

PNGase activity and free N-glycans in phloem fluid prepared from *Nerium oleander* (oleander tree)

Fuki Otaguro¹, Yoshinobu Kimura^{1,2,*}, and Megumi Maeda^{1,3,*}

¹Graduate School of Environmental, Life, Natural Science and Technology, Okayama University, Okayama, Japan

²Faculty of Food Culture, Kurashiki Sakuyo University, Tamashima, Kurashiki, Japan

³Faculty of Environmental, Life, Natural Science and Technology, Okayama University, Okayama, Japan.

*Correspondence: Megumi Maeda, mmaeda@okayama-u.ac.jp; Yoshinobu Kimura, yosh8mar@okayama-u.ac.jp

Abstract

Free N-glycans (FNGs) occur ubiquitously in growing plants. Recently, it was reported that these FNGs interact with auxin. In this study, we investigated whether PNGase activity responsible for producing the FNGs occurs in the extracellular fluid, where auxin is present during its polar transfer. Here, we report the occurrences of PNGase activity and FNGs in the phloem fluid.

Keywords: free N-glycans, phloem fluid, *Nerium oleander*, PNGase

Abbreviations: DEAE: diethylaminoethyl; ESI-MS: electrospray ionization mass spectrometry; FNG: free N-glycan; Gal: D-galactose; GlcNAc: N-acetyl-D-glucosamine; GN1M3FX: GlcNAc1Man3Xyl1Fuc1GlcNAc2; GN2M3FX: GlcNAc2Man3Xyl1Fuc1GlcNAc2; HPLC: high-performance liquid chromatography; M3FX: Man3Xyl1Fuc1GlcNAc2; Man: D-mannose; NeuNAc: N-acetyl-D-neuraminic acid; NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₁: NeuNAc₂-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuNAc₂-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc; NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₂: NeuNAc₂-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuNAc₂-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAc; PA: pyridylamino; PNGase: peptide: N-glycanase; SF-HPLC: size-fractionation HPLC; Xyl: D-xylose

Free N-glycans (FNGs), which are ubiquitous in plants, have been postulated to be involved in plant differentiation, growth, and fruit maturation, but their physiological functions have not yet been clarified. Recently, it has been reported that these FNGs interact with the plant hormone auxin (indole-3-acetic acid, IAA) *in vitro* (Iguchi *et al.* 2025), suggesting that these FNGs may interact with IAA in the extracellular fluid or in the cytosol of plants and affect phytohormone activity. These FNGs have been found in the extracellular fluids such as xylem sap or cell culture broth (Maeda *et al.* 2010; Tsujimori *et al.* 2019), and the plant specific peptides: N-glycanases (PNGases) (Takahashi and Nishibe 1978; Kimura and Ohno 1998; Chang *et al.* 2000; Hossain MA *et al.* 2010) has been assumed to be involved in the production of these FNGs. On the other hand, these PNGases are believed to function in the vacuole and the existence of the PNGase activity in the extracellular fluid, especially in phloem fluid that contains various hydrolytic enzymes and bioactive compounds, remains unknown. In this study, therefore, we prepared the phloem fluid and examined the occurrences of the PNGase activity and FNGs in the extracellular fluid.

Since phloem fluid has been reported to be efficiently collected from young branches of oleander, *Nerium oleander*, in this report, the phloem fluid was collected according to the following method described in a previous report (Abdolzadeh *et al.* 2008). The young oleander branches (growing in a field at the Faculty of Agriculture, Okayama University) were covered with parafilm, the covered area was wounded with a razor and the exudate was collected as phloem fluid. FNGs in the phloem fluid were partially purified by the method described in a previous paper (Tsujimori

