RESEARCH LETTER

Laccases produced by lichens of the order *Peltigerales*

Alexander V. Lisov[1], Anna G. Zavarzina[2], Alexey A. Zavarzin[3] & Alexey A. Leontievsky[1]

[1]Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (IBPM RAS), Pushchino, Moscow Region, Russia; [2]Faculty of Soil Science, Moscow State University, Moscow, Russia; and [3]Faculty of Biology and Soil Sciences, St Petersburg State University, St Petersburg, Russia

Correspondence: Alexander V. Lisov, Nauki, 5, Pushchino, Moscow Region 142292, Russia. Tel.: +7 4967 73 17 86; fax: +7 4967 956 33 70; e-mail: ssl206@rambler.ru

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Abstract

'Large' and 'small' fractions of laccase were found in the thalli of lichens *Solorina crocea* and *Peltigera aphthosa*. In both lichens, 'large', possibly dimeric, laccases were determined as 175 and 165 kDa (based on the gel filtration data), and 'small' ones were 76 and 97 kDa (according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis data), respectively. By their substrate specificity, pH optima, and thermostability, they were typical laccases. The fractions of 'small' laccases of 45 kDa from *S. crocea* and 55 kDa from *P. aphthosa* consisted of two enzymes.

Introduction

Laccase is a multicopper blue oxidase (benzenediol:oxygen oxidoreductase, EC1.10.3.2). It catalyzes a one-electron oxidation of appropriate substrates, such as substituted phenols, aromatic amines, nonphenolic compounds of a 'low' redox potential [e.g. syringaldazine, 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate], and some inorganic compounds (e.g. $K_4[Fe(CN)]_6$) by oxygen, which is reduced to water during the course of catalysis. The oxidized products of a laccase reaction with organic compounds can undergo spontaneous coupling, yielding polymers.

Laccases are produced by plants, fungi, bacteria, and insects (Mayer & Harel, 1987; Suzuki *et al.*, 2003; Asada *et al.*, 2004). The most profoundly studied are laccases produced by white-rot basidiomycetes (Baldrian, 2006). In nature, these enzymes participate in phenol metabolism, as well as in the synthesis and breakdown of lignin (Edens *et al.*, 1999; Boerjan *et al.*, 2003) and humic substances (Temp *et al.*, 1999; Zavarzina, 2006). Owing to a low substrate specificity and a high oxidizing potential, laccases are able to attack a wide range of persistent compounds (both natural and xenobiotic). This makes them attractive for biotechnological applications (Akin *et al.*, 1993; Abadulla *et al.*, 2000). The search for new sources of laccases has a more than 20-year history, interest focusing more and more on unusual laccase producers, such as nonbasidiomycetes and thermophylic microorganisms (Robles *et al.*, 2000; Palonen *et al.*, 2003; Miyazaki, 2005).

The laccase activity was recently discovered in lichens of different taxonomic and substrate groups (Laufer *et al.*, 2006; Zavarzina & Zavarzin, 2006). It is well known that lichens are symbiotic organisms representing associations of fungi (most often ascomycetes) and green algae and/or cyanobacteria. Depending on the host (higher plants, rocks, or soil), lichens can be divided into epiphytic, epilythic, or epigeic species. They are well known for tremendous abilities to adapt and survive under extreme conditions and for a rapid restoration of their metabolic activity (Purvis, 2000). Lichens are able to develop even in an oligothroph environment (foreign for other organisms); they constitute more than 8% of the total terrestrial vegetation.

High- and low-('large' and 'small')-molecular-weight (MW) laccases were isolated from the thalli of lichens *Solorina crocea* and *Peltigera aphthosa* (the order *Peltigerales*), studied, and characterized.

Materials and methods

Chemicals and materials

The substrates, superoxide dismutase and catalase, calibration kits, Tween-80, Pharmalytes, Coomassie Blue R, and G were commercially available from Sigma Aldrich, and Fluka.

The salts, acids, inhibitors, alkali and acetone were obtained from Reakhim (Russia). Materials and columns for chromatography were obtained from Millipore (DEAE-cellulose) and GE Biosciences (DEAE-Sepharose CL; Mono-Q HR 5/5 and HiLoad 26/60 Superdex 200). Chemicals for electrophoresis were obtained from Bio-Rad.

