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Occurrence of high tyrosinase activity in the non-Peltigeralean lichen *Dermatocarpon miniatum* (L.) W. Mann

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Abstract: In our earlier work, we demonstrated the presence of the multicopper oxidases tyrosinase and laccase in the cell walls of lichens from the *Peltigerales*, while these enzymes appeared to be absent in lichens from other orders. Likely roles for tyrosinase in lichens include melanin synthesis, the generation of quinones needed for laccase-mediated redox cycling, and the removal of harmful reactive molecules formed by this cycling. Non-Peltigeralean lichens will not need tyrosinase to detoxify laccase-generated radicals. However, many non-Peltigeralean lichens are often heavily melanized. Apparent absence of tyrosinase activity in these species prompted us to suggest that, in these lichens, melanins are probably synthesized by the polyketide pathway, which does not involve tyrosinase. Here, we surveyed intracellular tyrosinase activity in thallus homogenates from a range of lichens. Results showed that Peltigeralean species generally have much higher activities than species from other orders. However, the non-Peltigeralean lichen *Dermatocarpon miniatum* displays significant tyrosinase activity. In *D. miniatum*, tyrosinase differs from the corresponding enzyme from Peltigeralean lichens with respect to cellular location, substratum specificity, stability and pH optimum. Furthermore, unlike Peltigeralean lichens, in *D. miniatum* tyrosinase activity increased strongly following the rehydration of dry thalli. These differences are possibly a consequence of the role of tyrosinase in melanin synthesis rather than laccase-mediated redox cycling.

Key words: dessication, laccase, melanin, phenol oxidase, polyketide

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Introduction

Copper-containing enzymes occur in all organisms, and play important roles in the biological activation of oxygen, necessary for the oxidation of a great variety of different substrata (Solomon *et al*. 2001). Multicopper oxidases include mononuclear copper enzymes (e.g. amine oxidase), coupled binuclear copper enzymes (e.g. tyrosinase) and enzymes containing trinuclear copper clusters (e.g. laccase, pigment oxidase, ferroxidase and ascorbate oxidase) (Solomon *et al*. 2001; Nakamura & Go 2005). These enzymes have overlapping substratum specificities and can co-occur in the same samples, making their identification difficult (Ratcliffe *et al*. 1994; Baldrian 2006). Surveys of lichens using assays with intact thalli or leachates indicate that Peltigerlean lichens contain both laccases (EC 1.10.3.2) and tyrosinases (EC 1.14.18.1) (Laufer *et al*. 2006*a*, *b*; Zavarzina & Zavarzin 2006), and that phenol oxidase activity is largely absent in lichens from other orders.

Recently, progress has been made in establishing the structure and function of lichen laccases (Lisov *et al*. 2012), while lichen tyrosinases remain largely unstudied. In free-living fungi, a major role of tyrosinases is eumelanin synthesis (Bell & Wheeler 1986). Eumelanins are produced by a two-step reaction involving first the oxidation of tyrosine to *o*-dihydroxyphenylalanine (DOPA) by tyrosinase working as monophenol oxidase, and second by the conversion of DOPA to

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TABLE 1. *Total tyrosinase activity after collection (dry), and following rehydration in a range of lichen species. All measurements were made with 2 mM DOPA at pH 6 in 50 mM phosphate buffer.*

