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Measurement of ATP Hydrolytic Activity of Plasma Membrane H^+-ATPase from *Arabidopsis thaliana* Leaves

Masaki Okumura[1] and Toshinori Kinoshita[1, 2, *]

[1]Division of Biological Science, Graduate School of Science, Nagoya University, Nagoya, Japan; [2]Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Nagoya, Japan
*For correspondence: kinoshita@bio.nagoya-u.ac.jp

[Abstract] Plant plasma membrane H^+-ATPase, which is a P-type ATPase, couples ATP hydrolysis to H^+ extrusion and thereby generates an electrochemical gradient across the plasma membrane. The proton gradient is necessary for secondary transport, cell elongation, and membrane potential maintenance. Here we describe a protocol for measurement of the ATP hydrolytic activity of the plasma membrane H^+-ATPase from *Arabidopsis thaliana* leaves.
Keywords: *Arabidopsis thaliana*, ATP hydrolytic activity, Orthovanadate, P-type ATPase, Plasma membrane H^+-ATPase

[Background] Determination of the plasma membrane H^+-ATPase activity is important to elucidate its function and regulatory mechanism. However, it is sometimes difficult to determine the ATP hydrolytic activity of the plasma membrane H^+-ATPase, because plant cells contain many ATP hydrolytic enzymes. This protocol is developed based on the publications by Uemura and Yoshida (1986) and Kinoshita *et al*. (1995). We used KNO_3 as an inhibitor of V-type ATPases, ammonium molybdate as an inhibitor of acid phosphatases, oligomycin as an inhibitor of F-type ATPases, and NaF as an inhibitor of phosphatases (Shimazaki and Kondo, 1987; Kinoshita *et al*., 1995). Orthovanadate inhibits the P-type ATPase and thus can be used to measure the activity of the plasma membrane H^+-ATPase by assessing the vanadate-sensitive Pi release from ATP hydrolysis. The released Pi reacts with molybdate to form a blue complex which can then be quantified by measuring absorption at 750 nm.

Materials and Reagents

1. Ultracentrifuge tube (Beckman Coulter, catalog number: 349623)
2. Cuvette (100 µl) (Beckman Coulter, catalog number: 523270)
   Note: This product has been discontinued.
3. *Arabidopsis thaliana* ecotype Col-0
4. Dithiothreitol (DTT) (NACALAI TESQUE, catalog number: 14128-04)
5. Phenylmethylsulfonyl fluoride (PMSF) (NACALAI TESQUE, catalog number: 273-27)
6. Leupeptin (Wako Pure Chemical Industries, catalog number: 126-03754)
7. MOPS (NACALAI TESQUE, catalog number: 23415-54)
8. Oligomycin (Sigma-Aldrich, catalog number: 75351)

Vol 6, Iss 23, Dec 05, 2016
DOI:10.21769/BioProtoc.2044

9. Sodium chloride (NaCl) (Wako Pure Chemical Industries, catalog number: 191-01665)
10. Ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) (Dojindo Molecular Technologies, catalog number: N001-10)
11. Sodium fluoride (NaF) (NACALAI TESQUE, catalog number: 31420-82)
12. Tris (NACALAI TESQUE, catalog number: 35406-91)
13. 2-(N-morpholino)ethanesulfonic acid (MES) (NACALAI TESQUE, catalog number: 21623-26)
14. Magnesium sulfate ($MgSO_4$) (Wako Pure Chemical Industries, catalog number: 131-00405)
15. Potassium chloride (KCl) (Wako Pure Chemical Industries, catalog number: 163-03545)
16. Potassium nitrate (KNO_3) (Wako Pure Chemical Industries, catalog number: 160-04035)
17. Ammonium molybdate (Wako Pure Chemical Industries, catalog number: 016-06902)
18. Triton X-100 (Wako Pure Chemical Industries, catalog number: 169-21105)
19. ATP (NACALAI TESQUE, catalog number: 10406-61)
20. Sodium orthovanadate (Sigma-Aldrich, catalog number: S6508)
21. SDS (NACALAI TESQUE, catalog number: 31607-65)
22. Sodium molybdate (Wako Pure Chemical Industries, catalog number: 196-02472)
23. Sulfuric acid (H_2SO_4) (Wako Pure Chemical Industries, catalog number: 192-04696)
24. 1-amino-2-naphthol-4-sulfonic acid (ANSA) (NACALAI TESQUE, catalog number: 02212-12)
25. Sodium bisulfite ($NaHSO_3$) (Wako Pure Chemical Industries, catalog number: 190-01375)
26. Sodium sulfate (Na_2SO_4) (Wako Pure Chemical Industries, catalog number: 192-03415)
27. Potassium dihydrogen phosphate (KH_2PO_4) (Wako Pure Chemical Industries, catalog number: 169-04245)
28. DTT stock solution (see Recipes)
29. Protease inhibitor solution (see Recipes)
30. Oligomycin solution (see Recipes)
31. Homogenization buffer (see Recipes)
32. 2x ATPase buffer (see Recipes)
33. ATPase reaction buffer (see Recipes)
34. ATP solution (see Recipes)
35. Vanadate solution (see Recipes)
36. Stop solution (see Recipes)
37. ANSA solution (see Recipes)
38. Pi standard stock solution (see Recipes)

