Chapter 47
Phenol Oxidation

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1 Introduction

Lignins are major structural components of plant cell walls and therefore of plant litter (Sarkanen and Ludwig 1971). They are complex polymers of a small number of methoxylated phenolic compounds such as coumaryl alcohol, sinapyl alcohol, and coniferyl alcohol (Boerjan et al. 2003). Due to strong C-C linkages and alkyl-aryl ether bonds, lignins effectively resist chemical and enzymatic attack (Hagerman and Butler 1991). Therefore, lignin degradation requires phenol oxidation (Breznak and Brune 1994). Other important phenolic litter constituents include condensed tannins (Harrison 1971; Savoie and Gourbiére 1989; see Chap. 19), which are regularly structured polymers of flavan-3-ols and flavan-3,4-diols that are linked through C-C bonds between the monomers (Swain 1979; Hagerman and Butler 1991). As with lignins, the degradation of condensed tannins begins with oxidation. Hydrolyzable tannins, in contrast, are glucose esters of gallic acid or ellagic acid units and are hence subject to hydrolysis by esterases.

The degradation of the lignin moiety of lignocellulose (see Chap. 46) is strongly dependent on microbial activity (Breznak and Brune 1994). However, not every microbial species involved in decomposition is capable of degrading lignocellulose (Ljungdahl and Eriksson 1985). In contrast to brown- and white-rot fungi, which are

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primarily terrestrial, the litter-degrading soft-rot and other fungi (mostly Ascomycetes and Deuteromycetes) prominent in aquatic environments are only weakly adapted to degrading lignin (Rabinovich et al. 2004).

Numerous enzymes are involved in phenol oxidation (Sinsabaugh 2010). Laccases (EC 1.10.3.2) have been found in plants and many fungi (Mayer 1987), but only in one bacterium (Faure et al. 1995). Tyrosinases (EC 1.14.18.1) are known from fungi (Wood 1980; Claus and Filip 1990), actinomycetes (Claus and Filip 1990), and plants (Summers and Felton 1994). Both laccases and tyrosinases may be important in wood and leaf-litter decomposition (Wood 1980; Thurston 1994). Catechol oxidase (EC 1.10.3.1) has a similar function as laccases in many plants and fungi (Mayer 1987), and is involved in the oxidative polymerization of phenolics. Phenol-oxidizing enzymes of invertebrate origin are mostly considered to be involved in moulting, wound-healing, and immune response, but there is evidence suggesting that hemocyanin of crustaceans can be activated into a phenoloxidase-like enzyme (Jaenicke et al. 2009) that might serve in the oxidative breakdown of ingested lignins and other phenolic compounds (Cragg et al. 2015). Along this line of argument, Yoruk and Marshall (2003) suggest adding SDS (up to 2 mM) to assays of phenol oxidase activity to activate these enzymes. It should be kept in mind, however, that the outcome of such measurements will not reflect the natural conditions in the environment.

Determining the activity of phenol-oxidizing enzymes can be based on (1) the decrease in the concentration of a particular substrate, or (2) the increase in oxidation products, which potentially covers the combined activities of several enzymes with different substrate affinities when using a common substrate. Much more frequently than in decomposition studies, phenol oxidation is measured in the context of invertebrate immune response (e.g., Laughton and Siva-Jothy 2011). The corresponding techniques cannot be transferred directly to decomposition studies, but the basic approach, relying on the brownish color of oxidation products of most phenolic compounds, is the same. It is virtually impossible to determine individual activities of any of the various phenol-oxidizing enzymes in an environmental sample.

Since the oxidation products of phenolic compounds (called quinones) are not clearly defined, no specific extinction coefficient can be determined. The method therefore yields only relative phenol oxidation capacity (ΔA mg^{-1} h^{-1}), and, thus, comparison of the data is confined to using the same substrate. These quinones are prone to further cross-reaction and polymerization, eventually resulting in undefined melanins. To prevent the quinones formed upon oxidation of, for example, catechol as an often-used substrate to determine phenol oxidase activity, from further chemical change, Perucci et al. (2000) suggest adding L-proline to the assay to stabilize quinone and the corresponding red-brownish coloration of the assay.

