Assays for Determination of Acetylesterase Activity and Specificity Using pNP-acetyl and Acetylated Polysaccharides as Substrates

Gennady Pogorelko and Olga A. Zabotina*

Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, USA
*For correspondence: zabotina@iastate.edu

[Abstract] The acetylesterases are hydrolytic enzymes which in plants cleave acetyl groups from acetylated cell wall components, primarily polysaccharides. To estimate acetylesterase activity in plant apoplast, two assays can be used. First assay is a direct measurement of the acetylesterase activity in protein extract using synthetic substrate, pNP-acetyl. In this assay, amount of pNP released after hydrolysis of pNP-acetyl is determined by measuring the intensity of developed yellow color using spectrophotometer. The absorbance of reaction mixture is directly proportional to the activity of acetylesterases in the reaction mixture. Second assay is a determination of acetylesterase activity and its specificity towards natural polysaccharides and based on interaction between ferric perchlorate and acetyl residues resulting in ferric acetohydroxamic complex that can be quantified using spectrophotometer. In this assay, commercially available acetylated polysaccharides (xylan from Birchwood for acetylxylan esterase; pectin from citrus fruit for rhamnogalacturonan acetylesterase; or any other available polysaccharide of interest) incubated with apoplastic extract and amount of acetyl residues released from this polysaccharide is estimated using ferric perchlorate reagent (protocol was modified from McComb and McCready, 1957). The absorbance of produced colored complex is directly proportional to the amount of acetyls released from acetylated polysaccharide.

Materials and Reagents

1. Plant material
2. 4-Nitrophenyl acetate (Sigma-Aldrich, catalog number: N8130)
3. 4-Nitrophenyl (Sigma-Aldrich, catalog number: N1048)
4. Tris-HCl
5. EDTA
6. $MgCl_2$
7. Xylan from Birchwood (Sigma-Aldrich, catalog number: X4252)
8. Pectin from citrus fruit (Sigma-Aldrich, catalog number: P9436)
9. Sodium phosphate
10. Sodium hydroxide

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11. Hydroxylamine hydrochloride (Acros Organics, catalog number: 5470-11-1)
12. Perchloric acid (Sigma-Aldrich, catalog number: 311421-50ML)
13. Absolute methanol
14. Glucose-penta-acetate (Sigma-Aldrich, catalog number: G2354-25G)
15. Deionized water
16. Bio-Rad Protein Assay (Bio-Rad Laboratories, catalog number: 500-0006) (optional)
17. Extraction/reaction buffer (see Recipes)
18. Re-suspension buffer (see Recipes)
19. Acid-alcohol solution (see Recipes)
20. Ferric Perchlorate reagent (MP Biomedicals, catalog number: 215875) (see Recipes)

Equipment

1. Sharp razor blade
2. pH meter
3. Microtiter plate reader
4. Microcentrifuge
5. Centrifuge compatible with 15 ml vials
6. 96-well microtiter plate
7. Parafilm
8. Sharp razor blade
9. 10 ml syringe
10. Light water flow laboratory vacuum
11. SpeedVac dryer
12. Vortex
13. NanoDrop spectrophotometer (optional)
14. Shaker for microtubes

Procedure

A. Apoplast protein extraction from plant material

Note: Acetylesterase activity assay can be applied not only for apoplast proteins but also for total proteins extracts and purified enzymes.

1. Harvest 2-5 g of aerial parts (stems, leaves, siliques, flowers, and buds) of plants and immediately cut into 5 mm segments using a sharp razor blade.
2. Place plant material into a 10 ml syringe which had its tip sealed with Parafilm (Figure 1A-B).

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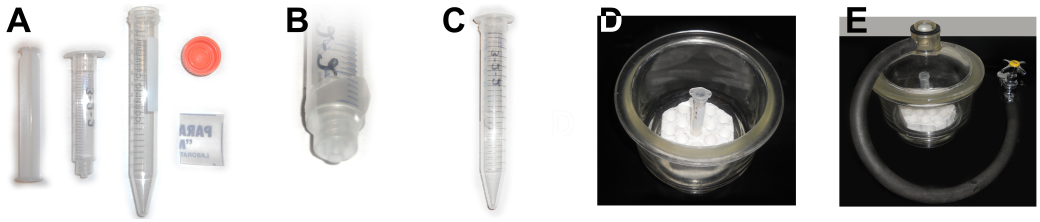

Figure 1. Apoplast extraction tools. A. Initial set: 5 ml syringe, 15 ml centrifuge tube, parafilm. B. Syringe tip sealed with parafilm. C. Syringe with sealed tip placed into centrifuge tube. D. Syringe with sealed tip in centrifuge tube placed into vacuum exicator. E. Vacuum exicator connected to the vacuum line.

3. Add 5 ml of pre-cooled extraction/reaction buffer.
4. Place the syringe into a centrifuge tube (tip is showing downwards, Figure 1C) and treat under vacuum in exicator (Figure 1D-E) twice for 15 min with a 5 min break in a cold room at 10 °C.
5. Open the tip of syringe, carefully drain the buffer by gravity flow and discard it.
6. Centrifuged at 1,000 *x g* for 10 min at 10 °C.
7. Place apoplast extracts accumulates at the bottom of the tube on ice.
8. Estimate protein concentration by NanoDrop or by Bradford assay described at Bradford Protein Assay (He, 2011).