Species		Enzyme Activity (units g^{-1} dry mass) after collection and slow drying	after rehydration
Peltigerales			
Leptogium saturninum (Dicks.) Nyl.	Monk's Cowl, RSA	4·3±0·1	4·2±0·5
Lobaria pulmonaria (L.) Hoffm.	Nottingham Road, RSA	2·5±0·3	1·9±0·3
L. scrobiculata (Scop.) DC.	Nottingham Road, RSA	7·0±0·4	5·3±0·0
Peltigera didactyla (With.) J. R. Laundon	Zittau, Germany	82·0±5·9	83·6±6·8
P. malacea (Ach.) Funck	Kazan, Russia	36·2±1·6	ND†
P. membranacea (Ach.) Nyl.	Kazan, Russia	13·5±0·4	ND
P. polydactylon (Necker) Hoffm.	Kazan, Russia	12·3±0·4	18·9±5·0
Pseudocyphellaria aurata (Ach.) Vain.	Nottingham Road, RSA	3·7±0·1	1·9±0·3
Sticta limbata (Sm.) Ach.	Nottingham Road, RSA	3·2±0·1	5·7±0·0
S. sublimbata (J. Steiner) Swinscow & Krog	Nottingham Road, RSA	4·8±0·3	4·0±0·2
Non-*Peltigerales*			
Cladonia portentosa (Dufour) Coem.	Zittau, Germany	0·4±0· 0	0·4±0·1
C. rangiformis Hoffm.	Kazan, Russia	0·0±0·0	0·0±0·0
Dermatocarpon miniatum (L.) W. Mann	Zittau, Germany	2·5±0·0	9·6±0·3
Cetraria islandica (L.) Ach.	Kazan, Russia	0·0±0·0	0·0±0·0
Lasallia pustulata (L.) Mérat	Zittau, Germany	0·5±0·0	0·6±0·1

* Data given ± standard deviation, $n = 5$ preparations
† not determined.

dopaquinonone by tyrosinase working as a diphenol oxidase. Dopaquinone then undergoes spontaneous cyclization to form brown or black pigments. By contrast, allomelanins are formed by the oxidation of di-(DHN) or tetrahydroxynaphthalene, via the pentaketide pathway (Plonka & Grabacka 2006). It is not known which forms of melanins occur in lichens (Muggia *et al.* 2009), but melanins are widely distributed in lichens, and are found in both Peltigeralean and non-Peltigeralean species. Melanins are believed to protect the photobiont again excessive light and UV-B, as melanin concentrations increase following exposure to UV-B (Nybakken & Julkunen-Tiitto 2006; McEvoy *et al.* 2007; Nybakken *et al.* 2007; Larsson *et al.* 2009). Based on the known distribution of tyrosinases in lichens, it could be predicted that lichens from the *Peltigerales* synthesize melanin using tyrosinases, while others use polyketide synthases, enzymes that are present in most lichens (Muggia & Grube 2010).

As part of an ongoing detailed microcharacterization of lichen tyrosinases, a range of both Peltigeralean and non-Peltigeralean species were tested for cytosolic rather that cell wall activity. While in general cytosolic tyrosinases activities were consistent with earlier reports based on assays of cell wall bound enzymes, unusually high cytosolic tyrosinase activity was found in the non-Peltigeralean genus *Dermatocarpon*. The aim of the work presented here was to study *Dermatocarpon* tyrosinase in more detail, and to determine whether its properties differ from those of Peltigeralean tyrosinases. In addition, to test whether tyrosinase may play a role in desiccation tolerance in lichens, we compared activities in dry and rehydrated thalli.

Materials and Methods

The species of lichens examined in this study and their collection localities are listed in Table 1. Specimens were collected from the field either dry or hydrated. If hydrated, they were allowed to dry slowly between sheets

TABLE 2. *Comparison of tyrosinase activity from* Dermatocarpon miniatum (Verrucariales, *Eurotiomycetes*) *with that from* Peltigera polydactylon (Peltigerales, *Lecanoromycetes*) *with and without 3-methyl-2-benzothiazolinone hydrazone (MBTH). Experiments carried out with 2 mM o-dihydroxyphenylalanine (DOPA), 10 mM phenol or 5 mM tyramine in 50 mM phosphate buffer pH 6.*

