Structural Analysis of Free N-Glycans in Bamboo (Phyllostachys heterocyclo) Shoots

Yoshinobu Kimura and Tatsuya Ueyama
(Department of Bioresources Chemistry)

Free N-glycans, the high-mannose-type and the plant complex-type, have been found in bamboo shoots. These free N-glycans were coupled with 2-aminoypyridine and purified by gel filtration, C18 Sepharose affinity chromatography, reversed-phase HPLC, and size-fractionation HPLC. The structures of these pyridylaminated free N-glycans were identified by two-dimensional sugar chain mapping, exomannosidase digestions, and ion-spray tandem mass spectrometry. The structural analyses showed that the various free high-mannose type sugar chains having one GlcNAc (Man9, GlcNAc1) and free xylene/fucosyl containing sugar chains having the xylotriose segment occur in the developing bamboo shoots, suggesting that an endo-β-N-acetylglucosaminidase should produce the former structures, and a peptide: N-glycanase should produce the latter structures.

Key words: free N-glycan, plant N-glycan, endo-β-N-acetylglucosaminidase, peptid/N-glycanase, Phyllostachys heterocyclo

Introduction

Recently, the physiological functions, such as promoting activity for tomato fruit ripening, of the free N-glycans derived from plant glycoproteins have been deduced by Priem et al.4, Indeed, several free N-glycans have been found in pericarp tissues of ripening tomato fruit, in culture medium of suspension cultured-cells of white campion3, or hypocotyls of pea seedlings6. Although both high-mannose type and xylose-containing type free N-glycans were found in the pea seedlings4, the concentration of free high-mannose type N-glycans in the hypocotyls was approximately 20 times higher than in that of complex type. This observation led us to a hypothesis that free N-glycans, especially high-mannose type structures derived from storage glycoproteins in the seedlings, could play a critical role(s) in growth and/or differentiation of plant cells beside fruit ripening. If our working hypothesis is a reasonable concept, such free N-glycans should be widely distributed in growing or differentiating plant cells. Therefore, we started to survey the ubiquitous occurrence of free N-glycans in seedlings, shoots, and developing seeds. For this purpose, in this report, we selected

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Abbreviations

RP-HPLC, reversed-phase HPLC; SF-HPLC, size-fractionation HPLC; 2D sugar chain map, two dimensional sugar chain map; LS-MS, ion-spray mass spectrometry; MS/MS, tandem mass; PA, pyridylamine; Endo-β-GlcNAc-ase, endo-β-N-acetylglucosaminidase; PNGase, peptide: N-glycanase; QN2M3FX, GlcNAc1-2Man1-6GlcNAc1-2Man1-3 (Xyl1-2Man1-1-4GlcNAc1-1-4Fuc1-3GlcNAc-PA; M03FX, GlcNAc1-2Man1-6GlcNAc1-2Man1-3 (Xyl1-2Man1-1-4GlcNAc1-1-4Fuc1-3GlcNAc-PA; M03FX, GlcNAc1-2Man1-6GlcNAc1-2Man1-3 (Xyl1-2Man1-1-4GlcNAc1-1-4Fuc1-3GlcNAc-PA; M2FX, Man1-6Xyl1-2Man1-1-4GlcNAc1-1-4Fuc1-3GlcNAc-PA;
bamboo shoots, since the shoots have been well known to grow last, suggesting occurrence of the putative biologically active oligosaccharides.

Materials and Methods

Materials — Bamboo (Phyllostachys heterococca; Hachiko-takenoko) shoots produced in May of 1996 in Okayama Prefecture were used. A Cosmosil 5C18-AR column (0.6×25 cm) was purchased from Naolai Tesque, Inc., and an Asahipak NHPLP-50 column (0.46×25 cm) from Showa Denko Co. a Mannosidase (jack bean) was purchased from Sigma. Hydratine anhydrous was purchased from Pioce. Authentic PA-sugar chains were prepared as described in our previous paper.42.