Lichens

The thalli of *P. aphthosa* and *S. crocea* were taken from Russia: the Khibiny mountains (the Kola peninsula, Murmansk region) in the summer of 2005, spring and autumn of 2006; some samples of *P. aphthosa* were taken from the Karelian Isthmus (Leningrad region) in the autumn of 2006. *Peltigera polydactyloon*, *Peltigera neopolydactyla*, *Peltigera praetextata*, *Peltigera canina*, *Peltigera horizontalis*, and *Peltigera rufescens* were taken from the Karelian Isthmus in spring 2006. The thalli were air-dried at the sites of sampling and then stored under the same conditions for 2 months before the treatment.

Crude enzyme preparations

The air-dried lichen thalli (1 g) were washed for 1 h at 30 °C by distilled water (50 mL) without agitation; the resulting leachats were cleared by filtration (Extract 1). The washed thalli were homogenized in a mortal and washed under the same conditions; the supernatant was centrifuged (5000 ***g***, 30 min) to obtain Extract 2. The precipitate of grinded thalli was washed in 0.1% Tween-80 in water with agitation (200 r.p.m., 1 h, 30 °C) and centrifuged; the supernatant was used as Extract 3. The enzyme preparations were concentrated by ultrafiltration on a micro-concentrator Vivaspin 2 (exclusion limit 10 kDa, Sartorius group, Germany).

Enzyme purification

A purification scheme consisted of three subsequent anion-exchange chromatography and final gel-filtration steps: (1) Extract 3, obtained from 100 g of air-dried thalli (pH 5.5), was loaded on DEAE-cellulose equilibrated with 20 mM Na-acetate buffer, pH 5.5 (buffer A), the column was washed with buffer A and the sorbed material was eluted by 0.5 M NaCl in buffer A. (2) Fractions containing the laccase activity were collected, dialyzed, and loaded on DEAE-Sepharose CL column under the same conditions with elution by a gradient of 0–1.0 M NaCl in buffer A. (3) This step was in general the same as the previous one, with the only exception that the column was replaced by Mono-Q HR 5/5. (4) The active fractions were collected, concentrated to 2 mL, and subjected to gel filtration on a HiLoad 26/60 Superdex 200 column using 0.1 M NaCl in buffer A as an eluent.

Enzyme assay

The laccase activity was determined by the rate of 2,2-azino-bis-(3-ethylbenzthiazolin-6-sulfonate) (ABTS) oxidation at 25 °C. The reaction mixture contained 0.5 mM ABTS in buffer A and the enzyme preparation. The absorption at 420 nm ($\varepsilon 420 = 36\,000\ M^{-1}\ cm^{-1}$ (Heinfling *et al.*, 1998) was monitored on a spectrophotometer Shimadzu UV-1650PC. A laccase activity unit (U) was expressed as the amount of the enzyme oxidizing 1 μm of a substrate per 1 min.

To study the laccase substrate specificity, the following substrates were used: 0.5 mM *p*-phenylenediamine, $\lambda = 460$ nm; pyrogallol, $\lambda = 296$ nm; 2,6-dimethylphenol, $\lambda = 420$ nm; ABTS, $\lambda = 420$ nm; syringaldazine, $\lambda = 545$ nm; 2,6-dimethoxyphenol, $\lambda = 469$ nm; guaiacol, $\lambda = 470$ nm; and veratryl alcohol, $\lambda = 310$ nm. The enzyme activity was expressed in arbitrary units (AU), namely $\Delta A_\lambda\ min^{-1}\ mL^{-1}$.

Enzyme characterization

The MW of purified laccases was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmly (1970) 12% gel, standard proteins: phosphorylase Â 97 kDa, bovine serum albumin 66 kDa, ovalbumin 45 kDa, carboanhydrase 29 kDa, tripsin inhibitor from soybean 20 kDa, and lactalbumin 14.2 kDa).

A MW of native enzymes was determined by gel filtration on a HiLoad 26/60 Superdex 200 column calibrated with a Molecular Weight Marker Kit 29 000–700 000 kDa (Sigma).

A native PAGE electrophoresis of acidic proteins was performed as described above but in the absence of SDS and dithiothreitol in solutions and without boiling of the samples. Laccase was stained in the gel by 1 mM guaiacol or 0.5 mM ABTS in buffer A.