Phenol	MBTH	Effector	Tyrosine activity (μmol min^{-1} g^{-1} dry mass)	
			Dermatocarpon miniatum	*Peltigera polydactylon*
DOPA	−	−	2·22 ± 0·07*	14·07 ± 0·24
DOPA	−	1 mM SDS	0·17 ± 0·08	23·87 ± 5·06
DOPA	+		1·80 ± 0·06	5·24 ± 0·04
DOPA	+	1 mM KCN	0·17 ± 0·02	0·88 ± 0·14
DOPA	+	40 mM NaF	0·09 ± 0·03	0·40 ± 0·05
Phenol	+		0·11 ± 0·03	1·49 ± 0·04
Tyramine	+		1·46 ± 0·16	1·83 ± 0·05
K_m DOPA†	−		0·45 ± 0·06 mM	1·55 ± 1·14 mM
Ratio monophenolase (phenol) / diphenolase (DOPA)			6%	28%

* Figures given ± standard deviation, $n = 5$ preparations.
† Concentration range for determining K_m of DOPA was 0·2 to 2 mM.

of newspaper. Lichens were stored refrigerated at 5°C for up to two weeks before use. In all cases, large collections of lichens were made, and the thalli used were randomly sampled from those that appeared most healthy. Lichens (typically up to 1 g dry mass) were thoroughly ground in 50 mM phosphate buffer pH 7 using a little aluminium oxide to facilitate grinding, and centrifuged at 5000 g for 20 min. Tyrosinase activity was estimated in the resulting supernatant by following the oxidation of 2 mM L-dihydroxyphenylalanine (DOPA; Sigma) to 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome; Horowitz *et al*. 1970) in 50 mM phosphate buffer pH 6. The extinction coefficient of the product measured at A_{475} is 3·6 mM^{-1} cm^{-1}. Activities were expressed as units g^{-1} dry mass. To test whether tyrosinase activity is affected by water stress, activity was first measured in dried lichen thalli. Replicate samples were then slowly rehydrated by storage at 10°C in darkness, in air at 100% relative humidity (RH) for 24 h, followed by storage for a further 24 h under the same conditions, but on wet non-cellulosic cloth. Hydrated lichens were then ground and tyrosinase activity measured as for desiccated lichens.

To further characterize the tyrosinase from *D. miniatum*, activity was compared with that from extracts of *Peltigera polydactylon* (hydrated material was used in both cases). The effect of the known tyrosinase effectors 1 mM sodium dodecyl sulphate (SDS), 1 mM KCN and 40 mM NaF on enzyme activity was tested in both species. To test the ability of the tyrosinases to oxidize the monophenols tyramine and phenol, the 'hydrazone' method of Rodrígues-López *et al*. (1994) was employed, involving the reaction of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with quinone produced by the oxidation of monophenols. The effect of pH on enzyme activity was also tested using McIlvaine's citrate-phosphate buffer (50 mM) from pH 3 to 6·5.

Results

While total activities of tyrosinase were usually much higher in Peltigeralean than in non-Peltigeralean lichens (Table 1), the non-Peltigeralean species *Dermatocarpon miniatum* displayed anomalously high tyrosinase activity. Rehydrating dry thalli had no consistent effect on tyrosinase activity in Peltigeralean lichens, but significantly increased activity in *D. miniatum*. Comparing tyrosinase from *D. miniatum* and *Peltigera polydactylon* revealed some interesting differences (Table 2). Both enzymes readily metabolized DOPA, with the enzyme from *D. miniatum* having a lower K_m. While the classic tyrosinase inhibitors KCN and NaF strongly inhibited activity in both species, the detergent SDS stimulated activity in *P. polydactylon*, but inhibited activity in *D. miniatum*. Both enzymes metabolized the monophenol tyramine, but the enzyme from *Peltigera* was much more effective at metabolizing phenol. The ratio of monophenol to diphenol oxidase activities (expressed as the rate of metabolism of phenol divided by the rate of metabolism of DOPA) was almost five times higher in *Peltigera* compared with *Dermatocarpon*. Unfortunately, attempts to concentrate the enzyme from *Dermatocarpon* to enable further characterization by chromatography or elec-

trophoresis were unsuccessful, as the enzyme rapidly lost almost all its activity (data not shown). The pH optimum of the tyrosinase from *Dermatocarpon* was lower than that from *Peltigera* (Fig. 1)

