Short Communication

Occurrence of Free N-Glycans in Pea (Pisum sativum. L) Seedlings†

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Free N-glycans have been found in pea seeds. These free N-glycans were coupled with 2-aminopyridine and purified by gel filtration, Con A-Sepharose affinity chromatography, and size fractionation HPLC. These structures of pyridylaminated free N-glycans were analyzed by exomannosidase digestions and ion-spray tandem mass spectrometry. The structural analyses showed that the several oligomannose-type sugar chains having one GlcNAc residue at the reducing-end side occur in the seedlings, suggesting the endo-β-N-acetylgalactosaminidase PS [Y. Kimura et al., Biosci. Biotech. Biochem., 60, 228-232 (1996)] should be involved in the release of oligomannose-type N-glycans from the storage glycoproteins [Y. Kimura et al., Biosci. Biotech. Biochem., 60, 1841-1850 (1996)] during the germination of pea seeds.

Key words: free N-glycans; pea seedling; endo-β-N-acetylgalactosaminidase; peptide: N-glycanase; ion-spray mass spectrometry

Recently, the physiological functions of the free N-glycans derived from plant glycoproteins were shown by Gross et al.[1,2] And Priem et al. also showed the presence of oligomannose type and xylose-containing type N-glycans in free forms in the tomato pericarp.3 These facts suggested that the N-glycans themselves may have some physiological functions in the plant cells. To release N-glycans from the plant glycoproteins, endo-β-N-acetylgalactosaminidase (endo-β-GlcNAc-ase) or peptide: N-glycanase (PNGase) should be required. Therefore, for identification of the physiological functions of N-glycans and N-glycan releasing enzymes in plant cells, we started to purify and characterize the plant-origin endoglycosidases involved in the release of N-glycans.4,5 The endo-β-GlcNAc-ases from pea seeds (endo-PS) and Ginkgo biloba seeds (endo-GB) were shown to effectively hydrolyze the chitobiose linkage of several oligomannose-type N-glycans having α-1,2-mannosyl residue(s), such as Man₅₋₆GlcNAc2. And more recently we found that several kinds of oligomannose-type N-glycans (Man₅₋₆GlcNAc2) which could be released by endo-PS occur in the storage glycoproteins of mature pea seeds.5 However, it has been uncertain whether free N-glycans released by the endo-β-GlcNAc-ase do occur in the pea seedlings. In this paper, we report the structural analyses of free N-glycans in the pea seedlings.

The seeds of Pisum sativum L. cv. Midoriusui (Takayama Seed Co., Kyoto, Japan) were soaked in running tap water overnight and sown on vermiculite in plastic container. Seedlings were grown at 25°C for 10 days in the darkness. The stems (hypocotyls and epicotyls, 166 gram wet weight) obtained from the etiolated pea seedlings were homogenized in 500 ml of 50 mm Tris·HCl, pH 8.5. The pH of the homogenates were adjusted to near 8.5 by addition of 0.1 N NaOH. It has already been confirmed that endo-β-GlcNAc-ase in pea seeds (endo-PS) is inactive at this pH.4,6 The resulting homogenates were dialyzed against deionized water (5 liters) and the outer solution was concentrated to be approximately 40 ml by the rotary evaporator. After centrifugation, the supernatant (10 ml each) was put on Sephadex G-10 column (2.8 × 45 cm) in deionized water to remove the salts and other low molecular substances. After hydrolysis of the oligosaccharide fraction, free N-glycans were pyridylaminated by the method of Kondo et al.[7] After gel filtration (Sephadex G-25 superfine (1.8 × 180 cm)) to remove the excess reagents, the PA-sugar chains were separated by RP-HPLC on a Cosmosil 5C18-AR column (6 × 250 mm).8 As shown in Fig. 1A, almost all the PA-derivatives were recovered in the run-through fraction (P-I) and one PA-sugar chain (P-II) was eluted at the elution position of Man3Fuc1XyllGlcNAc2-PA (M3FX). The elution positions of P-II on both the Cosmosil 5C18-AR column and the Asahipak NH2-P-30 column were the same as those of the authentic M3FX. A single charged ion [M+H]+ of P-II was observed at m/z 1268.0 on ion-spray mass spectrometry (Perkin Elmer Sciex API-III),6 which agreed with the expected mass (1267) for M3FX (Fig. 2A). The sugar component analysis of P-II showed that this PA-sugar chain consists of mannose (3.0), xylose (0.9), fucose (0.8), GlcNAc (1.0), and PA-GlcNAc (0.7). And the relevant signals observed by IS-MS/MS analysis9 of P-II could be reasonably assigned as fragment ions derived from the M3FX (m/z 1122.0 (Man3XyllGlcNAc2-PA), m/z 990 (Man3GlcNAc2-PA)), m/z 960.0 (Man2XyllGlcNAc2-PA), m/z 828.0 (Man2GlcNAc2-PA), m/z 665.0 (ManGlcNAc2-PA), m/z 503.0 (GlcNAc-GlcNAc-PA), m/z 446.0 (FucGlcNAc-PA), m/z 300 (GlcNAc-PA) (Fig. 2C). These results showed that the fucose- and xylose-containing type free N-glycan having two GlcNAc residues at the reducing-end side occurs in the pea seedlings. The concentration was approximately 80 μmol/g fresh weight. Since Plummer et al.9 reported that a peptide: N-glycanase (PNGase) that can release the complex-type N-glycans from polypeptide chains exists in pea seeds, the free M3FX should be released by the PNGase rather than endo-PS.

