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LC/MS-based Detection of Hydroxyproline *O*-galactosyltransferase Activity

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[Abstract] Arabinogalactan proteins (AGPs) are plant-specific extracellular glycoproteins regulating a variety of processes during growth and development. AGP biosynthesis involves *O*-galactosylation of hydroxyproline (Hyp) residues followed by a stepwise elongation of the complex sugar chains. The initial Hyp *O*-galactosylation is mediated by Hyp *O*-galactosyltransferase (HPGT) that catalyzes the transfer of a D-galactopyranosyl residue to the hydroxyl group of Hyp residues of peptides from the sugar donor UDP-α-D-galactose (Figure 1). Here we describe a LC/MS-based method for the detection of HPGT activity *in vitro*.

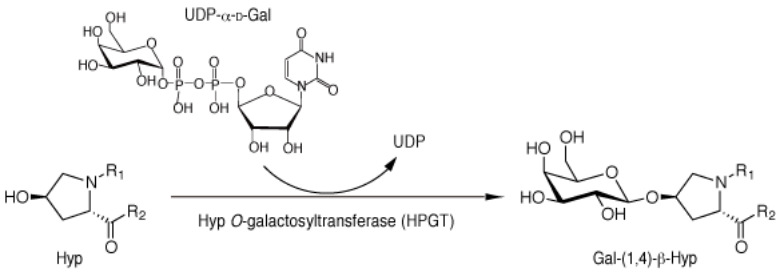

Figure 1. Reaction scheme for Hyp galactosylation by HPGT. HPGT catalyzes the addition of a D-galactopyranose from an UDP-α-D-Gal to the hydroxylgroup of Hyp residues.

Materials and Reagents

1. 1-week-old *Arabidopsis* T-87 cells (50 g fresh weight)
2. Bio-Rad Protein Assay (Bio-Rad Laboratories, catalog number: 5000006JA)
3. 2 mM synthesized substrate peptide [*e.g.*, $(OAOSOT)_3S$] [using standard Fmoc solid-phase synthesis chemistry on a 431A peptide synthesizer (Life Technologies)]
4. 2 mM Uridine 5'-diphosphogalactose disodium salt (Sigma-Aldrich, catalog number: U4500)
5. 1 M MOPS-KOH (pH 7.0)
6. 10 mM $MnCl_2$
7. 10% TX-100
8. 1% Formic acid
9. Acetonitrile (HPLC grade) containing 0.1% formic acid
10. Water (HPLC grade) containing 0.1% formic acid

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11. Tris-HCl (pH 7.0)
12. $MgCl_2$
13. Dithiothreitol
14. Leupeptin
15. Phenylmethanesulfonyl fluoride
16. Sucrose
17. Extraction buffer (see Recipes)
18. Suspension buffer (see Recipes)

Equipment

1. Waring blender
2. Miracloth (Merck Millipore Corporation, catalog number: 475855)
3. Ultracentrifuge
4. 30 °C incubator
5. Micro centrifuge
6. Semi-micro HPLC system (JASCO International Co., model: Micro21LC)
7. LCQ Deca XP-plus ESI ion-trap mass spectrometer (Thermo Fisher Scientific)
8. TSK-gel Amide-80 (3 μm) column (2 x 150 mm) (Tosoh Bioscience LLC, catalog number: 21865)

Procedure

A. Preparation of *Arabidopsis* microsomal membranes
   1. *Arabidopsis* T-87 cells are maintained on a 1-week culture interval under continuous darkness at 22 °C with shaking at 120 rpm.
   2. Suspend 1-week-old *Arabidopsis* T-87 cell (50 g fresh weight) in 20 ml Extraction buffer.
   3. Cool off to 4 °C on ice.
   4. Homogenize at 20,000 rpm for 5 min at 4 °C in a Waring blender.
   5. Cool off to 4 °C on ice.
   6. Filter the slurry through two layers of Miracloth.
   7. Centrifuge the filtrate at 3,000 *x g* for 15 min at 4 °C.
   8. Centrifuge the supernatant at 100,000 *x g* for 30 min at 4 °C.
   9. Suspend the pellet (microsomal membranes: approximately 150 μg/μl) in 500 μl Suspension buffer by gentle pipetting.
   10. Determine the protein concentration by conventional Bradford assay according to the manufacturer's protocol (Bio-Rad Protein Assay).

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B. Hyp *O*-galactosyltransferase activity assay

1. Set up 20 µl HPGT assay reactions in 0.5 ml microcentrifuge tube as follows.

HPGT assay components	Amount per reaction
1 M MOPS-KOH (pH 7.0)	2 µl
2 mM UDP-α-D-galactose	5 µl
10 mM $MnCl_2$	2 µl
10% TX-100	2 µl
2 mM substrate peptide	1 µl
Arabidopsis T-87 microsomal membranes	30 µg protein equivalent
Water	Total 20 µl

2. Incubate at 30 °C, 1 h.
3. Add 2 µl 1% formic acid to stop reaction.
4. Add 80 µl acetonitrile.
5. Centrifuge at 20,000 *x g* for 5 min.