The isoelectric points of enzymes (*p*I) were determined by isoelectrofocusing in a density gradient of sucrose with Pharmalytes (LKB 8100 column, Sweden), pH range 2.5–5.0.

An oxygen uptake during the course of the laccase ($12\ \mu g\ mL^{-1}$) reaction with 2 mM 2,6-dimethoxyphenol was measured with a Clark oxygen electrode in a 2 mL reaction chamber using buffer A.

The pH optimum of laccase activity was determined within the range of pH values from 2.7 to 6.3 with ABTS and 2,6-dimethoxyphenol as substrates. The 0.1 M Britton and Robinson buffer was made by mixing equal amounts of 0.1 M boric, 0.1 M orthophosphoric, and 0.1 M acetic acids and by pH adjustment to the required value using 1 N NaOH.

The enzyme thermostability was determined by an ABTS assay after a 1-h incubation in buffer A at 35, 50, and 60 °C.

The absorption spectra were recorded in buffer A, at room temperatures, on a Shimadzu 1650PC.

The protein concentration was measured according to Bradford (1976).

Results

Laccase production by different lichen species PAGE

The laccase activity in water extracts from the intact thalli of seven lichens species of the genus *Peltigera* was examined. It varied from 0.3 to 1.2 Arb U mL^{-1} (compare with 6.0 AU mL^{-1} of the *S. crocea* extract) (Fig. 1). After homogenization of the thalli, the laccase activity in all the extracts increased 10–12 times, except for *P. aphthosa*, where the activity increased 20-fold. Thus, *S. crocea* and *P. aphthosa* were chosen as the best candidates for isolation of laccases.

Purification of laccases from *S. crocea* and *P. aphthosa*

The dark orange–brown coloring of crude enzyme preparations, obtained by extraction of homogenized thalli, complicated the protein assay procedure. The pigment was removed by three consequent anion-exchange chromatography procedures. In *S. crocea* and *P. aphthosa*, the laccase activity was separated into two fractions and residual pigments were removed at the gel-filtration step (Fig. 2a and b).

The concentrated preparation of purified Sc1 laccase (1 mg mL^{-1}) was bright blue; in the absorption spectrum, the 'blue' maximum was observed at 610–615 nm, and a shoulder was seen at 330 nm. Concentrated Pa1 laccase was yellowish and had no blue maximum in the absorption spectrum (Fig. 2c).

The SDS-PAGE electrophoresis showed that laccase preparations Sc1 and Pa1 had single protein bands with MWs of 76 and 97 kDa, respectively (Fig. 3a and b). Specific staining of these samples after a nondenaturating electrophoresis also revealed single bands in gels (Fig. 3c and d). The apparent MWs of laccases determined by gel filtration were 175 kDa for Sc1 and 165 kDa for Pa1, thus pointing to a possible dimeric nature of these forms of lichens laccases.

After SDS electrophoresis, laccase preparations Sc2 and Pa2 had more than two protein bands (data not shown). Guaiacol staining of polyacrylamide gels after a native electrophoresis of these samples revealed the presence of two laccase forms: Sc2-1 and Sc2-2 in *S. crocea*, and Pa2-1 and Pa2-2 in *P. aphthosa* (Fig. 3c and d). After gel filtration, the apparent MWs of Sc2 and Pa2 fractions were 46 and

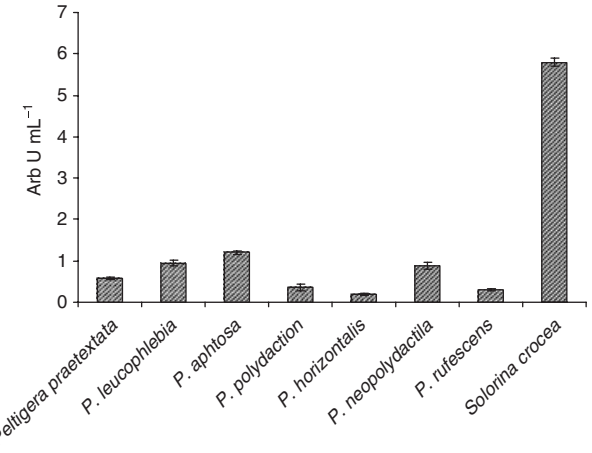

Fig. 1. Actitivty of laccase from water extracts (Extract 1) of lichens of the *Peltigerales* order. Error bars indicate the SD, $n = 3$.