Since it has been reported that the pyridylaminated N-glycans having only one GlcNAc residue at the reducing-end side run through on RP-HPLC,9 Con-A affinity chro-
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Fig. 1. HPLC Profiles of PA-Derivatives Prepared from Pea Seedlings.
A: RP-HPLC of PA-derivatives on Cosmosil 5C18-AR (6.0 x 250 mm) column. The PA-derivatives were eluted by increasing 1-butanol content in 0.5% 1-butanol/0.1 M ammonium acetate buffer, pH 4.0, as described in our previous report. M3FX indicates the elution position of authentic Man1→6(Man1→3)(Xyl1→2)Man1→4GlcNAcβ1→R(Fucα1→3)GlcNAc-PA.
B: SF-HPLC profile of Con-A bound PA-sugar chains in P-I fraction on a Asahipak NH2P-50 column. The PA-derivatives were eluted by increasing the water content in the water-acetonitrile mixture as described in our previous report.

Fig. 2. IS-MS Analyses of Free N-Glycans in Pea Seedlings.
The samples were dissolved in 50% acetonitrile/water (containing 0.05% formic acid) and infused through the ion-spray interface at a flow rate of 5.0 μl/min. The scan was done with a step size of 0.5 Da.
A, IS-MS spectrum of P-II in Fig. 1A; B, IS-MS spectrum of Con-A bound PA-sugar chains in P-I fraction; C, MS/MS spectrum of P-II; D, MS/MS spectrum of Man6GlcNAc-Pa (m/z 1273.0 in B).
matography was used to partially purify some oligomannose-type N-glycans having only one GlcNAc residue. The run-through fraction (P-I) on RP-HPLC was put onto a Con-A Sepharose column (1.5 × 20 cm) equilibrated with 25 mm Tris–HCl, pH 8.0, containing 5 mm CaCl₂ and 0.1 M NaCl. The Con-A bound PA-sugar chains were eluted by 0.1 M methyl-α-mannoside. After desalting by Sephadex G-10 column in deionized water, the PA-sugar chains were separated and analyzed by size-fractionation HPLC on an Asahipak NH₂-P column (4.6 × 250 mm, Showa Denko Co.).\(^6\)

As shown in Fig. 1B-I, the elution positions of the PA-sugar chains obtained by Con-A affinity chromatography did not correspond to any authentic oligomannose-type PA-sugar chains having two GlcNAc residues at the reducing-end side. However all these PA-sugar chains were converted to Manβ1-4GlcNAc-PA by α-mannosidase (jack bean, Sigma) digestion (Fig. 1B-II), indicating that all these PA-sugar chains were typical oligomannose-type N-glycans. As shown in Fig. 2B, several signals were detected at \(m/z\) 1111.0, 1273.0, 1435.0, and 1597.5 on the ion-spray mass spectrum. These observed molecular masses agreed with Man5GlcNAc-PA (MW = 1110.0), Man6GlcNAc-PA (MW = 1272.2), Man7GlcNAc-PA (MW = 1434.3), and Man8GlcNAc-PA (MW = 1596.5), respectively. The MS/MS analyses of these signals also indicated that these PA-sugar chains consist of several numbers (5 to 8) of hexose (mannose) and one pyridylaminated N-acetylgalactosamine (PA-GlcNAc). From the parent signal at \(m/z\) 1111.0, the fragment ions at \(m/z\) 948.5 (Man4GlcNAc-PA), \(m/z\) 786.5 (Man3GlcNAc-PA), \(m/z\) 624.5 (Man2GlcNAc-PA), \(m/z\) 462.0 (Man1GlcNAc-PA), and \(m/z\) 300.0 (GlcNAc-PA) were derived. From the parent signal at \(m/z\) 1273.0, the fragment ions at \(m/z\) 1110.5, \(m/z\) 948.5, \(m/z\) 786.5, \(m/z\) 624.5, \(m/z\) 462.0, and \(m/z\) 300.0 were derived. From the parent signal at \(m/z\) 1435.0, the fragment ions at \(m/z\) 1272.5, \(m/z\) 1110.5, \(m/z\) 948.5, \(m/z\) 786.5, \(m/z\) 624.5, \(m/z\) 462.0, and \(m/z\) 300.0 were derived. From the parent signal at \(m/z\) 1597.5, the fragment ions at \(m/z\) 1435.0, \(m/z\) 1272.5, \(m/z\) 1110.5, \(m/z\) 948.5, \(m/z\) 786.5, \(m/z\) 624.5, \(m/z\) 462.0, and \(m/z\) 300.0 were derived. Among these MS/MS analyses of the oligomannose-type free N-glycans, a spectrum obtained from the parent ion at \(m/z\) 1273.0 is shown in Fig. 2D as a typical example. These results clearly showed that the oligomannose-type N-glycans of the storage glycoproteins\(^6\) in pea seeds are released by endo-PS\(^4\) and the resulting free N-glycans do occur in the developing epicotyls and hypocotyls of the seedlings. The total amount of oligomannose-type free N-glycans in epicotyls and hypocotyls of pea seedlings was estimated to be approximately 1.5 nmol/g fresh weight. This concentration of free N-glycan in epicotyls and hypocotyls of pea seedlings is slightly lower than that of free N-glycans in the mature tomato pericarp tissue (5-6 µg/g fresh weight, which is roughly equivalent to 3-4 nmol/g).\(^3\)

Our findings also showed that oligomannose-type N-glycans should be unconjugated by endo-β-N-acetylgalactosaminidase (endo-PS) but not PNGase, which can release complex-type, hybrid-type, and oligomannose-type N-glycans. PNGase would serve as a N-glycan releasing enzyme that especially releases the fucose and/or xylose containing complex-type N-glycan in the plant cells.

References

5. Y. Kimura and S. Takagi, Abstracts of Papers, the Annual Meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry, Kyoto, April, 1996, p. 165.