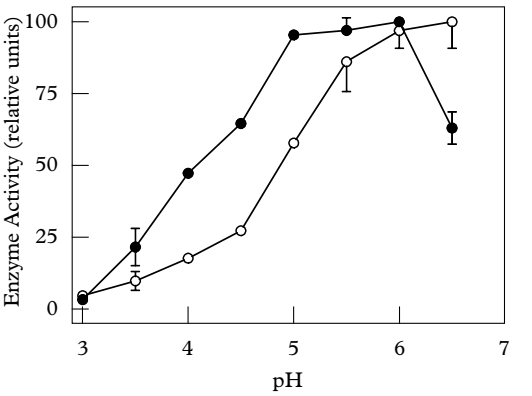

FIG. 1. Effect of pH on the activity of tyrosinases extracted from hydrated material of *Dermatocarpon miniatum* (closed circles) and *Peltigera polydactylon* (open circles) with 2mM DOPA as a substrate at 20°C. Above pH 7 DOPA precipitated. Activities expressed as a percentage of maximum activities (see Table 2 for actual values). Error bars indicate the standard deviation, $n = 3$ preparations, overlapping error bars removed.

Discussion

Results presented here clearly demonstrate that the lichen *Dermatocarpon miniatum* displays intracellular tyrosinase activity at rates comparable to those found in many Peltigeralean species. Previous surveys of cell wall tyrosinase activity in lichens have consistently shown that high activities occur only in the *Peltigerales* (Laufer *et al.* 2006*a*; Zavarzina & Zavarzin 2006; Beckett & Minibayeva 2007). Specifically, Beckett & Minibayeva (2007) found that cell surface tyrosinase activity is absent in *D. miniatum*. However, these earlier surveys did not include measurements of cytosolic tyrosinase activity. Full information on the cellular location of tyrosinases is available for only two Peltigeralean species (from the genera *Pseudocyphellaria* and *Peltigera*) and two non-Peltigeralean species (from the genera *Cetraria* and *Cladonia*) (Laufer *et al.* 2006*a*). This study reported strong cytosolic tyrosinase activity in both Peltigeralean lichens, but no activity in the non-Peltigeralean species. While results presented in Table 1 are broadly consistent with these findings, the non-Peltigeralean species *D. miniatum* is an exception, as it displays strong intracellular tyrosinase activity. Further investigation revealed that the kinetic properties of *Dermatocarpon* tyrosinase differ from those of *Peltigera* tyrosinase (Table 2). For example, stimulation of tyrosinase activity by the detergent SDS is common in tyrosinase from Peltigeralean lichens (Laufer *et al.* 2006*a*), but SDS inhibits *Dermatocarpon* tyrosinase. Stimulation of tyrosinase activity by SDS also occurs in free-living fungi (Espìn & Wichers 1999), but the mechanism of action is unknown. In addition, *Dermatocarpon* tyrosinase displays relatively poorer monophenol oxidase activity compared with *Peltigera* (Table 2), and the enzyme has a lower pH optimum (Fig. 1). While *Dermatocarpon* clearly possesses strong intracellular tyrosinase activity, significant differences exist between this enzyme and the enzyme that occurs in *Peltigera*.

Tyrosinase appears to play little role in desiccation tolerance in Peltigeralean lichens, as rehydrating desiccated thalli had no consistent effect on enzyme activity (Table 1). These results are in agreement with earlier reports showing no stimulation during rehydration of surface tyrosinase activity in *Pseudocyphellaria aurata* (Laufer *et al.* 2006*a*). By contrast, tyrosinase activity in *D. miniatum* was strongly stimulated by rehydration (Table 1). The greater increase in enzyme activity in *D. miniatum* found here could in part have been caused by the dry material having a slightly lower water content than the Peltigeralean species, as it was collected from an exposed rocky outcrop. As conversion of inactive to active forms of tyrosinase appears not to occur in *D. miniatum* (Table 2), the enzyme is probably upregulated during rehydration. In *D. miniatum*, tyrosinase may have a minor role in desiccation tolerance, for example by polymerizing reactive molecules formed during rehydration stress, or to strengthen cell walls against pathogen attack by synthesizing melanins. However, more

work is needed to establish the role of tyrosinases in desiccation tolerance in *D. miniatum*.