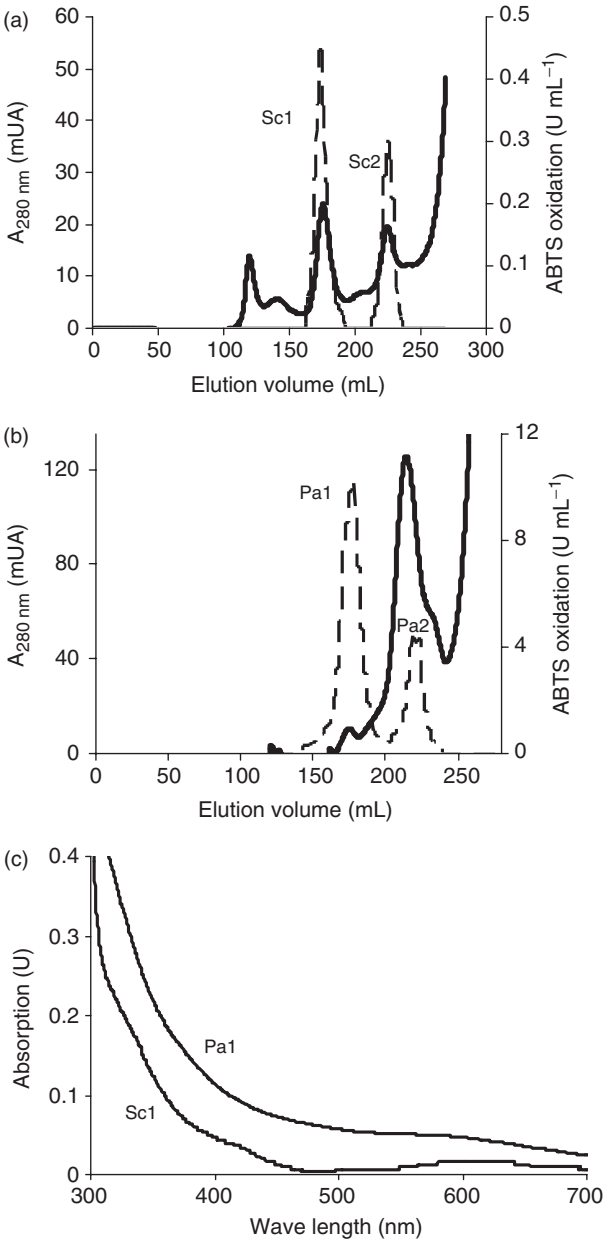

Fig. 2. Gel filtration of laccase preparations from lichens *Solorina crocea* (a) and *Peltigera aphthosa* (b). The solid line denotes absorption at 280 nm, the dotted line shows laccase activity; Sc1, Sc2, Pa1, and Pa2 are laccase fractions. (c) The absorption spectra of Sc1 and Pa1 laccases.

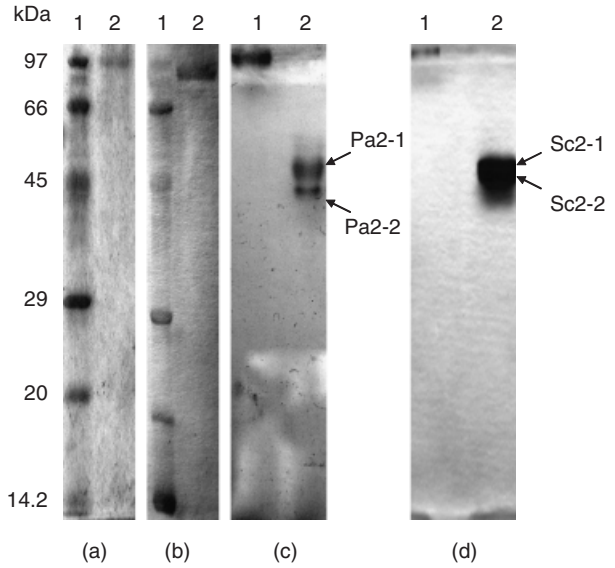

Fig. 3. SDS-PAGE (a, b) and native electrophoresis (c, d) of *Solorina crocea* and *Peltigera aphthosa* laccases. (a) Line 1, protein standards; Line 2, Pa1 laccase. (b) Line 1 as in (a); Line 2, Sc1 laccase. (c) Line 1, Pa1 laccase; Line 2, Pa2 laccases. (d) Line 1, Sc1 laccase; Line 2, Sc2 laccases.