Preparation of oligosaccharide fraction from bamboo shoots — Chopped bamboo shoots (75g) were homogenized in 25mM Tris-HCl, pH 8.8, containing 0.2M NaCl (11 liters). The homogenate was dialyzed against deionized water (5 liters) at 4°C for 10h and the resulting outer solution was concentrated to about 50ml by the rotary evaporator. After centrifugation, the supernatant (10ml each) was applied to Sephadex G-10 column (2.8×40 cm) in 0.1M NH4OH to remove the salts of other low molecular substances. The oligosaccharide fractions (elution volume: 40-180ml) were concentrated and applied to the Sephadex column again, and then the resulting salt-free oligosaccharide fraction was lyophilized (250 mg).

Pyridylation of free N-glycans — Pyridylation of free N-glycans was done by the method of Kondo et al.44. Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus equipped with a Jasco 820-FL Intelligent Spectrophotometer, using a Cosmosil 5C18-AR column (0.6×25 cm) or an Asahipak NHPLP-50 column (0.46×25 cm) as described in our previous paper.42.

Con A-Sepharose Chromatography — A run-through fraction (F-I in Fig. 1) on RP-HPLC of PA-derivatives prepared from free oligosaccharides was dissolved in 25mM Tris-HCl, pH 7.8, containing 0.1M NaCl (5 ml) and applied to Con A-Sepharose column (1.5×10 cm) equilibrated with the same buffer. After washing the column with above 5ml of the same buffer, the Con A-bound Con A(+) PA-sugar chains were eluted by addition of 0.1 M methyl-α-mannoside. PA-sugar chains in each fraction were monitored by a Fluorescence Spectrophotometer (Excitation 330 nm, Emission 380 nm, Hitachi 650 ILS). The Con A(+) fraction was concentrated and desalted by gel-filtration on Sephadex G-10 column (2.8×45 cm) in water.

- Mannosidase digestion — a-Mannosidase digestion and the HPLC analysis of the product were done as described in our previous report.42.

Ion-spray mass spectrometry — MS and MS/MS analyses of PA-oligosaccharides were performed as described in our previous report,42 using a Perkin Elmer Sciex API III, triple-quadrupoles mass spectrometer with an atmospheric-pressure ionization ion source.

Results and Discussion

Parification of pyridylaminated (PA- ) free N-glycans. PA-derivatives of oligosaccharide fraction prepared from the extract of bamboo shoots were first separated by RP-HPLC. Since we found in a previous report that the high-mannose type N-glycans with only one GlcNAc residue at reducing-end side run through the ODS column, the run-through fraction (Fig. 1, F-I) was subject to a Con A affinity chromatography to further purify the high-mannose type sugar chains (data not shown). The Con A-bound Con A(+) PA-oligosaccharides eluted by 0.1 M methyl-α-mannoside were desalted through Sephadex G-10 column in water and then used in the following structural analysis.

Structural analysis of high-mannose type free N-glycans — The Con A(+) fraction was further purified and analyzed by SF-HPLC as shown in
Fig. 2-1. The elution positions of four main PA-sugar chains did not correspond to any authentic high-mannose type sugar chains (Man$_{4}$GlcNAc$_{2}$-PA), however, $α$-mannosidase digestion of this PA-oligosaccharide mixture caused a new peak at the elution position of Man$\cdot$GlcNAc$\cdot$PA (Fig. 2-1). These results clearly suggested these PA-oligosaccharides were typical high-mannose type but slightly different in the reducing-end structure from authentic high-mannose type structures. As shown in Fig. 3, IS-MS analysis of these PA-oligosaccharide fractions showed the fraction contained at least four PA-oligosaccharides (Peak A, B, C, and D). [M+H]$^+$ = m/z 1110.4 for Peak A (Man$_{4}$GlcNAc$\cdot$PA), [M+H]$^+$ = m/z 1272.8 for Peak B (Man$_{4}$GlcNAc$_{2}$-PA), [M+H]$^+$ = m/z 1434.8 for Peak C (Man$_{4}$GlcNAc$_{3}$-PA), [M+H]$^+$ = m/z 1596.8 for Peak D (Man$_{4}$GlcNAc$_{4}$-PA). These deduced structures were further confirmed by MS/MS analysis of each PA-oligosaccharide as shown in Table 1. These four PA-oligosaccharides gave two characteristic fragments at m/z 3000 (GlcNAc-PA) and m/z 462.5 (Man$_{4}$GlcNAc$_{2}$-PA) by the fragmentation of each parent ion. These results also indicated that these four N-glycans are lacking is the innermost GlcNAc residue involved in the

