



# STANDARD OPERATING PROCEDURES

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## PLANT PEROXIDASE ACTIVITY DETERMINATION

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### 1.0 SCOPE AND APPLICATION

The objective of this standard operating procedure (SOP) is to describe procedures for determining the peroxidase (POD) activity of plant tissues. Peroxidases are found throughout the plant kingdom. An increase in peroxidase activity has been associated with environmental stresses on plants.<sup>(1-3)</sup> This analysis, in conjunction with other plant physiological and toxicological techniques, will be used to assess the impact of contaminants on plants.

These are standard (i.e., typically applicable) operating procedures which may be varied or changed as required, dependent upon site conditions, equipment limitations, or limitations imposed by the procedure. In all instances, the ultimate procedures employed should be documented and associated with the final report.

Mention of trade names or commercial products does not constitute U.S. Environmental Protection Agency (U.S. EPA) endorsement or recommendation for use.

### 2.0 METHOD SUMMARY

In this method, one gram of fresh plant tissue is ground with a calcium chloride solution. The resulting slurry is clarified by centrifugation. An assay mixture is prepared with two solutions, one containing peroxide as the substrate and a buffer at a pH that has been determined to be optimal for peroxidase activity for the specific sample type. The other solution contains reagents that react with the products of the enzyme action on the peroxide to produce a color change. The crude extract is added to the assay mixture and absorbance readings are monitored for two minutes in a spectrophotometer. The absorbance change indicates the level of POD activity and this data is compared among samples.

### 3.0 SAMPLE PRESERVATION, CONTAINERS, HANDLING AND STORAGE

Tissue samples must be frozen in liquid nitrogen (-196°C) to prevent peroxidase degradation; this can be accomplished by placing these samples in proper sample containers and into a liquid nitrogen Dewar. If it is not practical for tissue samples to be placed under liquid nitrogen conditions immediately after collection, harvested tissue samples should be immediately placed into labeled plastic bags and into a cooler with ice until tissues can be frozen in liquid nitrogen. No more than one-half hour should pass from the time of sample collection to the time of sample freezing. Samples frozen in liquid nitrogen should not be held longer than fourteen days before analysis.

### 4.0 INTERFERENCES AND POTENTIAL PROBLEMS

If the handling and storage precautions detailed above are not carried out, results will be inaccurately low, and valid comparisons cannot be made. Inaccurate pipetting in sample dilution will cause magnification of errors. Additionally, personnel expected to perform this analysis must have experience in basic laboratory procedures and training in this SOP.



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### 5.0 EQUIPMENT/APPARATUS

Equipment required for collection of tissue in the field includes shears or a knife, appropriate protective gloves, resealable plastic bags, wet ice, coolers, liquid nitrogen Dewar, and associated cryogenic sample containers, sample labels, markers, logbook, data sheets, Chain of Custody records, and custody seals.

Equipment required for the laboratory analysis will include:

- a balance and weighing containers
- a pH meter and standard buffers
- mortars and pestles
- ice bucket/container with ice
- test tubes with 10 milliliter (mL) capacities
- tabletop/clinical centrifuge
- 12-mL borosilicate glass centrifuge tubes
- tube racks
- mechanical pipette(s) capable of dispensing volumes from 200 microliter ( $\mu$ L) to 2 mL
- Pasteur pipettes and bulbs
- waterbath (set at 25°C)
- a visible light spectrophotometer [set at 510 nanometers (nm)] with an optically matched set of cuvettes (1-cm cell size)
- timer
- microspatula (optional)
- all glassware necessary to prepare and hold reagents
- laboratory markers/labeling tape
- laboratory bench protection (optional)
- lint-free paper wipes
- logbook(s)

### 6.0 REAGENTS

The following reagents are needed for peroxidase extraction and determination:

- 0.5 Molar (M) calcium chloride solution
- Buffer solutions:
  - M 2-(N-Morpholino)ethanesulfonic acid (MES) solutions at pHs 5.5, 6.0, and 6.5
  - M N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) at pHs 7.0, 7.5, and 8.0
- Solution A: phenol-aminoantipyrene solution
- Solution B: hydrogen peroxide in buffer solution

Preparation of 0.5 M calcium chloride solution is as follows:

5.55 grams (g) of calcium chloride are required for every 100 mL of solution made. Place the calcium chloride in a graduated glass container and add distilled or deionized (DI) water to the



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final volume. At least 10 mL of this solution is necessary for every sample assayed. This solution may be stored refrigerated for up to two months prior to use. Cool this solution on ice prior to use in assay.

Preparation of the MES buffer solutions are as follows:

Make 0.02 M MES buffer solution by dissolving 293 milligrams (mg) in 75 mL of DI water. Separate this solution into three 25 mL quantities and adjust the pH of each individually to 5.5, 6.0, and 6.5. Adjust the pH upwards slowly with 1 M sodium hydroxide until the desired pH is reached.

Preparation of the HEPES buffer solutions are as follows:

Make 0.02 M HEPES buffer solution by dissolving 357 mg in 75 mL of DI water. Separate this solution into three 25 mL quantities and adjust the pH of each individually to 7.0, 7.5, and 8.0. Adjust the pH upwards slowly with 1 M sodium hydroxide until the desired pH is reached.

Preparation of Solution A is as follows:

Phenol (810 mg) and 4-aminoantipyrene (25 mg) are placed in a graduated glass container and brought to 50 mL with DI water. Prepare this solution fresh each assay day. Prepare and store this solution under proper ventilation using proper personal protective equipment.

