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Role of laccases and peroxidases in saprotrophic activities in the lichen *Usnea undulata*

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ARTICLE INFO

Article history:
Received 22 August 2014
Revision received 1 December 2014
Accepted 1 December 2014
Available online
Corresponding editor:
Darwyn Coxson

Keywords:
Laccase
Lichens
Peroxidase
Saprophyte
Usnic acid

ABSTRACT

Lichens produce various oxidoreductases including heme-containing peroxidases and the copper-containing phenol oxidases tyrosinase and laccase. Our earlier findings suggested that significant oxidoreductase activity occurs mainly in lichens from the order Peltigerales. Here we show that the non-Peltigeralean lichen *Usnea* can display significant activities of peroxidases and laccases. Strong evidence for the involvement of peroxidases and laccases in saprotrophic activities comes from the observation that their activities are induced by "starvation" due to prolonged dark storage, and also by treatment with soluble cellulose and lignin breakdown products. We also show that, given a quinone and chelated Fe, *Usnea* can produce hydroxyl radicals; these radicals contribute to the break down of carbohydrates or lignin. However, hydroxyl radical production is independent of laccase and peroxidase activity. Laccases and peroxidases are involved in other aspects of lichen biology; here we show that peroxidases, but not laccases, can break down lichen substances. Reduction in the amounts of lichen substances will reduce photoprotection, which will increase the photosynthetic capacity of thalli during winter when light intensities are low.

Introduction

Lichens produce various metal-containing oxidoreductases such as the copper-containing phenol oxidases tyrosinase and laccase, and heme peroxidases (Beckett et al., 2013b). Our earlier work showed that significant oxidoreductase activity only occurs in lichens from the order Peltigerales (Beckett et al., 2012, 2103a); here we used freshly collected material that was slowly dried and stored for no more than 4 weeks. In Peltigeralean lichens the average laccase, tyrosinase and peroxidase activities were 19.7, 17.0 and 12.7 units g^{-1} dry mass, while the corresponding activities from non-Peltigeralean species were 0.2, 0.7 and 0.1 respectively. However, our later work showed that significant laccase and peroxidase activities can be induced in some non-Peltigeralean lichens by hydrating them for several days in the dark (Beckett et al., 2014), suggesting that the occurrence of these enzymes may be more widespread. The first aim of the present study

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http://dx.doi.org/10.1016/j.funeco.2014.12.004

was to investigate the basic properties of laccases and peroxidases from a non-Peltigeralean lichen using *Usnea undulata* as a model system, and to compare them with the enzymes from Peltigeralean lichens.

The second aim of this study was to investigate the physiological roles of laccases and peroxidases in lichen biology. Here we considered three possible roles: first, a role in saprotrophic activities, second, a role in reactive oxygen species (ROS) scavenging, and third, a role in the metabolism of lichen secondary metabolites. A first role, by analogy with free-living fungi, could be to allow lichens to participate in facultative saprotrophy. Such activity may provide an additional carbon supply to the mycobiont during periods of low photosynthate supply or during intensive growth periods, as suggested for mycorrhizal fungi (Talbot et al., 2008). In saprotrophic free-living fungi, lignin breakdown products often induce laccase activity (Strong, 2011), and so the ability of the lignin breakdown product xylidine to increase enzyme activity was tested. Laccases and peroxidases have been implicated in cellulose breakdown (Martinez et al., 2009), so we also tested whether soluble cellulose can increase enzyme activity. While laccases and peroxidases can directly metabolize a wide variety of carbon containing substrates, it has been proposed that they can also work indirectly by producing ROS by, for example, using extracellular redox cycling reactions (Gómez-Toribio et al., 2009a,b). *U. undulata* was, therefore, manipulated to display different activities of laccase and peroxidases, and then tested for ability of thalli to produce hydroxyl radicals. A second role of oxidoreductases, particular peroxidases, is ROS scavenging. We tested whether treating lichens with the artificial ROS-generating agent menadione increases enzyme activity, as in free-living fungi (Ruiz-Dueñas et al., 1999). Finally, the ability of lichen laccases and peroxidases to metabolize lichen secondary metabolites was tested. Lichens often contain a variety of secondary metabolites, for example in *U. undulata* mainly usnic acid. The mechanism of usnic acid breakdown by lichens is currently unknown. A major role of usnic acid may be to screen excess sunlight (Solhaug and Gauslaa, 2012). Lichens typically contain lower concentrations of usnic acid in winter compared with summer (Bjerke et al., 2005), presumably because excessive protection when light levels are low will reduce photosynthetic capacity. We therefore tested the ability of lichen laccases and peroxidases to break down usnic acid extracted from *U. undulata*.