55 kDa, respectively. Because Sc1 and Pa1 fractions were electrophoretically homogenous, they were chosen for further investigations.

The isoelectric points of Sc1 and Pa1 were 4.8 and 4.2, respectively.

Characterization of 'large' Sc1 and Pa1 laccases

Sc1 and Pa1 enzymes catalyzed oxidation of different phenols, *p*-phenylenediamine, ABTS, syringaldazine, but did not catalyze the oxidation of tyrosine and veratryl alcohol (Table 1). Treatment of Sc1 and Pa2 by 1.0 mM NaN_3 or 10 mM 1,10-phenantroline resulted in total loss of the enzyme activity.

Table 1. Substrate specificity of laccases Sc1 and Pa1

	Arb U mg^{-1}		
Substrate	Wavelength (nm)	Laccase Sc1	Laccase Pa1
p-Phenylendiamine	460	3.3 ± 0.05	8.6 ± 0.07
Tyrosine	*	No change	No change
Pyrogallol	296	8.3 ± 0.1	10.7v0.08
2,6-Dimethylphenol	420	0.42 ± 0.05	1.1 ± 0.03
ABTS	420	44 ± 0.15	10.4 ± 0.07
Syringaldazine†	545	0.49 ± 0.02	0.55 ± 0.01
2,6-Dimethoxyphenol	469	10.6 ± 0.2	18.7 ± 0.2
Guaiacol	470	0.16 ± 0.01	3 ± 0.05
Veratryl alcohol	340	0	0

*Oxidation was observed as changes in the absorption spectrum after 5 min.

†Enzyme activity was measured in buffer containing 40% ethanol.

± SD, $n = 5$.

During oxidation of 2,6-dimethoxyphenol by laccases Sc1 or Pa1, oxygen consumption was detected. Subsequent addition of superoxide dismutase and catalase to the reaction mixture did not affect the oxidation rate. This indicated the absence of superoxide radical or hydrogen peroxide formation during the reaction, and suggests that oxygen was reduced to water.

The pH optimum for laccase Sc1 determined with ABTS and 2,6-dimethoxyphenol was 4.2 for both substrates. Laccase Pa1 had a more acidic pH optima: 3.6 for ABTS and below 2.7 for 2,6-dimethoxyphenol (Fig. 4a and b).

Laccase Pa1 was more thermostable. The enzyme preserved 85% of its activity during a 1-h incubation at 35 °C, whereas during the same period, the activity of laccase Sc1 declined to $< 50\%$. The half-time inactivation at 50 °C was a few minutes for Sc1 and 25 min for Pa1. Nevertheless, both Sc1 and Pa1 enzymes had a low thermal stability at 60 °C (Fig. 4c and d).

Occurrence of different laccase forms in *S. crocea* and *P. aphthosa*

Specific staining of laccases in the gels after native electrophoresis revealed fractions of 'large' (Pa1) and 'small' (Pa2-1 and Pa2-2) laccases in Extracts 1 and 3; 'small' laccases were not found in Extracts 2 (*P. aphthosa* enzyme preparation) irrespective of the period and place of thalli sampling (Fig. 5a and b).

In case of *S. crocea*, a similar distribution of laccase fractions ('large' in Sc1 and 'small' in Sc2-1, Sc2-2) was observed in Extracts 1–3 of the thalli sampled in the Khibiny in summer 2005. Extracts 1–3 of *S. crocea* thalli, sampled in spring and autumn 2006 also in the Khibiny mountains, lacked fractions of 'small' laccases (Fig. 5c). The distinctions could be a result of seasonal variations or physiological peculiarities of different lichens.

Discussion

The authors showed that two lichens of the order *Peltigerales* have two types of laccase. One of them is distinguished by an unusually high MW. SDS-PAGE electrophoresis of 'large' Sc1 and Pa1 laccases made it possible to consider them as homodimeric proteins. The most fungal laccases reported hitherto are monomeric proteins with a MW of 60–80 kDa (Baldrian, 2006). However, recently, the laccase from the lichen *Peltigera malacea* has been described as a homotetrameric protein with a MW of 340 kDa (Beckett *et al.*, 2005; Laufer *et al.*, 2006). Some authors wrote about fungal laccases consisting of several subunits: e.g. in ascomycetes such as *Podospora anserine* (MW of a tetramer ~390 kDa, MW of a subunit ~80 kDa) (Durrens, 1981);

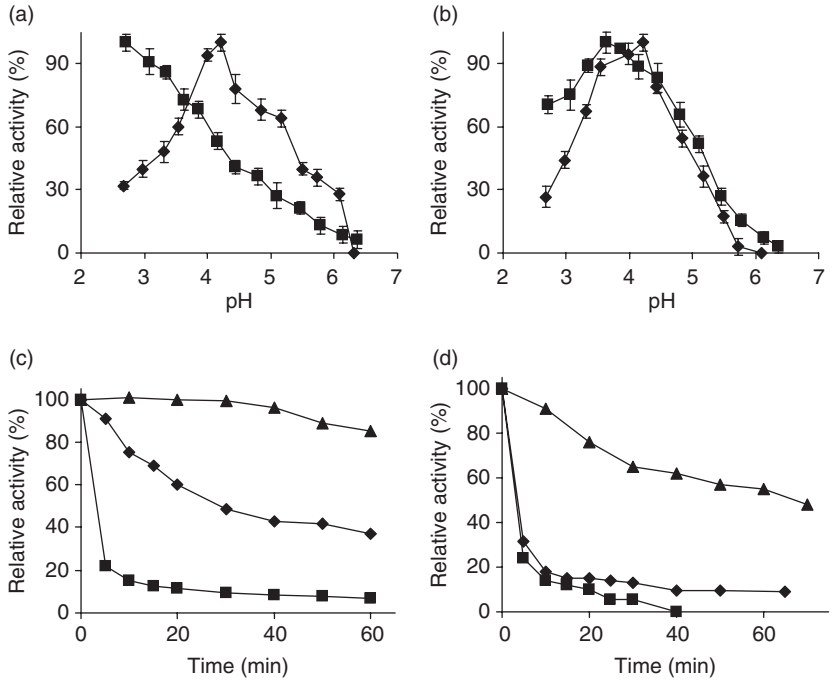

Fig. 4. pH optima (a, b) and thermostability (c, d) of Sc1 and Pa1 laccases. (a) 2,6 DMP oxidation. (b) ABTS oxidation. (◆) Laccase Sc1; (■) laccase Pa1. Error bars indicate the SD, $n = 3$. Thermostability of Pa1 (c) and Sc1 (d) laccases. (▲) 35 °C; (◆) 50 °C; (■) 60 °C. The averaged data of triplicate experiments are presented.

Gaeumannomyces graminis (MW of a homodimer ~190 kDa, MW of a subunit ~70 kDa) (Edens *et al.*, 1999); basidiomycetes such as *Phellinus ribis* (MW of a homodimer ~140 kDa, MW of a subunit ~76 kDa) (Min *et al.*, 2001); *Cantharellus cibarius* (MW of a homodimer ~92 kDa, MW of a subunit ~46 kDa) (Ng & Wang, 2004); and *Trametes villosa* (MW of a homodimer ~130 kDa, MW of a subunit ~60 kDa) (Yaver *et al.*, 1996). However, these data need careful interpretation. Laccase is a glycoprotein; its standard sugar content is 10–20%. This can easily distort the determination of its apparent MW (Thurston, 1994). For instance, by analytic centrifugation and gel filtration, the MW of laccase from *Agaricus bisporus* was determined to be 100 kDa (Wood, 1980); however, the SDS-PAGE analyses indicated it to be 65 kDa (Perry *et al.*, 1993). The laccases of lichen can be associated with some unknown compounds available in the thallus; they can increase the MW of laccases, thus making them appear as dimers. Probably, this is the reason for the bad correlation between the MW of the Pa1 homodimer (MW 165 kDa) and its subunit (MW 97 kDa).