Equipment

1. Mortar (90 mm diameter) and pestle
2. Refrigerated centrifuge (TOMY DIGITAL BIOLOGY, model: MX-307)
3. Ultracentrifuge (Beckman Coulter, model: Optima™ TLX)
4. Vortex (Scientific Industry, model: SI-0286)

Vol 6, Iss 23, Dec 05, 2016
DOI:10.21769/BioProtoc.2044

5. Heat block (TAITEC, model: e-ThermoBucket ETB)
6. Spectrophotometer (Beckman Coulter, model: DU 730)
   Note: This product has been discontinued.

Procedure

A. Preparation of microsomal membranes
   1. Grow *Arabidopsis thaliana* in soil for 3 weeks at 23 °C under white light (50 µmol photons m^{-2} s^{-1}) with a 16-h-light/8-h-dark cycle.
   2. Homogenize rosette leaves (about 100 mg) with a mortar and pestle in 2 ml ice-cold homogenization buffer and keep on ice.
   3. Centrifuge the homogenate at 13,000 *x g* for 10 min at 4 °C.
   4. Ultracentrifuge the supernatant at 100,000 *x g* for 1 h at 4 °C.
   5. Resuspend the pellet in 100 µl ice-cold homogenization buffer by pipetting up and down.
   6. Quantify the protein concentration by Bradford assay (Bradford, 1976).
   7. Protein concentration is adjusted to 0.45 µg/µl with homogenization buffer.
      Note: Keep microsomal membranes on ice until use.

B. Measurement of vanadate-sensitive ATP hydrolytic activity
   1. Mix 100 µl of microsomal membranes with 100 µl ATPase reaction buffer, split the mixture in two tubes, 90 µl each (20 µg protein), and keep on ice.
   2. To determine vanadate-sensitive ATPase activity, add 2 µl vanadate solution to one tube and an equal volume of 1x ATPase buffer to the other tube.
   3. Add 10 µl ATP solution and gently vortex.
   4. Incubate at 30 °C for 30 min. Gently vortex once after 15 min of incubation.
   5. Add 1 ml stop solution.
   6. Add 50 µl ANSA solution and gently vortex.
   7. Incubate at 24 °C for 30 min. Gently vortex once after 15 min of incubation.
   8. Measure absorption at 750 nm by a spectrophotometer using a cuvette.
      Note: Do not centrifuge the samples.

C. Preparation of Pi standard curve
   1. Prepare Pi dilution series as shown in Table 1.

Vol 6, Iss 23, Dec 05, 2016
DOI:10.21769/BioProtoc.2044

Table 1. Template for the preparation of the Pi standard curve

Amount of Pi (nmol)	Volume of Pi standard stock (µl)	Volume of H_2O (µl)
0	0	50
5	1	49
50	10	40
100	20	30
150	30	20
200	40	10
250	50	0

2. Add 50 µl ATPase reaction buffer and 1 ml stop solution.
3. Add 50 µl ANSA solution and incubate at 24 °C for 30 min. Gently vortex once after 15 min of incubation.
4. Measure absorption at 750 nm using a cuvette and make a standard curve (Figure 1).

