PHENOL OXIDASE AND PEROXIDASE ASSAYS
CENTER FOR DEAD PLANT STUDIES
15 September 2000

The purpose of this assay is to measure the activity of enzymes that can oxidize phenols. Such enzymes are classified by what they use as an oxidant. Oxygenases use molecular oxygen; peroxidases use hydrogen peroxide. Other oxidases may use other electron acceptors. Phenol oxidases and peroxidases are involved in the breakdown of lignin and other aromatic compounds and in the production and degradation of humic substances.

TEST TUBE ASSAY FOR SOILS AND LITTER

1. Prepare 5 mM solution of L-3,4-dihydroxyphenylalanine (DOPA) in 50 mM, pH 5.0, acetate buffer. The solution may be unstable (i.e. it may slowly darkens over several hours as the DOPA is oxidized by O_2) and should be made fresh daily.

NOTE: The pH optimum for this reaction is about 8, so assays should be run at that pH, using Tris buffer if the goal is to estimate the maximum potential activity. The pH 5 acetate buffer is the "general purpose" buffer we use to assay suites of soil enzyme activity as it is generally similar to the pH of that environment.

2. Phenol Oxidase Assay: Place 2.0 mL of sample suspension (soil, litter or sediment homogenzied in acetate buffer) and 2.0 mL of L-DOPA solution in a 5 mL polypropylene tube. Use 2.0 mL of sample suspension and 2.0 mL of acetate buffer as a background control. Do four analytical replicates for the sample and two replicates for the control.

3. Peroxidase Assay: Place 2.0 mL of sample suspension, 2.0 mL of L-DOPA solution. and 0.2 mL of 0.3% hydrogen peroxide in a 5 mL tube. Do four analytical replicates for each sample. Use 2.0 mL of sample homogenate, 2.0 mL of acetate buffer, and 0.2 mL of 0.3% hydrogen peroxide as a background control. Do two replicates of each control.

NOTE: The sample is usually a suspension of fine particles or a homogenate of larger particles. When using suspensions, place the sample bottle on a stir plate, mix vigorously, withdraw 2 ml aliquots using a P5000 pipetter. To prevent clogging of the tip, snip off the end to make an opening about 0.5 cm in diameter.

4. Vortex all the tubes and place them in the platelet mixer at 20 C for 1 h.

5. Centrifuge all the tubes and withdraw 2.0 mL of supernatant.

6. Measure the absorbance of the supernatants at 460 nm, using distilled water to zero the spectrophotometer.

7. Compute activity as µmol substrate converted per hour per g organic matter of sample as follows:

Phenol Oxidase:

OD = Sample ABS - Control ABS

Activity (µmol / h / g) = OD / (1.66 / µmol) (incubation time, h) (g sample / ml of sample homogenate)

Peroxidase:

NOTE: Peroxidase activity is the increment in activity between samples incubated with and without hydrogen peroxide.

OD = (Sample ABS) - (Control ABS) - (OD for phenol oxidase)

Activity (µmol / h / g) = OD / (1.66 / µmol) (incubation time, h) (g sample / mL of sample homogenate)

NOTE: 1.66 / µmol is the micromolar extinction coefficient for DOPA under the conditions of the assay. It is determined by mixing 1.0 mL of 1.00 µmol / mL DOPA (in acetate buffer) with 3 mL of Horseradish peroxidase (obtain from Sigma, dissolve lyophilate in acetate buffer) then monitoring OD@460 nm until it peaks. The product of this reaction is 3-dihyroindole-5,6-quinone-2 carboxylate (DIQC) which reportedly has a molar extinction coefficient of 37,000 according to H.S. Mason, 1948, The chemistry of melanin III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase, J. Biol. Chem. 172:83-99. Our empirical determination generates a number that is lower by a factor of 5-6.

NOTE: The activity of oxidative enzymes does not generally follow Michaelis-Menton kinetics. Activity may not be linear with time. Whether the assays do or do not follow pseudo- first order kinetics depends on the sample, the time frame of measurement and the level of activity. For soil and litter we generally standardize incubation at one hour. If you decide to run them for longer periods, it will be necessary to run a substrate control (2 mL of 5 mM DOPA solution + 2 mL of water) because the substrate will react slowly, and more or less linearly, with oxygen even in the absence of enzymatic activity.

NOTE: Bear in mind that these assays do not cleanly separate oxidase and peroxidase activities. A sample homogenate to which DOPA is added may contain multiple types of oxidases, oxygenases and peroxidases. Peroxidase activity will be limited by the activity of peroxide generating enzyme systems, e.g. glucose oxidase, but nothing precludes such activity. The addition of peroxide to the assay mix alleviates a peroxide limitation but does not preclude other types of oxidative activity. In general, phenol oxidase activity predominates in decomposing litter; the addition of peroxide may not generate any additional activity. In soils the situation reverses. In general, as particle size or carbon quality declines the relative abundance of peroxidase activity increases while phenol oxidase declines.

MICROPLATE PROTOCOL

FOR SOIL AND LITTER:

1. Place 0.5 g of chopped litter or 1.0 g soil in a 125 mL screw-cap Nalgene bottle. Thoroughly homogenize the sample in 50-60 mL of 50 mM acetate buffer, pH 5. Use a squirt bottle to wash residue from the polytron probe into the bottle. Fill the bottle with acetate buffer.

NOTE: The pH optimum for this reaction is about 8, so assays should be run at that pH if the goal is to estimate the maximum potential activity. A pH of 5 is chosen here because it approximates the bulk pH of many soils. Also at pH 5, the rate of the non-enzymatic reaction between DOPA and oxygen appears nil, so longer incubations are possible. At pH 8, incubations should probably be under 2 h to avoid significant DOPA degradation.