![Figure 1](image1.png)  
**Fig. 1** RP-HPLC of PA-derivatives of oligosaccharide fraction prepared from the extract of bamboo shoots.

![Figure 2](image2.png)  
**Fig. 2** SF-HPLC of ConA (+) fraction obtained from F-1 in Fig. 1. I. ConA (+) fraction from F-1 in Fig. 2. II. $α$-mannosidase digest of LMB-M1, Man$_{4}$GlcNAc$_{4}$-PA.

![Figure 3](image3.png)  
**Fig. 3** IS-MS spectrum of ConA (+) fraction obtained from F-1 in Fig. 1.
linkage to the specific asparaginyl residue in glycopeptides. Since Yamaguchi et al. have already reported that an endo β-N-acetylgalactosaminidase (endo P) occurs in bamboo shoots and this endoglycosidase degrades Man₉₋₈ GlcNAc blocks from several glycopeptides, the high-mannose type free N-glycans obtained in this study would be derived by endo P in the bamboo shoots. The amount of these high-mannose type free N-glycans in bamboo shoots was 2.5 nmol/gram fresh weight and this value was comparable with that of high-mannose type free N-glycans occurring in pea hypocotyl (1.5 nmol/gram fresh weight).

Structural analysis of plant complex type free N-glycans —F-I—F—VI obtained in Fig. 1 were applied to SF-HPLC for further purification and IS-MS analysis of pyridylaminated free N-glycans. From F-II and F-V, several peaks were separated by SF-HPLC; however, MS/MS analysis of all PA-derivatives did not show any signals at m/z 503 (GlcNAc-PAn, m/z 503 (GlcNAc-PAn), or m/z 565 (Man,GlcNAc-PAn), suggesting these two fractions did not contain any N-glycans. On the other hand, several PA-derivatives observed in F-III, IV, and VI by SF-HPLC were confirmed to be N-glycans, since these PA-derivatives produced some characteristic fragments for N-glycans by MS/MS analysis as described below.

The elution position of a pyridylaminated N-glycans as described below. From F-III, designated Peak F, on SF-HPLC corresponded to that for GlcNAc-Man,Fuc,XYl,GlcNAc-PAn (GN1M3FX). The molecular mass of Peak F (m/z 1470.5) also corresponded to that of GN1M3FX. As shown in Fig. 4-L, the relevant signals observed by IS-MS/MS analysis of Peak F reasonably assigned as fragment ions derived from the GN1M3FX, m/z 1245.5 (GlcNAc-Man,XYl,GlcNAc-PAn), m/z 1397.5 (GlcNAc-Man,Fuc,XYl,GlcNAc-PAn), m/z 1392.5 (GlcNAc-Man,GlcNAc-PAn), m/z 1182.0 (GlcNAc-Man,XYl,GlcNAc-PAn), m/z 1063.5 (GlcNAc-Man,GlcNAc-PAn), and m/z 959.5 (Man,XYl,GlcNAc-PAn). The molecular ions of m/z 466.0 (Fuc,GlcNAc-PAn), m/z 369.5 (Fuc,GlcNAc-PAn) m/z 297.0 (Man,XYl,GlcNAc-PAn), m/z 665.5 (Man,GlcNAc-PAn, m/z 619.5 (Fuc,GlcNAc-PAn) m/z 503.5 (GlcNAc-GlcNAc-PAn), m/z 446.5 (Fuc,GlcNAc-PAn), m/z 300 (GlcNAc-PAn). For the structure, two isomeric forms could occur; one is GlcNAcβ1-2Manα1-6Galα1-3 (XYlβ1-2Manα1-6GlcNAcβ1-4(Fucα1-3) GlcNAc-PAn (F3M3FX), the other is Manβ1-6GlcNAcβ1-2Manα1-3(Xylβ1-2Manα1-6GlcNAcβ1-4(Fucα1-3)GlcNAc-PAn (F3M5FX). It has been reported that on the OSM column the former structure is eluted before GN2M3FX and the latter structure is eluted after GN2M3FX. Considering the elution position of Peak F, which was eluted before authentic GN2M3FX on the OSM column, the structure of Peak F should be Manα1-6GlcNAcβ1-2Manα1-3(Xylβ1-2Manα1-6GlcNAcβ1-4(Fucα1-3)GlcNAc-PAn.

