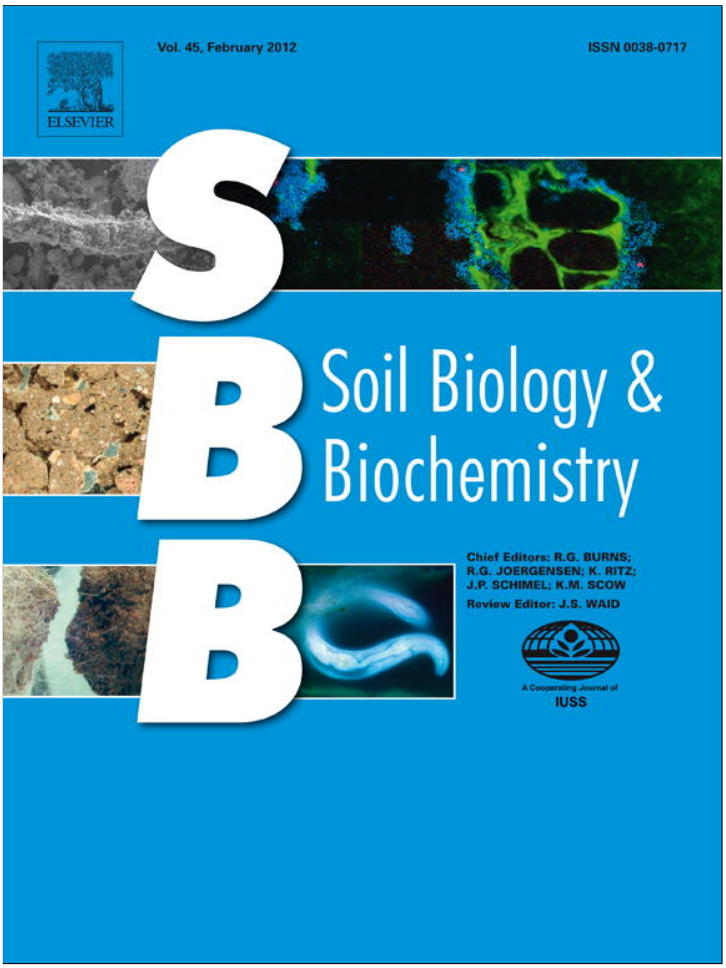

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Dimeric and monomeric laccases of soil-stabilizing lichen *Solorina crocea*: Purification, properties and reactions with humic acids

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ABSTRACT

Lichens form the dominant plant cover in extreme environments and participate in mineral weathering, fine-earth stabilization and primary accumulation of soil organic matter. However, biochemical role of lichens in soil processes has never been investigated. Recently, laccases and tyrosinases have been discovered in representatives of the order *Peltigerales* (Laufer et al., 2006a, b; Zavarzina and Zavarzin, 2006). Laccases from most species had unusually large molecular weights (Laufer et al., 2009). Together with oligomeric laccases, we have found monomeric enzymes in *Solorina crocea* and *Peltigera aphthosa* (Lisov et al., 2007). In the present work we have purified homodimeric (large) and monomeric (small) laccases of the soil-stabilizing lichen *S. crocea*, determined their physico-chemical and catalytic properties and studied their reactions with soil humic acids. Our results suggest that oligomeric nature of lichen laccases can be artifactual, because homodimeric laccase was transformed into the monomeric form following hydrophobic interaction chromatography. We hypothesize that large laccase consists of two monomeric enzymes, each of which is bound with additional hydrophobic component(s). Small laccase is similar in its properties to the laccases of basidiomycetes. It is more resistant to elevated temperature and storage than the large form, showed a higher oxidation potential, had different pH-optima in oxidizing substrates and was less inhibited by humic acids. Despite these differences, both laccases depolymerized and decolorized humic acids from soils at comparable rates, with small laccase being slightly more effective. This finding suggests that lichens have a potential to participate in transformation of soil organic matter.

