Protein Extraction, Acid Phosphatase Activity Assays, and Determination of Soluble Protein Concentration

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[Abstract] Acid phosphatases (APases) catalyze the hydrolysis of inorganic phosphate (Pi) from a broad range of Pi-monoesters with an acidic pH optimum. The liberated Pi is reassimilated into cellular metabolism via mitochondrial or chloroplastic ATP synthases of respiration or photosynthesis, respectively. Eukaryotic APases exist as a wide variety of tissue- and/or cellular compartment-specific isozymes that display marked differences in their physical and kinetic properties. Increases in intracellular (vacuolar) and secreted APase activities are useful biochemical markers of plant nutritional Pi deficiency. The protocols for protein extraction, APase activity determination and measurement of soluble protein concentration from plant tissues or cell suspension cultures are presented.

Materials and Reagents

1. Plant tissues
2. Sodium acetate (Bioshop, catalog number: SAA305)
3. EDTA (Bioshop, catalog number: EDT001)
4. Dithiothreitol (DTT) (Bioshop, catalog number: DTT 002)
5. Phenylmethyl sulfonyl fluoride (PMSF) (G-Biosciences, catalog number: 786-0555)
6. Thiourea (Sigma-Aldrich, catalog number: T-7875)
7. Polyvinyl (polypyrrodlidone) (PVPP) (Sigma-Aldrich, catalog number: P-6755)
8. β-nicotinamide adenine dinucleotide reduced form (NADH) (Bioshop, catalog number: NAD002)
9. Phosphoelnolpyryvate (PEP) (Biovectra, catalog number: 2552)
10. $MgCl_2$ ((Biolynx, catalog number: 18641)
11. Rabbit muscle lactate dehydrogenase (LDH) (Sigma-Aldrich, catalog number: L-2500)
12. para-nitrophenyl-phosphate (pNPP, phosphatase substrate) (Sigma-Aldrich, catalog number: P-4744)
13. Coomassie Brilliant blue G-250 (Serva, catalog number: 35050 C.!.42655)
14. Bovine gamma globulin (BGG) (2.0 mg/ml) (Thermo Fisher Scientific, catalog number: 23212)

15. NaOH
16. Extraction buffer (EB) (see Recipes)
17. Bradford working solution (see Recipes)
18. Acid phosphatase enzyme assay mix #1 (see Recipes)
19. Acid phosphatase enzyme assay mix #2 (see Recipes)
20. Bradford assay stock (see Recipes)

Equipment

1. Whatman #1 filter paper
2. 1.5 ml microfuge tubes
3. 10 and 25 µl Hamilton syringes
4. Pipetor
5. Small mortar and pestle
6. Eppendorf microfuge
7. 96 well polystyrene microtitre plate (flat bottom)
8. A computer supported microplate spectrophotometer (Spectromax Plus, Molecular Devices)

Procedure

A. Extraction

This protocol applies to extraction of intracellular (vacuolar) APases from plant tissues and suspension cell cultures (*e.g.*, Tran *et al*., 2010a; Veljanovski *et al*., 2006). Refer to Tran *et al*. 2010b and Robinson *et al*., 2012 for information on the isolation and analysis of plant cell wall localized and secreted APase isoforms.

1. Weigh tissue and freeze in liquid N_2. Store at -80 °C until use.
2. Place a small spatula of sea sand into the mortar, add small amount of liquid N_2, followed by frozen tissue, and grind to a powder. Carefully add more liquid N_2 if needed to keep frozen.
3. Invert tube with extraction buffer (EB) to mix and add to sample at a ratio of 1:2, w/v (*e.g.* 0.5 g powdered tissue + 1.0 ml EB) although this may need to be increased to 1:3 for leaf tissue and 1:4 for roots. Grind for several minutes and place in 1.5 ml microfuge tubes.
4. Centrifuge 5 min at 4 °C and 11,000 *x g.* Remove supernatant (clarified extract) to a fresh microfuge tube and keep it on ice. Measure APase activity immediately at room temperature (24 °C). Aliquots of clarified extracts can be snap frozen in liquid N_2 and stored at -80 °C for future use.