Differences between the tyrosinases in *Dermatocarpon* and *Peltigera* are probably a consequence of differences in the roles of the enzyme in the different species. Peltigeralean lichens almost all contain strong cell wall laccase activity (Zavarzina & Zavarzin 2006). Surveys have shown that the ratio between tyrosinase activity and laccase activity is remarkably similar in a range of species (Laufer *et al*. 2006*b*). Even in genera such as *Leptogium* where laccases are absent, peroxidases appear to take over their function (Liers *et al*. 2011). Extracellular laccases and peroxidases are probably involved in producing reactive oxygen species (ROS) that play several important roles in lichen biology, for example assisting lignocellulosic breakdown or in pathogen defence (Zavarzina *et al*. 2011). ROS can be produced via an indirect mechanism involving extracellular redox cycling, which has been described in detail in free-living fungi (Gómez-Toribio *et al*. 2009*a*, *b*). Briefly, laccases and peroxidases catalyze the conversion of hydroquinones to quinone radicals, which then spontaneously form quinones, producing superoxide ($O_2^{\cdot -}$) or other radicals as byproducts. Hydroquinones can be regenerated from the quinones using a plasma membrane quinone reductase. The possession of tyrosinases may be important for lichens with strong laccase or peroxidase activity, firstly because tyrosinases can synthesize quinones from simple phenolics precursors, which are known to be produced in large quantities by Peltigeralean lichens (Zagoskina *et al*. 2011). Secondly, in saprophytic free-living fungi it has also been suggested that tyrosinases protect fungi by removing (via polymerization) reactive phenols and quinones produced as by-products during lignocellulosic breakdown (Sinsabaugh 2010). Non-Peltigeralean lichens display almost no laccase activity, which may explain the absence of cell wall tyrosinases in these lichens. As discussed in the Introduction, a major role for tyrosinases in free-living fungi is considered to be melanin synthesis. Melanin synthesis in free-living fungi appears to occur in the cytoplasm (Bell & Wheeler 1986; Henson *et al*. 1999). In lichens that are heavily pigmented, but that display low or no tyrosinase activity (e.g. *Lasallia pustulata*) melanins are probably synthesized via the 'DHN' or polyketide synthase pathway (Muggia *et al*. 2009). However, in *Dermatocarpon*, which has a heavily pigmented lower cortex, tyrosinase is presumably involved in melanin synthesis rather than extracellular ROS metabolism. These differences may explain the almost exclusively cytosolic location and rather different biochemical characteristics of *Dermatocarpon* tyrosinase compared with the corresponding enzyme from *Peltigera*.

In future work, we hope to further characterize *D. miniatum* tyrosinase. Unfortunately, our attempts to concentrate the enzyme were unsuccessful, as activity was rapidly lost even following a mild dialysis procedure. By contrast, we have found that tyrosinases from *Peltigera* are remarkably stable following isolation. In conclusion, it is clear that lichens from orders other than the *Peltigerales* can display significant intracellular tyrosinase activity. While in the *Peltigerales* extracellular tyrosinases appear to have functions associated with laccase-mediated redox cycling, these functions are not needed in clades of lichens without significant laccase activity. However, melanization reactions occur in many diverse lichens, both Peltigeralean and non-Peltigeralean, and in both groups tyrosinases may be involved. In future studies, we plan to clarify the structure, properties, relationship of this enzyme to forms occurring in free-living fungi, and the specific roles of tyrosinases in various aspects of lichen biology such as desiccation tolerance.

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