccharide from PL-B consists of two α-mannosyl residues, one β-xylosyl residue, one α-fucosyl residue, and one β-mannosyl residue, and chitobiose-PA. From these results, the structure of PA-oligosaccharide from PL-B was deduced to be Manz₁→6(Manz₁→3)(Xylβ₁→2)Manβ₁→4GlcNAcβ₁→4(Fucz₁→3)GlcNAc.

**Table**

<table>
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<tr>
<th>Reporter groups</th>
<th>Residue</th>
<th>PL-B</th>
<th>M3FX</th>
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<tr>
<td>H-1 of</td>
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<tr>
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<td>(8.9)'</td>
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<tr>
<td>Fuc</td>
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<td>5.060</td>
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</tr>
<tr>
<td>(4.0)'</td>
<td>(4.2)'</td>
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</tr>
<tr>
<td>H-2 of</td>
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</tr>
<tr>
<td>4'</td>
<td>3.996</td>
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</table>

1) Coding of sugar residues of M3FX

4 Manz₁→6 3 2 1 Manβ₁→4GlcNAcβ₁→4GlcnAc
Manz₁→3 2 3
4 Xylβ₁→2 Fucβ₁

2) Chemical shifts were measured by reference to internal acetone (δ = 2.255 ppm).

* Data for 21 C.

† Reference 4.

J₁₂ values are shown in the angled brackets.
To confirm this structure, the PA-oligosaccharide was analyzed by IS-MS MS. As shown in Fig. 2, all relevant signals could be assigned as fragment ions derived from the M3FX structure. The ion peak at $m/z$ 1268.0 was a parent ion for [M+H]$^+$, and agreed with the calculated molecular mass (1267.2) for M3FX. The locations of the fragment ions at $m/z$ 1121.5, 989.5, 827.5, 665.5, 502.5, and 300 were consistent with the successive loss of each monosaccharide from the parent molecule in the order of Fuc, Xyl, Man, Man, Man, and GlcNAc. The location of the fragment ions at $m/z$ 959 and 798.5 were consistent with the loss of one mannosyl residue and two mannosyl residues from the fragment ion at $m/z$ 1121.5. The fragment ion at $m/z$ 446.5 corresponding to Fuc-GlcNAc-PA indicated that a fucosyl residue is bound to the innermost GlcNAc residue. The fragment ions at $m/z$ 690.0 and 366.0 should be derived from the fragment ions at $m/z$ 989.5 and 665.5, respectively, by the cleavage of GlcNAc-GlcNAc linkage. A summary of the IS-MS MS analysis of PA-oligosaccharide from PL-B is shown in Fig. 2.

More detail structure of PA-oligosaccharide from PL-B was further analyzed by $^1$H-NMR in D$_2$O. As shown in the Table, all structural reporter signals agreed well with those of M3FX.$^4$ The chemical shifts of the Xyl H-1 doublet ($\delta = 4.452$ ppm) and the Fuc H-1 ($\delta = 5.063$ ppm), in combination with the coupling constant $J_{1,2}$ (8.0 Hz) and (4.0 Hz), are indicative of the $\beta$- and $\alpha$-configuration of the linkages to Man-3 and GlcNAc-1, respectively.

From these results, the structure of the N-linked oligosaccharide of PL-B was established to be

$$\text{Man}z\text{I} \rightarrow 6 \text{Xyl}$$

$$\text{Man}z\text{I} \rightarrow 3 \rightarrow 4 \text{GlcNAc}$$

$\rightarrow 4 \text{GlcNAc}$

To identify the oligosaccharide-linked asparagine residue, PL-B (8 mg) was digested with proteases and the resulting glycopeptides were isolated and sequenced. Reduced and S-pyridylethylated (RPE) PL-B, prepared by the method of Friedman et al.$^7$ was digested with 1.5% (w/w) lysylendopeptidase in 4 M urea-50 mm Tris-HCl buffer, pH 8.6, at 37 C for 3 h, and the resulting peptides were separated by gel filtration on a Biogel P-30 column (1.3 x 133 cm) in 30% acetic acid solution, yielding 5 peptides (L1-L5). The carbohydrate-containing peptide L1 was further digested with 1.5% (w/w) TPCK-trypsin in 0.1% ammonia solution at 37 C for 3 h, and the resulting peptides were separated by RP-HPLC with a YMC-Gel C4 column (4.6 x 150 mm) by a linear gradient of acetonitrile from 0 to 30% in 0.1% trifluoroacetic acid solution, yielding 5 peptides (L1-T1-L1-T5) (Fig. 3-I), of which L1-T2 and L1-T4 were obtained in the yields of 52.4% and 40.8%, respectively, and contained almost equal amounts of carbohydrate. Their amino acid compositions were Asp$_1$, Gly$_{2,5}$, Val$_{2,1}$, Phe$_{1,10}$, Arg$_{1,0}$, and Asp$_{9,0}$, Thr$_{2,2}$, Ser$_{1,0}$, Glu$_{5,5}$, Pro$_{1,0}$, Gly$_{1,0}$, Val$_{1,5}$, Phe$_{1,1}$, Leu$_{0,9}$, Tyr$_{0,8}$, Phe$_{0,9}$, Ser$_{1,0}$, Trp$_{2,0}$, Arg$_{1,0}$, respectively. Peptide L1-T4 was further digested with 1.0% (w/w) TLCK-chymotrypsin in 0.1% ammonia solution at 37 C for 2 h, and the resulting peptides were separated by RP-HPLC as described above, yielding 3 peptides (L1-T4-C1-L1-T4-C3) (Fig. 3-II), of which L1-T4-C2 contained carbohydrate and its amino acid composition was Asp$_{1,0}$, Gly$_{2,6}$, His$_{2,3}$, Arg$_{6,5}$, Val$_{1,9}$, Phe$_{1,0}$, Ser$_{1,0}$, Phe$_{1,0}$, Trp$_{1,1}$, Trp$_{2,1}$, Tyr$_{0,8}$, Phe$_{0,9}$, Ser$_{1,0}$, Arg$_{1,0}$, respectively. Amino acid sequences of L1-T2 and L1-T4-C2 were estimated to be Cys-Gly-Val-Asp-Phe-Gly-X-Arg and Cys-Gly-Val-Asp-Phe-Gly-X-Arg, respectively, by manual Edman degradation using the DABTC PITC double coupling method.$^9$ The neutral carbohydrate content of PL-B was 4.33% as mannose by the phenol-sulfuric acid method.$^{10}$ This value was close to the value, 4.23%, calculated from the two oligosaccharides having M3FX structure.

From these results, it was found that the two N-linked oligosaccharides in PL-B have an M3FX structure and link to the asparagine residues in different domains of the PL-B molecule.

References