et al. 2019). Briefly, the fluid (1.6 mL) was mixed with the same volume of 0.1 M NH₄OH to denature glycosidases. FNGs in the phloem fluid were partially purified by a combination of gel-filtration using Sephadex G-25 superfine column (0.1 M NH₄OH as a solvent, 2.7 × 34 cm) and ion-exchange method using both Dowex 50 × 2 and Dowex 1 × 2 resins. The run-through fraction obtained by the ion-exchange step was desalted by gel-filtration using the same column. The oligosaccharides were detected by the phenol-sulfuric acid method (Dubois *et al.* 1951), and the oligosaccharide-fraction obtained (elution volume, 70–120 mL) was concentrated and then lyophilized. The oligosaccharides containing FNGs were pyridylaminated by the method previously described (Natsuka and Hase 1998). After gel filtration to remove excess 2-aminopyridine (PA), PA sugar chains were partially purified by RP-HPLC using a Cosmosil 5C18 AR column (0.6 × 25 cm, Nacal Tesque, Kyoto), and structures of FNGs were analyzed by SF-HPLC using Shodex Asahipak NH2P-50 4E columns (0.46 × 25 cm, Showa Denko, Tokyo) as described in our previous paper (Tsujimori *et al.* 2019). As shown in Figure 1, 3 peaks (peak-a, -b, -c) were observed, and the elution position of peak-a, -b, and -c coincided with those of M3FX, GN1M3FX, and GN2M3FX. Furthermore, peak-b and -c were converted to peak-a by a digestion with partially purified *Aspergillus saitoi* β-GlcNAc'ase (Amano and Kobata 1986), suggesting that peak-b, and -c are GN1M3FX, and GN2M3FX, respectively. The total amount of plant complex-type FNGs (M3FX, 16%; GN1M3FX, 43%; GN2M3FX, 41%) in the phloem fluid was approximately 100 pmol/mL as a pyridylaminated glycan, but this is not an accurate quantitative value due to the small amount of fluid obtained in this study. Furthermore, consid-

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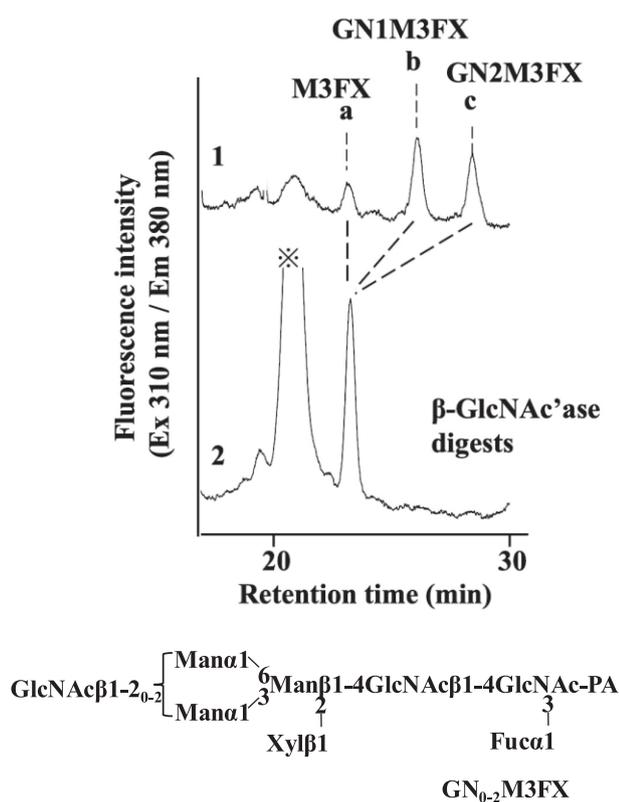


Figure 1. HPLC profiles of PA sugar chains prepared from oleander phloem fluid. 1, SF-HPLC profiles of PA-sugar chain obtained in RP-HPLC; 2, β -GlcNAc'ase digest of 1.

ering the reaction efficiency of fluorescent labeling, the amount of these FNGs present would be much higher. The total amount of plant complex-type FNGs in tomato xylem sap reported previously was approximately 375 pmol/mL (Tsuji-mori et al. 2019), and the amount of FNGs detected in the phloem fluid in this study was less than half of that. There were some differences in the structural features of FNGs detected between the phloem fluid and tomato xylem sap, which could be attributed to differences in the exoglycosidase activity expressed in each. However, the predominant occurrence of the plant complex type FNGs in these two extracellular fluids is common.