Preparation of Solution B is as follows:

Hydrogen peroxide (0.5 mL of 30% H<sub>2</sub>O<sub>2</sub>) is added to the MES or HEPES buffer solution after pH adjustment, and the volume is brought to 50 mL to make a 3% H<sub>2</sub>O<sub>2</sub> solution with a 0.01 M final buffer concentration. Always prepare this solution the day the POD assays are to be performed.

## 7.0 PROCEDURES

After preparing the reagents, the first step in the POD activity determination is to prepare crude enzyme extract (Section 7.1) for the determination of optimal pH conditions for the particular sample type (Section 7.2). Once the optimal pH is determined, the tissue samples are ground to prepare an enzyme extract and then assayed spectrophotometrically for POD activity (Section 7.3). Repeat POD activity assay as necessary to complete all samples required. Sample data is then statistically analyzed.

### 7.1 Preparation of Crude Enzyme Extract

Follow this procedure to produce a crude enzyme extract:

1. Place one gram of fresh or frozen plant tissue into a cold mortar with 5 mL of ice cold 0.5 M calcium chloride solution.
2. Macerate tissue with a cold pestle to extract POD.
3. Centrifuge this slurry at 1000 x gravity for 8 minutes. Pour supernatant (containing POD) into a clean and labeled test tube and store on ice.



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4. Resuspend the cell wall pellet remaining in the centrifuge tube with 2.5 mL of ice cold calcium chloride solution (0.5 M) and centrifuge again. Pour supernatant in with the first supernatant collected. Repeat this step once.
5. Store supernatant (crude extract) on ice for up to two hours prior to conducting the POD assay.

The total amount of calcium chloride solution used was 10 mL, thus a 10:1 volume: mass dilution was made. Adjustments can be made to the dilution factor for a particular tissue sample assayed as long as all of the samples to be compared with each other are diluted by the same factor.

### 7.2 Selection of Optimal pH

The pH optima of different POD are not identical. Therefore, the pH most suitable for the POD in the given samples must be determined.

In order to test for optimal pH conditions for a particular plant species and tissue, buffers from a pH range of 5.5 to 8.0 are used. Acidic conditions are tested with MES buffer solutions at pHs 5.5, 6.0, and 6.5. Conditions at or above neutral are tested using HEPES buffer solutions at pHs 7.0, 7.5, and 8.0. The pH buffer solution that provides the greatest POD activity is chosen.

Once the buffer solution with the optimal pH is selected, this buffer solution is then utilized in Solution B for all of the samples of that species and tissue type.

To standardize the assay, and report the results as units of POD activity, horseradish peroxidase of known activity (units/mg) is dissolved in calcium chloride and assayed in the same manner as the unknown samples.

### 7.3 Spectrophotometric Peroxidase Determination

This portion of the assay is as follows:

1. Bring buffer solution(s) to 25°C with a waterbath before using in assay. Place 1.4 mL of solution A and 1.5 mL of solution B into a 3-mL cuvette.
2. Immediately prior to measuring - place crude extract or standard horseradish POD (200 µl) into the cuvette and briefly stir cuvette contents with tip of Pasteur pipette or clean microspatula.
3. Measure the change in absorbance at 510 nm for 2 minutes in the spectrophotometer, recording the initial and ending absorbance readings.

## 8.0 CALCULATIONS

The following calculations will be made to ascertain peroxidase activity in a sample:

$$\text{Change in } A_{510} = A_f - A_i$$



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where:

$A_i$  = initial absorbance reading

$A_f$  = final absorbance reading

A regression equation is calculated from the standard horseradish peroxidase results, and the change in  $A_{510}$  for each sample is substituted into the equation to find the units of POD activity. Note that peroxidase activity will be expressed on a plant protein basis (refer to ERT/SERAS SOP# 2033, "Plant Protein Determination"). Units of POD are divided by the soluble protein in the sample (in micrograms) to give the specific activity.

### 9.0 QUALITY ASSURANCE/QUALITY CONTROL

A sampling plan, which includes appropriate sample size estimates, will be created prior to sampling and followed during sample collection.

All data must be documented on field data sheets and in logbooks. Samples will also be duplicated at a minimum rate of ten percent. The order of sample analyses will be randomized between the contaminated and reference area samples.

All instrumentation must be operated in accordance with the operating instructions supplied by the manufacturer, unless otherwise specified in the work plan. The spectrophotometer will be properly calibrated according to the manufacturer's operating manual. The instrument's zero adjustment will be checked with the solvent blank between readings. Equipment checkouts and calibration activities must occur prior to sampling/operation and they must be documented. Additionally, calculations will be rechecked at a rate of ten percent by another person.

### 10.0 DATA VALIDATION

The data generated will be reviewed according to the quality assurance/quality control considerations listed in Section 9.0. Multiple analyses will be carried out on each plant and data will be statistically analyzed.

### 11.0 HEALTH AND SAFETY

When working with potential hazardous materials, follow U.S. EPA, OSHA, and corporate health and safety procedures.

Appropriate protective gloves will be worn when collecting plant samples and when working in the laboratory. Additionally, those individuals preparing reagents and performing this assay should be familiar with the appropriate chemical material data safety sheets.

### 12.0 REFERENCES

- (1) Van Assche, F., C. Cardinaels, and H. Clijsters, "Induction of enzyme capacity in plants as a result of heavy metal toxicity: dose-response relations in *Phaseolus vulgaris* L. treated with zinc and cadmium", *Environmental Pollution*, vol. 52, 1988, pp. 103-115.



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- (3) Byl, T., Memphis State University, Personal Communication, December 19, 1991.