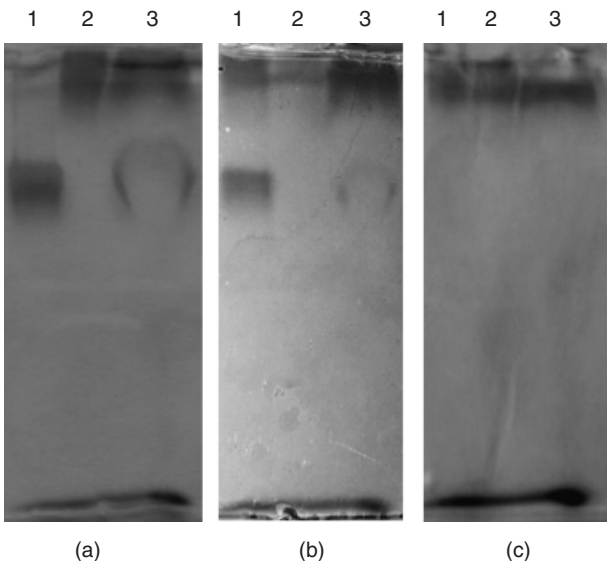

Fig. 5. Native electrophoresis of laccases in Extracts 1–3. (a) *Peltigera aphthosa* sampled in the Khibiny mountains in the autumn of 2006; (b) *P. aphthosa* sampled in the Karelian Isthmus in the autumn of 2006; (c) *Solorina crocea* sampled in the Khibiny mountains in the autumn of 2006. Lines 1, 2, and 3 denote Extracts 1, 2, and 3, respectively.

The absorption spectrum of the enzyme Sc1 of *S. crocea* with a maximum at 610 nm and a shoulder at 330 nm was typical of blue laccases (Baldrian, 2006). The absence of a 'blue' maximum in the absorption spectrum of laccase Pa1 from *P. aphthosa* resembled that of 'yellow' laccases described earlier from basidiomycets (Leontievsky *et al.*, 1997).

By their substrate specificity, reduction of oxygen to water during substrate oxidation, interaction with inhibitors, pH optimum, and thermal stability, the dimeric forms of Sc1 and Pa1 were typical laccases. They oxidized phenols, ABTS, syringaldazine, and *p*-phenylenediamine, but did not oxidize tyrosine and nonphenol compounds, as well as veratric alcohol, which usually is not a substrate for laccase. Both laccase activities were totally suppressed by typical laccase inhibitors sodium azide and chelator 1,10-phenantroline.

The pH optima of ABTS oxidation by laccases were in the acidic region, which is in general typical of laccases (Bollag & Leonowicz, 1984; Robles *et al*., 2002). The pH optimum of the 2,6-dimethoxyphenol oxidation by laccase Pa1 was unusually acidic and similar to ABTS oxidation (Fukushima & Kirk, 1995; Palonen *et al*., 2003). Fungal laccases are relatively thermostable proteins. Lichen-derived laccases Sc1 and Pa1 showed thermal stability comparable with that of typical fungal laccases. By its thermostability, the Pa1 approached the laccase from e.g. *Ganoderma lucidum,* which preserved 50% of its activity at 50 °C in 1 h (Ko *et al*., 2001).

The 'small' laccases can be easily extracted from intact thalli by their gentle washing with water (Extract 1). A subsequent homogenization of thalli increased the laccase activity significantly; however, 'small' laccases were not found in Extracts 2. They appeared only after the treatment of homogenized thalli with a detergent (Extract 3). The 'large' laccases were found in all the three extracts; they were, however, predominant in the extracts of the homogenized thalli. The 'large' laccases are either more firmly bound to certain lichen structures or are located somewhere deeper, e.g. inside the mycobiont.

A physiological role of laccases in lichens can be considered from the point of view of the metabolism of unique secondary compounds (lichen acids) and other phenols. For example, *S. crocea* produces solorinin, solorinic acid (responsible for the orange coloring of the thallus), averantin, and averythrin. They all are phenol compounds (Anderson *et al*., 1966; Ebizuka *et al*., 1970). Laccases released from the lichen 'tissues' can take part in delignification and in the synthesis of humic substances during the early stages of soil development (Beckett *et al*., 2005; Laufer *et al*., 2006; Zavarzina & Zavarzin, 2006).

Conclusions

The occurrence of different laccase forms produced by two lichens of the order *Peltigerales* was shown by electrophoresis and gel filtration. Purified dimeric enzymes of *S. crocea* and *P. aphthosa* were typical laccases as evidenced by their substrate specificity, thermal stability, and pH optima.

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