1. Introduction

Lichens are symbiotic organisms, comprising of a fungus (commonly Ascomycete) and the photobiont – an alga (usually *Trebouxia*) and/or a cyanobacterium (commonly *Nostoc*). Lichens form the dominant plant cover in extreme environments – mainly in tundra, Arctic and Antarctic regions, high mountain elevations and dryland crusts. A variety of physiological and chemical adaptations enable lichenized fungi to tolerate severe abiotic stresses such as desiccation, rapid rehydration, temperature extremes and high UV light intensities (Nash, 2008).

Lichens are often pioneers on rocky surfaces and are well known for accelerating mineral weathering. Lichen-induced changes include surface corrosion, disintegration, precipitation of amorphous Fe, Al and Si compounds, neoformation of crystalline metal oxalates and secondary minerals (Chen et al., 2000). In addition, lichens colonizing fine-earth or disturbed soil often act as stabilizers of their substrate preventing surface erosion. Lichens also contribute to the primary accumulation of soil organic matter via the products of thalli decomposition or as leached organic compounds. However, possible biochemical role of these symbiotic organisms in soil organic matter turnover is unknown.

As was recently reported lichens produce laccases, with representatives of the order *Peltigerales* being most active (Laufer et al., 2006a, b; Zavarzina and Zavarzin, 2006). Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is multicopper oxidase, which reduce O_2 to two molecules of water and simultaneously perform one-electron oxidation of various phenolic substrates and aromatic amines to phenoxy radicals and semiquinones (Thurston, 1994). These reactive species can be further oxidized to quinones and/or undergo spontaneous radical-generated reactions leading to substrate polymerization or depolymerization (Sarcanen and

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Ludwig, 1971). Depolymerization is favored at acidic pH and high oxygen supply if initial enzyme substrate is polymeric (Yaropolov et al., 1994; Rabinovich et al., 2004). Laccases are widespread in fungi, plants and bacteria but occur especially widely in white-rot and litter-decomposing basidiomycetes. There they participate in the degradation of lignin and humic substances (HS) together with ligninolytic peroxidases (Baldrian, 2006; Grinhut et al., 2007). Another function of laccase is to convert the phenolic products of lignin degradation into polymeric non-toxic compounds (Thurston, 1994). In free-living ascomycetes laccases are considered to be responsible for the partial oxidation of lignin; humic substances are formed as by-products (reviewed by Zavarzina et al., 2011). Thus, laccases play important role both in synthesis and degradation of HS – the dominant components of soil humus.

The role of laccase in lichenized ascomycetes is unknown. Laccases in lichens seem to be constitutively expressed (Laufer et al., 2006a). It is almost certain that the mycobiont is responsible for production of the enzyme; the lower (mycobiont) lichen surface changed its color during oxidation tests with laccase substrates (Zavarzina and Zavarzin, 2006). Besides, presence of laccases in algae and cyanobacteria is unknown except for a single report of a laccase-like enzyme in the coccoid green soil alga *Tetracystis aeria* (Otto et al., 2010). It is unlikely that laccases in lichens are used for saprotrophyte-related activities because mycobiont obtains all or most of its carbon from the photosynthetic partner and apparently have no need in additional organotrophic nutrition. However, it was found that continuous amounts of laccase can be washed out from intact lichen thalli by water. In peltigerous lichens washed-out activity comprise up to 5–10% of the total activity in the thalli (Zavarzina and Zavarzin, 2006). After cell homogenization and addition of 0.1% Tween-80 activities in the extracts increase by 10–12 times (Lisov et al., 2007). Thus, irrespective of the role of laccases in lichen physiology, once released or leached into the soil they have a potential to participate in extracellular reactions of humus synthesis or degradation.

Interestingly, laccases in lichens have unusually large molecular weights (135–200 kDa and up to 300–350 kDa) suggesting them being apparent dimers or even tetramers (Lisov et al., 2007; Laufer et al., 2009). Oligomeric lichen laccases had typical substrate specificities and catalytic properties, but rapidly lost activity during concentration or storage and were less thermostable than laccases from basidiomycetes. Together with oligomeric (large) laccases we have found monomeric (small) forms in *Solorina crocea* and *Peltigera aphthosa* (Lisov et al., 2007). They have not been purified and characterized so far first because their concentration in thalli is low, and second because they were not always present in replicate collections of the same species from the same locality. