Stem Microsome Preparation and Mannan Synthase Activity Assay

Yan Wang*

Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, USA
*For correspondence: yanwang@msu.edu

[Abstract] Mannans are hemicellulosic polysaccharides and are present in cell walls of all land plants. Mannan polysaccharides are synthesized by two enzymes, mannan synthase (ManS) for backbone (mannan or glucomannan) synthesis and galactomannan galactosyl transferase for side-chain (galactosyl) addition. Here, a method for ManS activity assay using microsomes freshly isolated from *Arabidopsis* stems is described. This method can be applied to isolation of microsomes from any tissues of *Arabidopsis* or any other plants.

Materials and Reagents

1. cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (F. Hoffmann-La Roche, catalog number: 11836170001)
2. GDP-[^{14}C]-Man (9.694 GBq/mmol, 262.0 mCi/mmol, 76.34 μM) (PerkinElmer, catalog number: NEC536050UC)
3. Bicinchoninic acid (BCA) Protein Assay Kit (Pierce Antibodies, catalog number: 23225 or 23227)
4. OptiPhase Supermix Cocktail (PerkinElmer, catalog number: 1200-439)
5. GDP-Man (Sigma-Aldrich, catalog number: G-7377)
6. Carob galactomannan (Megazyme International, catalogue number: P-GALML)
7. Sucrose
8. $MgCl_2$
9. Extraction buffer (EB) (see Recipes)
10. 4x ManS assay buffer (see Recipes)
11. 1 mM GDP-Man (see Recipes)
12. 1% carob galactomannan (see Recipes)

Equipment

1. Mortar and pestle
2. 13 ml Sarstedt tube (Sarstedt, catalog number: 60.540.500)
3. Centrifuge

4. 4 ml plastic liquid scintillation counting vial (PerkinElmer, catalog number: 1200-421)
5. 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer, catalog number: 1450-024)

Procedure

1. Grow Arabidopsis plants in a growth chamber under standard growth conditions (20 °C, 100 μmo/m^2/s, 16 h light/8 h dark, 60% humidity) for 6-7 weeks.
2. Harvest the whole stem (including secondary stems) from a single plant, and remove leaves, flowers and siliques. The stem should be big enough (at least 200 mg of fresh weight) but not very old or hard (without any yellow siliques).
3. Weigh the stem and cut it into approximately 1 cm segments.
4. Immediately transfer the stem segments into a mortar pre-chilled on ice, and add ice-cold EB (~1 ml EB/100 mg stem).
5. Grind the stem segments in EB on ice with a mortar and pestle until the tissue is homogenized well (~5 min).
   I usually grind the tissue in the cold room.
6. Transfer stem homogenate to a pre-chilled 13 ml Sarstedt tube using a 1 ml wide-bore pipetman tip (with the sharp end cut off), and centrifuge it at 3,000 *x g* at 4 °C for 10 min.
7. Transfer the supernatant to a new pre-chilled 13-ml Sarstedt tube, and centrifuge it at 17,000 *x g* at 4 °C for 20 min.
8. Centrifuge the supernatant from step 7 at 100,000 *x g* at 4 °C for 90 min to pellet microsome membranes.
9. Resuspend the membrane pellet in EB (0.5 μl EB/mg stem) by pipetting up and down using a 200 μl tip. The membranes are pelleted tightly, so it takes some time to resuspend the pellet. I usually do it in the cold room.
   Aliquot a small volume of the microsome membranes, diluted it by 10 fold with EB, and store it at -20 °C. The diluted sample will be used for quantifying protein concentration using the BCA Protein Assay Kit.
10. Transfer microsome suspension to a 1.5 ml Eppendorf tube, vortex briefly, and incubate on ice for 5 min to let large particulates settle down.
11. Aliquot 20 μl of microsomes (do not pipet the precipitate on the bottom) into 1.5 ml Eppendorf tubes pre-chilled on ice. Boil 2-3 tubes of microsomes at 100 °C for 10 min, and the boiled samples will be used as a boiled control.
12. Prepare reaction cocktail by adding and mixing the following reagents.

4x ManS assay buffer	10 μl	(final concentration in reaction: 1x)
1 mM GDP-Man	0.85 μl	(final concentration in reaction: 21.18 μM)

GDP-[^{14}C]-Man (76.34 μM)	2 μl	(final concentration in reaction: 3.82 μM)
Deionized H_2O	7.15 μl	

Make master mix by scaling up the volumes based on the numbers of reaction tubes.