B. APase activity assay 1

1. Conveniently measure APase activity by coupling the hydrolysis of phosphoenolpyruvate (PEP) to pyruvate to the lactate dehydrogenase (LDH) reaction at 24 °C and using a spectrophotometer to continuously monitor NADH oxidation at 340 nm. PEP seems to be an excellent APase substrate for since it occupies the highest position on the thermodynamic scale of phosphorylated intermediates (and thus its P atom is an excellent leaving group). For every PEP molecule hydrolyzed to pyruvate, one NADH molecule is oxidized to NAD^+ by LDH as shown in Figure 1.

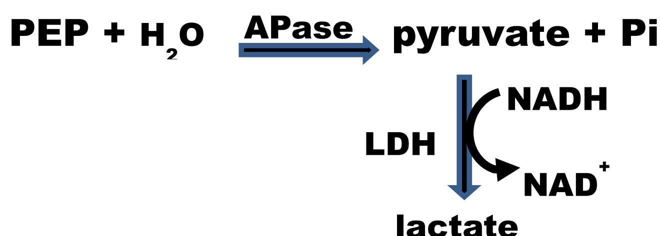


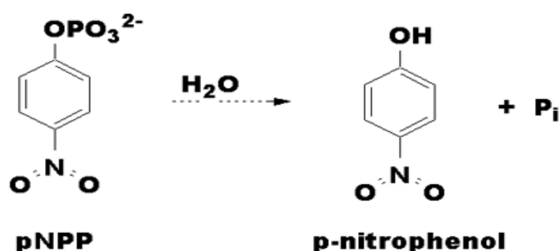
Figure 1. APase activity can be conveniently determined by coupling the hydrolysis of PEP to pyruvate to the lactate dehydrogenase (LDH) reaction and using a spectrophotometer to continuously monitor NADH oxidation to NAD^+ at 340 nm

2. Accurately pipette 1-10 µl of clarified extract into a microplate well. We prefer to use a 10 or 25 µl Hamilton syringe as opposed to automated pipetors for accurate pipetting of enzyme protein extracts into wells of the microtitre plate.
3. Use repeat pipetor to add 200 µl APase assay mix #1 to each well and immediately place in microplate spectrophotometer. Continuously monitor NADH oxidation to NAD^+ as a decline in absorbance at 340 nm (A_{340}), taking readings every 5-10 sec for up to 5 min.
4. Correct for background NADH oxidation by omitting PEP from the reaction mixture. Ensure that the decline in A_{340} (amount of NADH being oxidized; ϵ340 = 6,220 $M^{-1}cm^{-1}$) is proportional to assay time and concentration of enzyme assayed. Dilution of clarified extract in extraction buffer (lacking PVPP) may be necessary for samples containing abundant APase activity.

   Note: One international unit (U) of enzyme activity is defined as the amount of enzyme resulting in the hydrolysis of 1 µmol of substrate (e.g. one µmol of NADH oxidized to NAD^+) per min at 24 °C. APase activity in (U/ml clarified extract) = (ΔA_{340}/min x clarified extract dilution factor)/6.22. Thus, if 2.0 µl of clarified extract mixed with 200 µl of APase reaction mixture yields a ΔA_{340}/min of 0.1 at 340 nm, then the APase activity = (0.1 x 100)/6.22 U/ml = 1.6 U/ml.

C. APase activity assay 2

This is a 'stopped-time' APase assay based upon the hydrolysis of the synthetic substrate, para-nitrophenyl-phosphate (pNPP), to para-nitrophenol (pNP) and Pi (Figure 2). The pNP product forms a yellow color at alkaline pH (λ_{max} = 410 nm; extinction coefficient = є410 = 18.2 $mM^{-1}cm^{-1}$; meaning a 1 mM solution of pNP should have an A_{410} of 18.2). The amount of yellow color formed is thus directly proportional to the amount of pNP produced and is therefore an indicator of the APase activity. This assay tends to be more popular in the APase literature. However, care needs to be taken to ensure that the amount of pNP being formed is proportional to the assay time and volume of clarified extract being assayed.