Since the finding of plant complex-type FNGs in the phloem fluid suggested the occurrence of PNGase activity in the extracellular fluid, we investigated the activity in the extracellular fluid. The PNGase activity was assayed according to our previous report (Yamamoto et al. 2021). Since the crude enzyme was used in this assay system, sialylated N-glycopeptides were used as substrates for the determination of PNGase activity. The sialylated N-glycopeptides are not present in plants, so it is possible to distinguish between the endogenous FNGs in the crude extracellular fluid or PNGase-products (GN2-FNGs). Since plant acidic PNGases have been reported to be active against a variety of N-glycopeptides (with high mannose, plant complex, and sialylated

animal complex type glycans), the usage of the glycopeptide carrying animal-specific N-glycans should be a good substrate for analyzing the plant PNGase activities in plant (Kimura and Ohno 1998; Hossain MA et al. 2010).

Briefly, the oleander phloem fluid was dialyzed against 1 L of distilled water twice for overnight at 4 °C and the dialysate was used as a crude enzyme. A mixture of the substrate egg yolk glycopeptide carrying NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₂ (approximately 25 μ g) and the internal standard PA-sugar chain (NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₁-PA; approximately 10 nmol) was treated with the crude enzyme (100 μ g protein) in 300 μ L of various buffer (0.1 M acetate buffer for pH 4.0 and 5.0, 0.1 M MES buffer for pH 6.0, 0.1 M HEPES buffer for pH 7.0 and pH 8.0) for 1 h at 37 °C. The reaction mixture was boiled for 5 min and centrifuged at 20600 \times g for 10 min at 4 °C. The resulting supernatant was freeze-dried and pyridylaminated (Natsuka and Hase 1998). After gel-filtration, the resulting PA sugar chains were subjected to DEAE-HPLC to obtain sialylated PA-FNGs (NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₂-PA and NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₁-PA), and the sialylated PA-FNGs were analyzed by RP-HPLC using Cosmosil 5C18-AR-II columns (0.6 \times 25 cm). As shown in Figure 2, the PNGase products (NeuNAc₂Gal₂GlcNAc₂Man₃GlcNAc₂-PA) were observed and the structure of the PNGase products was confirmed by ESI-MS analysis (Figure SI and SII). Furthermore, when the optimum pH of the detected PNGase activity was examined, the PNGase found in the oleander phloem fluid showed strong activity in the pH range 4-7, and the pH of the phloem fluid is 6, suggesting that this PNGase may be involved in FNGs production in the phloem fluid. Although PNGase activity appears to be constant at pH 4-7, the amount of crude enzyme used in this reaction (100 μ g) was very small relative to the amount of substrate, so this result is not likely due to an excess amount of enzyme. The optimum activities of plant PNGases reported so far are at pH 5 for PNGase from almond seeds and soybean seeds, pH 4-5 for PNGase from rice seeds (Takahashi and Nishibe 1978; Kimura and Ohno 1998; Chang et al. 2000), and pH 4.5 for tomato PNGase (Hossain et al. 2010), with reduced activity in the neutral pH range. These PNGases are sometimes classified to be acidic PNGases residing in the vacuoles or protein bodies, but the phloem fluid PNGase found in this study has relatively high activity even at the neutral pH, suggesting that its molecular structure may be different from these acidic PNGases found so far. Since the amino acid sequence information of this phloem fluid PNGase was not available in the present study, the molecular correlation with acidic PNGases residing in the vacuoles or protein bodies is unknown.

On the other hand, there is another PNGase that is involved in the quality control system of nascent proteins in the cytosol (Masahara-Negishi et al. 2012; Shiai et al. 2021), but it has been reported that the cytosolic PNGase is only active under reducing environment such as in the cytosol. Since cytosolic PNGase is not detected in oxidative environments such as extracellular fluid, it is likely that the phloem fluid PNGase activity detected in this study should be an extracellular PNGase activity, but not the cytosolic PNGase leaking from the cytosol.