The elution position of a pyridylaminated N-glycans from F-IV, designated as Peak F on the 2D sugar chain map corresponded to that for M3FX, and the molecular mass [M+H]+ was 1267.5, which agreed with the calculated molecular mass of 1267 for M3FX. Furthermore, as shown in Fig. 4-H, the relevant signals observed by MS/MS analysis of Peak F could be reasonably assigned as fragment ions derived from the

<table>
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<th>Fragment ion</th>
<th>m/z (g)</th>
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<th>B</th>
<th>C</th>
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<tr>
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<td>Man,GlcNAc-PAn</td>
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<td>Man,GlcNAc-PAn</td>
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<tr>
<td>Man,GlcNAc-PAn</td>
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<tr>
<td>Man,GlcNAc-PAn</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Man,GlcNAc-PAn</td>
<td>665.5</td>
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<tr>
<td>Man,GlcNAc-PAn</td>
<td>665.5</td>
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<td>Man,GlcNAc-PAn</td>
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The amount of these plant complex type free N-glycans was approximately 31 pmol/gram fresh weight. The structures of the reducing-end side of these complex type free N-glycans indicate that these oligosaccharides should be derived from glycopeptides or glycoproteins by PNGase instead of endo-β-GlcNAc-ase, however, PNGase has not been purified from bamboo shoots yet.

In this report, we have revealed that free N-glycans also occur in developing bamboo shoots and the amount of high-mannose type structure (Man₉, GlcNAc₃-P) overwhlems that of complex type structure. Such high mannose type free N-glycans may be derived by the endo-β-GlcNAc-ase (endo-P); however, the endogenous substrate(s) for the endoglycosidase or the origin of such free N-glycans in bamboo shoots are obscure at this moment. For the next step to understand the physiological function(s) of free N-glycans in plant cells, the construction of a transgenic plant in which the expressions of endo-β-GlcNAc-ase or PNGase are controlled seems to be necessary. For this purpose, the purification and characterization of the N-glycan releasing enzymes (endo-β-GlcNAc-ase and PNGase) from several plant materials are in progress.
タケノコ (ハチク) 中に存在する遊離 N-グリシンの構造解析

木村 吉伸・上山 達也

(生物資源研究所)

植物細胞の分化・成育における遊離 N-グリシンの植物ホルモン様作用が報告されている。そこで、分化・成育中の細胞における遊離 N-グリシンの関与性を立証する研究の一環として、成長速度の早い早生稲で知られるタケノコから遊離 N-グリシンを精製し、それらの構造解析を行った。タケノコ抽出液の透析処理から、調製したオリゴ糖液を2-アミノピリアルでエラクム試験後の遊離 N-グリシンを確認し、HPLCおよびNMRで構造解析を行った。得られた遊離 N-グリシンの構造解析は α-マンノシダーゼ消化、含糖2次元NMR、ESI-MSおよびNMR分析を組み合わせることにより行った。その結果、タケノコ抽出液にはエンド-β-N-アセチルグルコサミノーゼにより誘導されたと考えられるハイマノース型糖鎖（Mannoside）が検出された。