Measurement of NADPH Oxidase Activity in Plants

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[Abstract] NADPH oxidase is a membrane-bound enzyme that generates (O^{2-}) by transferring electrons from NADPH to molecular oxygen O_2. $O^{2-}$is spontaneously dismasted to the more stable form H_2O_2. Both $O^{2-}$and H_2O_2 are forms ofreactive oxygen species (ROS), which are involved in regulation of many cellular activities such as transcription, intracellular signaling, and host defense. The NADPH oxidase - dependent generation of O^{2-} in total membrane fraction of plant tissue has been determined by the reduction of the tetrazolium salt XTT by O^{2-}. In the presence of O^{2-}, XTT generates a soluble yellow formazan that can be quantified spectrophotometrically.

Materials and Reagents

1. Sucrose
2. HEPES
3. EDTA
4. DTT
5. L-cysteine
6. $MgCl_2$
7. PVP
8. Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (F. Hoffmann-La Roche, catalog number: 04693159001)
9. BSA
10. Bio-Rad Protein Assay (Bio-Rad Laboratories, catalog number: 500-0006)
11. Tris-HCl
12. Sodium 3,3'-(-[(phenylamino)carbonyl] -3,4-tetrazolium)-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT) (Sigma-Aldrich, catalog number: X4626)
13. NADPH (Sigma-Aldrich, catalog number: N1630)
14. Protein extraction working solution (see Recipes)

Equipment

1. Microtiter plate reader (Infinite M200 Pro, Tecan)

2. Microcentrifuge (AqquSpin Micro R) (Thermo Fisher Scientific)
3. Ultracentrifuge (Optima TLX, Beckman)
4. Microtiter plate (BD Biosciences, catalog number: 353075)

Procedure

A. Protein extraction and separation of membrane fraction from plant tissues

1. Harvest tissue in liquid nitrogen. If not used immediately, keep at -80 °C until processing.
2. Grind tissue in liquid nitrogen and weigh out 0.5 g of the ground tissues in empty Falcon tube that has been pre-chilled in liquid nitrogen and used to tare the scale.
3. Add 6 ml of ice-cold protein extraction buffer to ground tissues on ice.
4. Vortexat room temperature to mix thoroughly.
5. Filter homogenized tissue through four layers of cheese cloth and transfer filtrate (flow-through) to 2-ml microcentrifuge tubes, on ice.
6. Centrifuge at 10,000 *x g* for 45 min at 4 °C and transfer supernatant to ultra- centrifuge tube.
7. Separate total membrane fractions by ultra-centrifugation at 203,000 *x g* for 60 min at 4 °C.
8. Discard supernatant and resuspend pellet in 1 ml ice-cold 10 mM Tris-HCl (pH 7.4).

B. Protein estimation using Bradford microassay (160 µl)

9. Prepare BSA standards ranging from 5 µg-25 µg/ml as follows:

Standard concentration	Volume of BSA (100µg/ml)	Volume of water
5 µg/ml	8 µl	152 µl
10 µg/ml	16 µl	144 µl
20 µg/ml	32 µl	128 µl
25 µg/ml	40 µl	120 µl

These standards will be used to generate a standard curve.

10. Use 96 well microtiter plate to prepare reaction mix.
11. Prepare blank byadding 160 µl of water to one well in triplicates.
12. Prepare test samples by adding 2 µl of supernatant (from section 1) to 158 µl of water
13. Add 40 µl of Bradford Assay reagent to BSA standards, blank and test samples.
14. Mix and incubate at room temperature for 5 min and read absorbance at 595 nm (A_{595}) on plate reader spectrophotometer.

    Note: If spectrophotometer does not include a software to generate standard curve to automatically estimate protein content, generate a BSA standard curve by plotting known

protein concentration (X-axis) vs. Absorbance (in Y-axis). Protein concentration for a given unknown sample is estimated by plotting the A_{595} absorbance of the unknown (in the y-axis) and determining the intersection point with the BSA standard curve and then find the concentration associated with that particular point (in the x-axis). If using excel, after plotting concentration vs A_{595}, obtain the trendline and use the equation for the line and the A_{595} of the unknown to resolve the unknown concentration.

C. NADPH oxidase activity assay

15. Prepare fresh solution of 1 mM XTT and 1 mM NADPH.
16. Prepare two different assay solutions A and B:

Stock solution	Assay solution A	Assay solution B
1M Tris-HCl (pH 7.5)	12.5 µl	12.5 µl
1mM XTT	125 µl	125 µl
1mm NADPH	25 µl	----------
Water	77.5 µl	102.5 µl

Note: Because membrane fraction can spontaneously reduce XTT, even in the absence of substrate (NADPH), it is necessary to prepare two blanks, one without NADPH, to correct for this background levels of activity.

17. Prepare blanks:
    Blank 1: Add 10 µl of water to 240 µl of Assay solution A.
    Blank 2: Add 10 µl of membrane fraction to 240 µl Assay solution B.
18. Prepare samples by adding 10 µl of membrane fraction (from section 1) to 240 µl of Assay solution A. Read the absorbance at 492 nm (A_{492}) at 0 min and then 20 min intervals for one hour or until saturation point reached.
19. To get final A_{492} Blank reading, subtracts A_{492} Blank1 and A_{492} Blank 2.
20. Calculate rate of O^{2-} generation by using an extinction coefficient 2.16 x 10^4 cm (Jiang and Zhang 2002).
    $(\Delta_{A492nm}/min_{\ test} - \Delta_{A492nm}/min_{\ blank}) / (2.16X10^{4M^{-1}}C^{M^{-1}})$ (0.04)
    $\Delta_{A492nm}/min_{\ Test} = A_{492\ (sample\ X)}$ at saturation point - $A_{492\ (sample\ X)}$ at 0 min
    $\Delta_{A492nm}/min_{\ blank} = A_{492nm\ (blank)}$ at saturation point - $A_{492nm\ (blank)}$ at 0 min
    0.04 = dilution factor (10 µl/250 µl)
    To calculate specific activity, divide the value obtained in equation by the amount of protein present in the sample (converted to mg/ml).

Recipes

1. Protein extraction working solution

   0.25 M sucrose

   50 mM HEPES

   3 mM EDTA

   1 mM DTT

   3.6 mM L-cysteine

   0.1 mM $MgCl_2$

   0.6% PVP

   10 Tablets of Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets.

   Prepare the following stock solutions:

   1 M sucrose

   1 M HEPES (pH 7.2)

   0.25 M EDTA

   1 M DTT

   100 mM $MgCl_2$

   In 80 ml of water add the following reagents:

Stock solutions	Volume
1M Sucrose	25ml
1M HEPES (pH 7.2)	5ml
0.25M EDTA	1.2 ml
1M DTT	0.1 ml
L-cysteine	0.0632 g
100mM $MgCl_2$	0.1 ml
PVP	0.6 g

   Add 10 Tablets of Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets.

   Mix well and adjust volume to 100 ml

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References

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