The method by Zimmer and Topp (1998) described here provides an estimate of the overall phenol oxidation capacity based on the amount of oxidation products. To this end, a suitable phenolic substrate (see Faure et al. 1995) is mixed with the sample, and the change in absorbance resulting from the release of colored (brownish) oxidation products is followed over time. However, different substrates may be indicative of different types of phenol oxidases, such as *o*-diphenol oxidase

(catecholase), oxidizing, for example, catechol or L-3,4-dihydroxyphenylalanine (L-DOPA); monophenol oxidase (tyrosinase), oxidizing, for example, tyrosine to L-DOPA; laccase, oxidizing, for example, syringaldazine or the artificial substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) that is often used for determining phenol oxidase activity but is highly sensitive to the pH of the assay.

2 Equipment and Materials

2.1 *Equipment*

- Homogenizer (e.g., electronic disperser or mortar and pestle for leaf litter; rotation grinder, or ultrasonic disintegrator for gut and feces samples)
- Incubation tubes: glass tubes with screw caps (15–20 ml) for leaf litter and plastic reaction tubes (1.5 ml) for gut and feces samples
- Analytical balance
- Shaker
- Centrifuge (10,000 g).
- Micropipettes (100–1000 μl; 10–100 μl)
- Plastic cuvettes
- Spectrophotometer (340 nm)

2.2 *Materials*

- Leaf litter collected in the field
- Dissected guts of detritivores having fed on leaf litter (gut epithelium preferably removed)
- Feces of detritivores having fed on leaf litter

2.3 *Chemicals and Solutions*

- 0.05 M phosphate buffer: 415 ml 0.1 M KH_2PO_4 + 85 ml 0.1 M Na_2HPO_4 + 500 ml distilled water, pH 6.2; if prepared accurately, the pH does not need to be adjusted
- 50 mM catechol and 50 mM L-proline in 0.05 M phosphate buffer, pH 6.2
- Depending on the phenol oxidase investigated, phenolic substrates other than catechol may be more appropriate (e.g., gallic acid, L-DOPA, tyrosine, syringaldazine). The wavelength for photometric determination of oxidation products may have to be adjusted accordingly (Faure et al. 1995; Zimmer and Topp 1998); for the 4-(*N*-proline)-*o*-benzoquinone product of proline and the catechol-derived quinone, a wavelength of 525 nm is most appropriate (Perucci et al. 2000)

- Depending on the phenolic substrate, the addition of up to 20% ethanol may be necessary to dissolve the substrate (Faure et al. 1995; Zimmer and Topp 1998)

3 Experimental Procedures

3.1 *Extraction of Microbial Enzymes*

1. Weigh samples of leaf litter (corresponding to 50–100 mg dry mass), dissected guts (5–10 mg), or feces (5–10 mg).
2. Determine dry mass/fresh mass ratios to estimate dry mass of samples from fresh mass.
3. The appropriate method of enzyme extraction depends on the source of enzymes; with extracellular enzymes, thoroughly chopping up samples with a homogenizer is sufficient for accurate measurement of enzyme activity; with cell-bound enzymes, additional sonication is recommended to release enzymes into the supernatant.
4. Homogenize litter samples in 10 ml of 0.05 M phosphate buffer or gut or feces samples in 1 ml of 0.05 M phosphate buffer. Homogenization must be done on ice to avoid thermal denaturation of enzymes.
5. Homogenates may be stored frozen (−20 °C) until used for assays.
6. Centrifuge suspensions (5 min; ca. 10,000 g, depending on the available centrifuge and reaction tubes; 4 °C).

3.2 *Determination of Phenol Oxidase Activity*

1. Add 100 µl of the supernatant to 900 µl of 50 mM catechol solution and mix thoroughly.
2. Follow change in absorbance (ΔA) at 340 nm at 1-min intervals for the first 10 min.
3. Determine relative catechol oxidation as mean ΔA per min by linear regression analysis.
4. Calculate relative phenol oxidase activity (ΔA mg^{-1} h^{-1}) as:

$$\text{phenol oxidase activity} = \frac{\Delta A \times 60 \times \text{dilution factor}}{\text{sample dry mass}} \tag{47.1}$$

where the dilution factor = 100 for litter and 10 for guts and feces.

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