Data analysis

A typical Pi standard curve is shown in Figure 1. Calculate Pi content of samples using the standard curve. Vanadate-sensitive ATP hydrolytic activity is determined by subtracting Pi content in the presence of vanadate from that in the absence of vanadate, and is expressed as nmol Pi/h/mg of protein.

1. Calculate a slope of Pi standard curve.
   A_{750} = 0.0038 (Pi content [nmol]) + 0.0088
2. Determine Pi content of samples from the slope.
   (Pi content [nmol]) = (A_{750} - 0.0088)/0.0038
3. Subtract Pi content in the presence of vanadate from that in the absence of vanadate.
4. Divide by the amount of protein (mg), and reaction time (h). The following is an example of calculation.
   Pi content in the absence of vanadate = 10 nmol
   Pi content in the presence of vanadate = 5 nmol
   Reaction time = 0.5 h (30 min)
   Amount of protein = 0.02 mg (20 µg)
   Vanadate-sensitive ATP hydrolytic activity (nmol Pi/h/mg of protein)
   = ([Pi content in the absence of vanadate] - [Pi content in the presence of vanadate])/(reaction time)/(Amount of protein)
   = (10 - 5)/0.5/0.02 = 500 nmol Pi/h/mg of protein

Vol 6, Iss 23, Dec 05, 2016
DOI:10.21769/BioProtoc.2044

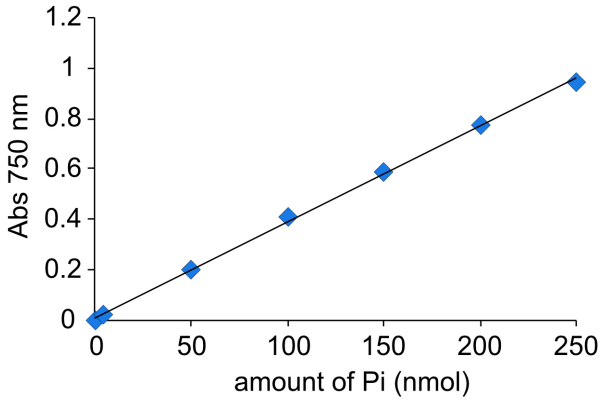

Figure 1. Standard curve generated by using known amounts of Pi. The absorption at 750 nm was measured.

Recipes

1. DTT stock solution
   1 M DTT in sterile water
   Store at -20 °C in small aliquots
2. Protease inhibitor solution
   200 mM PMSF and 4 mM leupeptin in DMSO
   Store at 4 °C in small aliquots
3. Oligomycin solution
   5 mg/ml oligomycin in DMSO
   Store at -20 °C in small aliquots
4. Homogenization buffer
   50 mM MOPS-KOH (pH 7.5)
   100 mM NaCl
   2.5 mM EDTA
   10 mM NaF
   5 mM DTT
   1 mM PMSF
   20 μM leupeptin
   Store at 4 °C. Add NaF, DTT, PMSF, and leupeptin just before use
5. 2x ATPase buffer
   60 mM Tris-MES (pH 6.5)
   6 mM $MgSO_4$
   100 mM KCl
   200 mM KNO_3
   Store at 4 °C

Vol 6, Iss 23, Dec 05, 2016
DOI:10.21769/BioProtoc.2044

6. ATPase reaction buffer (freshly prepared)
   2x ATPase buffer supplemented with:
   1 mM ammonium molybdate
   10 µg/ml oligomycin
   0.1% (w/w) Triton X-100
   0.5 mM PMSF
   10 µM leupeptin
7. ATP solution
   20 mM ATP in 1x ATPase buffer
   Solution should be aliquoted in small volumes to avoid freeze-thawing and can be stored at -80 °C for at least 6 months
8. Vanadate solution (freshly prepared)
   10 mM sodium orthovanadate in 1x ATPase buffer
9. Stop solution (freshly prepared)
   1.3% (w/v) SDS
   0.25% (w/v) sodium molybdate
   0.3 N H_2SO_4
10. ANSA solution (freshly prepared)
    0.125% (w/v) 1-amino-2-naphthol-4-sulfonic acid (ANSA)
    15% (w/v) $NaHSO_3$
    1% (w/v) Na_2SO_4
11. Pi standard stock solution (freshly prepared)
    5 mM KH_2PO_4

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Vol 6, Iss 23, Dec 05, 2016
DOI:10.21769/BioProtoc.2044

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