NOTE: A soil suspension of around 1 g / 100 mL gives a background absorbance of around 1.000.

2. Prepare 25 mM L-3,4-dihydroxyphenylalanine (DOPA) solution in acetate buffer.

3. Mix the sample suspension thoroughly and dump about half of it into a wide mouth container. Put the container on a stir plate and mix vigorously to assure an even dispersion of particles. While mixing, dispense 200 µL aliquots into each microplate well.

NOTE: There will be much more variation from well to well with suspensions than with water samples or extracts, so use at least eight replicate wells (one column) per sample. Even with good technique, the well to well variation will be 20% or more because of differences in the amount of material hitting each well and because you are measuring changes in absorbance against a high background. When scaling assays down from test tube to microplate, you sacrifice precision for economy. Depending on the nature of your samples and the magnitude of difference you consider significant, you may want to increase the number of replicate wells to 16.

NOTE: Strong mixing and good pipetting technique will minimize well to well variation. Also snipping off the ends of the tips to increase the diameter of the opening will improve uniformity of dispensing (alternatively Fisher sells pipette tips with wide openings).

4. Add 50 µL of DOPA solution to each well. The peroxidase plates are done the same except that each well also gets 10 µL of 0.3% H_2O_2 added.

5. To correct for possible substrate background (negative controls), dispense 200 µL of acetate buffer and 50 µL of DOPA solution into a series of wells. To correct for sample background (blanks), dispense 200 µL of sample suspension into a series of wells and add 50 µL of acetate buffer. For peroxidase plates, add 10 µL of 0.3% H_2O_2 to each well.

7. Mix the contents of the microplate on a plate mixer and let them incubate at 20 C.

NOTE: You may want to incubate at ambient temperature or at elevated temperature depending on your question.

NOTE: Litter activities are generally higher than soil activities on a dry mass basis; soil and litter activities may be comparable on an organic matter basis. In litter samples, phenol oxidase activity generally predominates peroxidase. In soil samples, peroxidase activity is generally predominant. At room temperature, incubations can go for 24 h at pH 5. Higher pH increases rate of non-enzymatic DOPA oxidation. But be aware that reaction rates will probably not be linear over time, especially for the peroxidase assay. The peroxide you add may be gone after a short time. Further additions of peroxide to the wells may yield higher activity numbers.

8. Read absorbance at intervals of one to several hours using a microplate spectrophotometer with a 460 nm filter.

9. Calculate results as described above, remembering that sample volume here is only 0.2 ml.

Final OD = (mean OD of assay wells) - (mean OD of blank wells) - (mean OD of negative control wells)

Activity (μmole h^{-1} g^{-1}) = Final OD (125 mL) / (7.9 μmole^{-1}) (incubation time, h) (0.2 mL) (g soil used to make sample suspension)

NOTE: 125 mL = volume of sample suspension; 7.9 = μmolar extinction coefficient, 0.2 = sample volume in microplate well

NOTE: The 7.9 extinction coefficient used here is empirically determined.
Mix 200 µL of 0.50 µmol / mL DOPA (in acetate buffer) with 50 µL of Horseradish peroxidase (obtain from Sigma, dissolve lyophilate in acetate buffer about 1 mg / mL). Then monitor OD@460 nm until it peaks. The product of this reaction is 3-dihyroindole-5,6-quinone-2 carboxylate (DIQC) which reportedly has a molar extinction coefficient of 37,000 according to H.S. Mason, 1948, The chemistry of melanin III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase, J. Biol. Chem. 172:83-99. Our empirical determination generates a number that is lower by a factor of 5-6.

FOR SURFACE WATERS

You can do this assay on surface waters. Activities are often hard to detect, but are easily measurable in waters with high phenol content. The protocol is similar to that above, using 200 µL aliquots of sample water rather than soil or litter suspensions.

1. Prepare 5 mM L-3,4-dihydroxyphenylalanine (DOPA) solution in deionized water.

NOTE: Substrate concentration generally does not have to be as high in a surface

water assay as it is in a soil or litter suspension to achieve saturation conditions. However, it is a good idea to check whether the substrate concentration you select is high enough.

2. Add 200 µL of water sample to each microplate well. Use at least three replicate wells per sample.

3. Add 50 µL of DOPA solution to each well. Negative control wells contain 50 µL of DOPA solution plus 200 µL of deionized water. Blank wells get 50 µL of deionized water plus 200 µL of sample water.

NOTE: Because you are not dealing with suspensions here, three replicate wells is usually sufficient. Use more if you like.

NOTE: This protocol assumes that activity is being measured at about ambient water pH. If you want to measure activity at a controlled pH, use an appropriate buffer to prepare the DOPA solution.

NOTE: There is no peroxidase assay in this procedure because added peroxide is likely to be detrimental to your sample. Try it if you like. You should probably run some preliminary tests at varying peroxide concentrations.

4. Incubate the plates at 20 C. An overnight incubation may be needed to measure phenol oxidase activity in most surface waters.

5. Read absorbance at intervals of one to several hours using a microplate spectrophotometer with a 460 nm filter.

6. Calculate results as described above, remembering that sample volume here is only 0.2 ml.

final OD = (mean OD of sample wells - mean OD of negative control wells - mean OD of blank wells)

Activity ($\mu mol\ h^{-1}\ mL^{-1}$) = final OD / ($7.9\ \mu mol^{-1}$)(0.2 mL)(incubation time, h)