Measurement of NADPH Oxidase Activity in Plants

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[Abstract] NADPH oxidase is a membrane-bound enzyme that generates (O²⁻) by transferring electrons from NADPH to molecular oxygen O₂. O²⁻ is spontaneously dismasted to the more stable form H₂O₂. Both O²⁻ and H₂O₂ are forms of reactive 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 O²⁻ in total membrane fraction of plant tissue has been determined by the reduction of the tetrazolium salt XTT by O²⁻. In the presence of O²⁻, 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. MgCl₂
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)
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. Vortex at 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 \( \times \) 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 \( \times \) 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:

<table>
<thead>
<tr>
<th>Standard concentration (μg/ml)</th>
<th>Volume of BSA (100 μg/ml)</th>
<th>Volume of water (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μg/ml</td>
<td>8 μl</td>
<td>152 μl</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>16 μl</td>
<td>144 μl</td>
</tr>
<tr>
<td>20 μg/ml</td>
<td>32 μl</td>
<td>128 μl</td>
</tr>
<tr>
<td>25 μg/ml</td>
<td>40 μl</td>
<td>120 μl</td>
</tr>
</tbody>
</table>

   These standards will be used to generate a standard curve.

10. Use 96 well microtiter plate to prepare reaction mix.
11. Prepare blank by adding 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:

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>Assay solution A</th>
<th>Assay solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Tris-HCl (pH 7.5)</td>
<td>12.5 μl</td>
<td>12.5 μl</td>
</tr>
<tr>
<td>1mM XTT</td>
<td>125 μl</td>
<td>125 μl</td>
</tr>
<tr>
<td>1mM NADPH</td>
<td>25 μl</td>
<td>—</td>
</tr>
<tr>
<td>Water</td>
<td>77.5 μl</td>
<td>102.5 μl</td>
</tr>
</tbody>
</table>

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).

\[
\frac{(\Delta A_{492\text{nm/min test}} - \Delta A_{492\text{nm/min blank}})}{(2.16 \times 10^{4} \text{M}^{-1} \text{cm}^{-1})} (0.04)
\]

$\Delta A_{492\text{nm/min test}}$ = $A_{492}$ (sample X) at saturation point - $A_{492}$ (sample X) at 0 min

$\Delta A_{492\text{nm/min blank}}$ = $A_{492}$ (blank) at saturation point - $A_{492}$ (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).
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 MgCl₂
   - 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 MgCl₂

In 80 ml of water add the following reagents:

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M Sucrose</td>
<td>25 ml</td>
</tr>
<tr>
<td>1M HEPES (pH 7.2)</td>
<td>5 ml</td>
</tr>
<tr>
<td>0.25M EDTA</td>
<td>1.2 ml</td>
</tr>
<tr>
<td>1M DTT</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0.0632 g</td>
</tr>
<tr>
<td>100mM MgCl₂</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>PVP</td>
<td>0.6 g</td>
</tr>
</tbody>
</table>

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

Mix well and adjust volume to 100 ml

**Acknowledgments**

This protocol has been adapted and modified to use in Arabidopsis from Jiang and Zhang (2002). This work was supported by the Samuel Roberts Noble Foundation.
References