C. LC/MS analysis

10 µl aliquots of the assay solution were analyzed by LC-MS using a micro HPLC (high pressure liquid chromatography) system connected to an LCQ Deca XP-plus ESI ion-trap mass spectrometer. Chromatographic separation is performed by normal-phase HPLC on a TSK-gel Amide-80 (3 µm) column (2 x 150 mm).

1. The mobile phase is composed of HPLC grade water containing 0.1% formic acid (eluent A) and HPLC grade acetonitrile containing 0.1% formic acid (eluent B). The column temperature is maintained at 25 °C.
2. The HPLC flow rate is 100 µl/min, and the elution gradient was 60 to 30% B over 15 min.
3. The HPLC eluate was introduced into an electrospray ionization (ESI) ion-trap mass spectrometer in the positive ionization mode.
4. MS source parameters are as follows [*e.g.,* $(OAOSOT)_3S$ peptide]:
   a. Capillary temperature: 200 °C
   b. Capillary voltage: 42 V
   c. Source voltage: 5 kV
   d. Source current: 8.5 µA
   e. Sheath gas flow: 50
   f. Aux gas flow: 0
   g. Sweep gas flow: 0
   h. The mass range: *m/z* 500-2,000
5. The mass spectra are obtained by selected ion monitoring. [*e.g.,* $(OAOSOT)_3S$: *m/z* 951.1, Galactosylated product: *m/z* 1032.2] (Figure 2).

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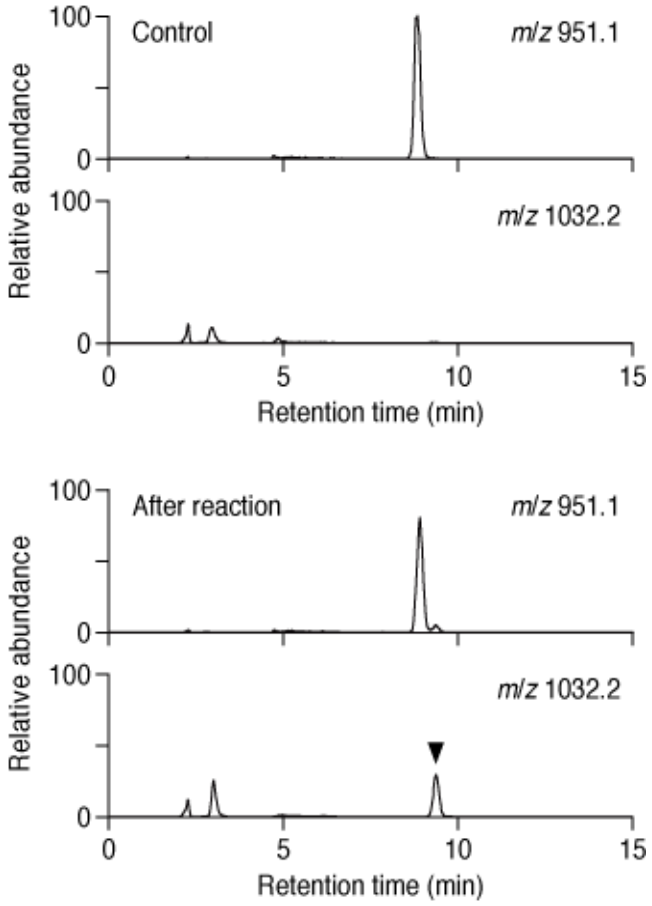

Figure 2. Selected ion chromatogram of substrate peptide and the galactosylated product. Substrate peptide was incubated with solubilized membrane fractions in the presence of UDP-α-D-galactose, then analyzed by LC-MS with selected ion monitoring of the substrate (*m*/*z* 951.1) and the galactosylated product (*m*/*z* 1032.2).

Recipes

1. Extraction buffer (prepare freshly and keep on ice)
   25 mM Tris-HCl (pH 7.0)
   10 mM $MgCl_2$
   2 mM dithiothreitol
   2 μM leupeptin
   2 mM phenylmethanesulfonyl fluoride
   250 mM sucrose
2. Suspension buffer
   10 mM Tris-HCl (pH 7.0)
   250 mM sucrose

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Acknowledgments

This is the detailed protocol for the detection of HPGT activity described by Ogawa-Ohnishi and Matsubayashi (2015). This research was supported by a Grant-in-Aid for Scientific Research (S) from the Ministry of Education, Culture, Sports, Science, and Technology (No. 25221105).

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