Glycolate Oxidase Activity Assay in Plants

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[Abstract] Glycolate oxidase is located in the peroxisome and is involved in the photorespiratory cycle which recovers some of the carbon loss during photosynthesis. Glycolate oxidase converts glycolate to glyxoylate with the concomitant production of H_2O_2.In this assay, the H_2O_2 generated, in the presence of HRP, oxidizes O-dianisidine into a colored O-dianisidine radical cation that can be quantified spectrophotometrically. The amount of color produces is directly proportional to the glycolate oxidase activity.

Materials and Reagents

1. Sucrose
2. HEPES
3. EDTA
4. DTT
5. L-cysteine
6. $MgCl_2$
7. PVP
8. BSA
9. Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (F. Hoffmann-La Roche, catalog number: 04693159001)
10. Bio-Rad Protein Assay (Bio-Rad Laboratories, catalog number: 500-0006)
11. Horseradish peroxidase(HRP) (Sigma-Aldrich, catalog number: P8375)
12. O-Dianisidine (Sigma-Aldrich, catalog number: D9143)
13. Sodium glycolate (Thermo Fisher Scientific, Acros Organics,catalog number: 351570250)
14. Potassium phosphate
15. Triton X-100
16. Protein extraction buffer (see Recipes)
17. Glycolate oxidase assay buffer (see Recipes)

Equipment

1. Microtiter plate reader (Infinite M200 Pro, Tecan)
2. Microcentrifuge (AqquSpin Micro R) (Thermo Fisher Scientific)
3. 96-well microtiter plate flat bottom(BD Biosciences, catalog number: 353075)
4. 2 ml-microcentrifuge tubes

Procedure

A. Total Protein extraction 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 andtransfer 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 new tubes on ice.
7. Use supernatant to estimate protein concentration and to measure glycolate oxidase activity.

B. Protein estimation using Bradford microassay (160 µl)

8. 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

9. Use 96 well microtiter plate to estimate protein content.
10. Prepare blank by160 µl of water to one well in triplicates.
11. Prepare test samplesby adding 2 µl of supernatant (from section 1) to 158 µl of water.
12. Add 40 µl of Bradford Assay reagent to BSA standards, blank and test samples.
13. Mix and incubate at room temperature for 5 min and read absorbance at 595 nm (A595) 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 A595 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 vsA595, obtain the trendline and use the equation for the line and the A595 of the unknown to resolve the unknown concentration.

C. Glycolate oxidase activity assay

14. Prepare blank by adding 10 µl of 0.1 M sodium phosphate buffer (pH 8.3), to 250 µl of glycolate oxidase assay bufferin 96 well microtiter plate.
15. Prepare test samples by adding 10 µl of supernatant (from section 1) to 250 µl of glycolate oxidase assay buffer.
16. Read blank and samples at 440 nm for 0 min and then at 20 min intervals for one hour or until saturation point reached, on plate reader spectrophotometer.
17. Calculate the generation of O-dianisidine radical using the following formula:
    $(\Delta A_{440nm}/\min_{test} - \Delta A_{440nm}/\min_{blank}))/ (11.60) (0.04)$
    $\Delta A_{440nm}/\min_{test} = A_{440\ (sample\ X)}$ at saturation point - $A_{440\ (sample\ X)}$ at 0 min

    $\Delta A_{440nm}/\min_{blank} = A_{440nm\ (blank)}$ at saturation point - $A_{440nm\ (blank)}$ at 0 min
    Where
    11.60 =extinction coefficient for O-dianisidine. (Macheroux *et al,* 1991)
    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 buffer
   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.
   Protein extraction buffer (100 ml)
   Use following stock solutions to make working solution:

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.

2. Glycolate oxidase assay buffer

   Working solution:

   10 µg/ml HRP

   0.4 mM O-dianisidine

   10 mM sodium glycolate

   in 0.1 M potassium phosphate (pH 8.3)

   Glycolate oxidase assay buffer (1 ml)

   PrepareGlycolate oxidase assay buffer using following stock solutions:

Stock solutions	Volume
1mg/ml Horseradish peroxidase (HRP)	10 µl
8mM - O- Dianisidine(dissolved in 20% Triton X-100)	50 µl
1M sodium glycolate	10 µl
0.1 M potassium phosphate pH 8.3	930 µl

Acknowledgments

This protocol has been adapted and modified to use in Arabidopsis from Macheroux *et al.* (1991). This work was supported by the Samuel Roberts Noble Foundation.

References

1. Macheroux, P., Massey, V., Thiele, D. J. and Volokita, M. (1991). Expression of spinach glycolate oxidase in *Saccharomyces cerevisiae*: purification and characterization. *Biochemistry* 30(18): 4612-4619.

2. Rojas, C. M., Senthil-Kumar, M., Wang, K., Ryu, C. M., Kaundal, A. and Mysore, K. S. (2012). Glycolate oxidase modulates reactive oxygen species-mediated signal transduction during nonhost resistance in *Nicotiana benthamiana* and *Arabidopsis*. *Plant Cell* 24(1): 336-352.