The overall aim of these experiments was to increase our understanding of the roles of these enzymes in lichen biology, including involvement in saprotrophic activities and photoprotection conferred by secondary metabolites.

Materials and methods

Lichen material

U. undulata, *Leptogium saturninum* and *Pseudocyphellaria aurata* were collected from Nottingham Road, South Africa (S29.412647, 29.913586). The collection site was classic Afromontane forest, characterized by warm wet summers and cold dry winters (Low and Rebelo, 1996). *Peltigera rufescens* was collected from boreal forest on the outskirts of Kazan, Russian Federation (N55.831613, 49.004731). Thalli were allowed to air dry slowly between sheets of newspaper, and then stored refrigerated for a maximum of 2 weeks before use.

Electrophoretic studies

The presence of laccases and peroxidases was tested electrophoretically in crude extracts of *U. undulata*, as described by Beckett et al. (2013a). A modified method of Laemmli (1970) was followed, using native 5 and 12 % polyacrylamide gels (PAGE). All chemicals were obtained from Sigma–Aldrich (St Louis, USA). Activity staining was done in Na-acetate buffer (0.25 M, pH 5.0) containing 10 % glycerol and 1 mM *o*-dianisidine for laccases. For peroxidases, the staining solution contained in addition 2 mM H_2O_2. Molecular mass markers were always run at the same time and stained with Coomassie Blue. Superoxide ($O_2^{\bullet -}$) production was tested in the presence or absence of NADH (0.4 mM) by staining with nitro blue tetrazolium chloride (0.5 mM NBT, López-Huertas et al., 1999). Specificity of the assay was checked by addition of superoxide dismutase (SOD, 100 units ml^{-1}).

Enzymatic reactions

Crude extracts were prepared by thoroughly grinding thalli in 50 mM phosphate buffer pH 7 with a little aluminium oxide to facilitate grinding. Extracts were centrifuged at 5 000 *g* for 20 min, and the supernatant taken for further analysis. Laccase activity was determined by following the oxidation of 2,2′-Azino-bis(3-ethylbenzthiazolin-6-sulfonat) (0.3 mM, ABTS, Eggert et al., 1996) at 420 nm ($\varepsilon_{420} = 36\ mM^{-1}\ cm^{-1}$) in Na-acetate buffer (100 mM, pH 4.5). Peroxidase activity was measured as the stimulation in activity following the addition of 0.1 mM H_2O_2. The effect of pH on enzyme activity was tested using 1 mM 2,6-dimethoxylphenol (DMP) as a substrate and measuring the production of coerulignone ($\varepsilon_{469} = 27.5\ mM^{-1}\ cm^{-1}$) (Gómez-Toribio et al., 2009a,b) in the pH range from 3.0 to 6.0 in 50 mM citrate-phosphate buffer. Laccases' and peroxidases' thermostability was tested by pretreating crude extracts at 40 °C for different periods, and then measuring enzyme activity with ABTS as described above. For comparison, the stability of the enzymes from the Peltigeralean lichens *L. saturninum* (peroxidase) and *P. aurata* (laccase) was also determined.

The effect of hydration and effectors on laccase and peroxidase activity

Activity was measured in dry material, and then the remaining material transferred to air at 100 % RH at 10 °C in the dark. Enzyme activities were measured after 1 and 2 d, and then the remaining material transferred to wet non-cellulosic cloth. Activity was measured after 1 d, and then at intervals until 17 d after the start of the experiment. At each time point, three replicates, comprising the equivalent of *c.* 0.5 g dry mass of lichens (each replicate containing thallus segments from several individual thalli), were sampled.

We tested the effect on enzyme activity of treating lichens with soluble cellulose (carboxymethyl cellulose, CMC), the classic laccase inducer 2,5-xylidine (2,5-dimethylaniline)