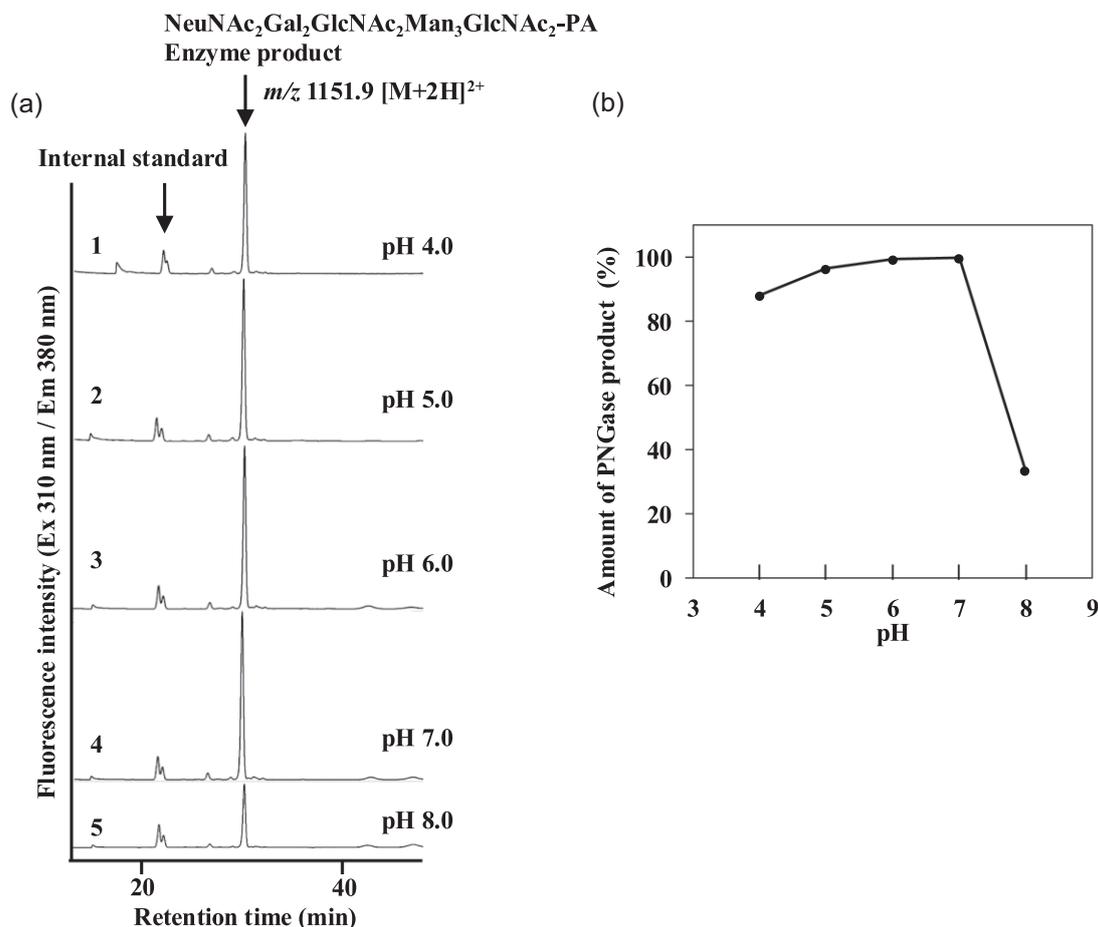


Figure 2. The optimum activity of the PNGase in the crude enzyme prepared from oleander phloem fluid. (a) RP-HPLC profiles of FNGs produced by the PNGase. The substrate glycopeptide was treated with the crude enzyme in the following buffers: 1, 0.1 M sodium acetate buffer, pH 4.0; 2, 0.1 M sodium acetate buffer, pH 5.0; 3, 0.1 M MES buffer, pH 6.0; 4, 0.1 M HEPES buffer, pH 7.0; 5, 0.1 M HEPES buffer, pH 8.0. (b) The amount of PNGase product. The presented data is an average of two independent experiments.

Supplementary material

Supplementary material is available at [Bioscience, Biotechnology, and Biochemistry](#) online.

Data availability

The data relevant to this article is available in *Biosci Biotechnol Biochem.* 2019;83:1310-1314, doi: 10.1080/09168451.2019.1608803., and in *Anal Biochem.* 2021;634:114367, doi: 10.1016/j.ab.2021.114367. Epub 2021 Sep 10.

Author contribution

M.M. shared responsibility for writing the manuscript with F.O and Y. K.; all the authors were responsible for the concept and design of the study; F.O. and M.M. performed all experiments; All authors contributed to critical revision of the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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