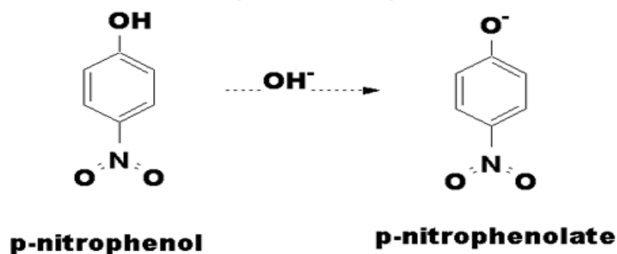


Figure 2. APase activity is often assayed spectrophotometrically at 410 nm by determining the amount of pNP produced following the hydrolysis of Pi from pNPP. Addition of NaOH after a specified assay time (*e.g.,* 10 min) serves to stop the APase reaction while simultaneously converting the product p-nitrophenol into the yellow colored p-nitrophenolate (λ_{max} = 410 nm).

1. Accurately pipette 1-10 µl of clarified enzyme extract into a well of the microtitre plate. Add 200 µl of APase assay mix #2 to each well containing enzyme extract and incubate for 10 min at room temperature (24 °C).
2. At t = 10 min, add 50 µl of 3 M NaOH to each well containing APase reactions. This stops the reaction (denatures APase) while simultaneously converting pNP product into the yellow colored p-nitrophenolate.
3. Determine ΔA_{410}/min for each well to determine the amount (µmol) of pNP formed per min.

D. Bradford Assay of Soluble Protein Concentration

1. Prepare standard curve using the template below:

Well #	Vol BGG (0.4 mg/ml)	Amt Protein (BGG)	Vol H_2O
	(µl)	(µg)	(µl)
1a	0	0	25 (blank)
1b	2	0.8	23
1c	4	1.6	21
1d	8	3.2	17
1e	12	4.8	13
1f	16	6.4	9
1g	20	8.0	5
1h	25	10.0	0
2a	0	0	25 (blank)

2. Pipette 2-20 µl of the clarified extract (dilute as necessary) and adjust final volume to 25 µl in each well with H_2O. Dilute sample if necessary to remain in linear range of standard curve. A 25 µl Hamilton syringe is more accurate than automated pipetors for pipetting of BGG standard and clarified extract into wells of the microtitre plate.
3. Add 250 µl of Bradford working solution to each well using a repeat pipetor and read A_{595} of protein standards and unknowns. Ensure that A_{595} values of clarified extract samples aliquot falls within range of A_{595} values of the BGG standards. The absorbance maximum for an acidic solution of Coomassie Brilliant blue G-250 shifts from 465 nm to 595 when protein binding occurs. Determine amount of protein in clarified extract aliquot from the standard curve (if 2 µl aliquot of clarified extract yields an A_{595} value equivalent to 5 µg of protein, then the extract would have a protein concentration of 2 mg/ml).

Recipes

1. Extraction buffer (EB, keep on ice)
   50 mM Na-acetate (pH 5.6)
   1 mM EDTA

1 mM DTT

1 mM PMSF (prepare 100 mM stock in absolute ethanol and store at -20 °C, add fresh to EB immediately prior to tissue extraction as PMSF is unstable in aqueous solution)

5 mM thiourea

1% (w/v) PVPP

2. APase assay #1 reaction mixture (prepare freshly and keep at room temperature)

   50 mM Na-acetate (pH 5.6)

   10 mM $MgCl_2$

   0.2 mM NADH

   5 mM PEP

   3 U/ml rabbit muscle LDH (Desalt LDH by centrifuging an aliquot 3 min at 11,000 *x g*. Discard supernatant and resuspend the pellet in an equal volume of EB).

3. APase assay #2 reaction mixture

   10 mM pNPP (sodium salt), dissolved in 50 mM acetate-KOH (pH 5.8)

   3 M sodium hydroxide (NaOH) also needed to stop the reaction

4. Bradford assay stock

   100 ml 95% EtOH

   200 ml 88% H_3PO_4

   350 mg Brilliant blue G-250

5. Bradford working solution

   30 ml Bradford Stock

   425 ml H_2O

   15 ml EtOH

   30 ml 88% H_3PO_4

   Filter through Whatman #1 filter paper and store in brown or dark glass bottle.

   Protein assay solutions are stable for months at room temperature.

6. Bradford Protein standard

   Dilute bovine Gamma Globulin with H_2O to 0.4 mg/ml and store 100 µl aliquots at -20 °C.

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