13. Pipet 20 μl of the reaction cocktail into each tube containing 20 μl of microsomes, and briefly vortex to mix. Allow ~5 sec staggering time between two samples.
14. Conduct reactions at room temperature for 1 h.
15. Stop the reactions by adding 1 ml of 70% ethanol containing 2 mM EDTA and 10 μl of 1% carob galactomannan. Precipitate assay products at -20 °C for at least 1 h.
16. Pellet the assay products by centrifuging at 16,000 *x g* at 4 °C for 10 min.
17. Wash the pellets with 70% ethanol containing 2 mM EDTA four times, and repeat centrifugation after each wash.
18. Resuspend each washed pellet in 300 μl of water.
19. Transfer suspension to a 4 ml plastic scintillation vial, and then add 3 ml OptiPhase Supermix Cocktail to the vial. Cap the vial and mix the sample well by vortexing briefly.
20. Perform liquid scintillation counting using a 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter.
21. Calculate the *in vitro* ManS enzymatic activities (shown as picomoles of GDP-Man incorporation per hour per mg protein) based on radioactivity of the assay products using the following formula.

Specific ManS activity = (CPM ÷ counting efficiency) ÷ 2,220,000 ÷ (GDP-[^{14}C]-Man radioactivity shown as μCi/μmol) x 1,000,000 x [(cold GDP-Man concentration + hot GDP-Man concentration) ÷ hot GDP-Man concentration] ÷ 1 h ÷ [20 x (protein concentration as μg/μl) ÷ 1,000]

Detailed explanations are as follows:

DPM (decaying per minute) = CPM (counting per minute) ÷ counting efficiency
(This formula is used to convert the detected radioactivity (cpm) of the assay products to the actual radioactivity (dpm) based on the detection efficiency of the of the liquid scintillation counting machine)

Hot GDP-Man incorporation (pmol) = DPM ÷ 2,220,000 dpm/μCi ÷ (GDP-[^{14}C]-Man radioactivity shown as μCi/μmol) x 1,000,000 pmol/μmol
[This formula is used to convert the radioactivity (dpm) of the assay products to pmol radio-labeled (hot) GDP-Man incorporation. Note the unit conversions: 1 μCi = 2,220,000 dpm; 1 μmol = 1,000,000 pmol.]

Total GDP-Man incorporation (pmol) = hot GDP-Man incorporation (pmol) x [(cold GDP-Man concentration + hot GDP-Man concentration) ÷ hot GDP-Man concentration]
[The total GDP-Man incorporation (pmol) is calculated by dividing hot GDP-Man incorporation (pmol) by the ratio of the hot GDP-Man concentration to the total (hot + cold) GDP-Man concentration]

Protein mass (mg) of 20 µl microsomes = 20 µl x (protein concentration as µg/µl) ÷ 1,000 µg/mg

Specific ManS activity (pmol GDP-Man incorporation/h/mg protein) = total GDP-Man incorporation (pmol) ÷ 1 h ÷ protein mass (mg)

For cold and hot GDP-Man reagents used in this protocol:
Specific ManS activity = (CPM ÷ counting efficiency) ÷ 2,220,000 ÷ 262 x 1,000,000 x [(21.18 + 3.82) ÷ 3.82] ÷ 1 h ÷ [20 x (protein concentration as µg/µl) ÷ 1,000]

Recipes

1. Extraction buffer (EB)
   50 mM HEPES-KOH (pH 7.5)
   0.4 M Sucrose
   10 mM $MgCl_2$
   Filter sterilize and store at 4 °C
   Before use, add 1 cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail Tablet per 10 ml buffer.
2. 4x ManS assay buffer
   200 mM HEPES-KOH (pH 7.5)
   10 mM DTT
   10 mM $MgCl_2$
   20 mM $MnCl_2$
   24% glycerol
   Store at -20 °C
3. 1 mM GDP-Man
   Dissolve 5 mg GDP-Man in 770 µl of 10 mM HEPES-KOH (pH 7.5) to a final concentration of 10 mM Dilute a small volume of 10 mM GDP-Man to 1 mM with H_2O.
4. 1% carob galactomannan

Dissolve 100 mg carob galactomannan in H_2O with a final volume of 10 ml. Heat to facilitate dissolution of galactomannan.

Acknowledgments

This protocol is adapted from Wang et al. (2013).

References

1. Wang, Y., Mortimer, J. C., Davis, J., Dupree, P. and Keegstra, K. (2012). Identification of an additional protein involved in mannan biosynthesis. *Plant J* 73(1): 105-117.