B. Total activity of acetylesterases

1. Dilute apoplast extract with extraction/reaction buffer to obtain 0.128 mg in 100 µl.
   Note: Amount of protein for assay will vary depending on the enzymatic activity and specificity of acetylesterases present in extract. Here we perform example made on Arabidopsis extracts.
2. Prepare blank negative control by adding of 1 µl of 200 mM pNP-acetyl to the 100 µl of boiled for 10 min apoplast extract containing 0.128 mg of protein.
3. Add 1 µl of 200 mM pNP-acetyl to 100 µl of diluted apoplast extract.
4. Incubate at room temperature for 0, 5, 15, 30, 60, 120, 180 min reading blank and samples absorbance at 460 nm in microplate reader at each time point.
5. Prepare standard curve by measuring absorbance at 460 nm of 100 µl pNP solution diluted in series of concentrations ranging from 0.1 mM to 2 mM. All dilutions prepare in 3 replicates. Calculate the slope of the line which will give STD1 value equal to absorbance of 0.1 µmoles of pNP.
6. Measure OD_{460} sample-OD_{460} blank for each time point and calculate the slope.
7. Calculate the amount of released pNP using the following formula:
   Slope/STD1*0.1/0.128

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Slope (from step B6)
0.1 and STD1 (from step B5)
0.128 (from step B2)
Obtaining valuey will indicate amount of released pNP µmoles per 1 min per 1 mg of apoplast proteins

C. Assay for acetylesterase activity using natural substrates (cell wall derived polysaccharides)
1. Apply 0.12 mg of apopalst protein in extraction/reaction buffer to 20 mg of natural substrate in total volume 0.6 ml.
2. Prepare blank negative control by adding 0.12 mg of boiled apoplast protein in extraction/reaction obuffer to 20 mg of natural substrate.
3. Incubate at room temperature for 0, 30 min, 1 h, 3 h, 6 h shaking at 130 rpm.
4. At each time point spin down polysaccharide material (1 min at 17,000 *x g*) and take an aliquot 100 µl of supernatant.
Note: After taking the aliquot vortex the pellet and continue incubation.
5. Dry the aliquot using SpeedVac.
6. Re-suspend dry material in 40 µl of Re-suspension buffer (use vortex).
7. Add 100 µl of deionized water and mix carefully.
8. Add 100 µl of acid-alcohol solution and mix carefully.
9. Add 260 µl of Ferric Perchlorate reagent and mix carefully.
10. Incubate at room temperature 15-30 min.
11. Measure the absorbance at 510 nm.
12. Measure OD_{510} sample-OD_{510} blank for each time point and calculate the slope.
13. Prepare standard curve using glucose-penta-acetate with a series of dilutions from 0.1 mM to 2 mM in 40 µl solution following by steps C7-12 paragraphs from above. Calculate slope which will give STD2 value equal to absorbance of 0.2 nmoles of acetyl residues.
14. Calculate the amount of released acetyls from natural substrates by following formula:
Slope*0.2/STD2/0.02
Slope (from step C12)
0.2 and STD2 (from step C8)
0.02 (from a. 0.18 mg of apoplast protein was used in total 6 measurements)
Determined value will indicate amount of released acetyls (nmoles) per 1 min per 1 mg of apoplast protein

Recipes

1. Extraction buffer

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25 mM Tris-HCl

50 mM EDTA

150 mM $MgCl_2$ (pH 7.4)

2. Re-suspension buffer

   0.25 M hydroxylamine hydrochloride

   1 M sodium hydroxide solution

3. Acid-alcohol solution

   5% perchloric acid in absolute methanol

4. Ferric Perchlorate reagent

   0.3% ferric perchlorate (non-yellow)

   0.5% perchloric acid dissolved in 88% methanol

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References

1. He, F. (2011). Bradford protein assay. *Bio-protocol* 1(6): e45.
2. McComb, E. and McCready, R. (1957). Determination of acetyl in pectin and in acetylated carbohydrate polymers. *Anal Chem* 29(5): 819-821.
3. Pogorelko, G., Lionetti, V., Fursova, O., Sundaram, R. M., Qi, M., Whitham, S. A., Bogdanove, A. J., Bellincampi, D. and Zabotina, O. A. (2013). *Arabidopsis* and *Brachypodium distachyon* transgenic plants expressing *Aspergillus nidulans* acetylesterases have decreased degree of polysaccharide acetylation and increased resistance to pathogens. *Plant Physiol* 162(1): 9-23.
4. Pogorelko, G., Fursova, O., Lin, M., Pyle, E., Jass, J. and Zabotina, O. A. (2011). Post-synthetic modification of plant cell walls by expression of microbial hydrolases in the apoplast. *Plant Mol Biol* 77(4-5): 433-445.

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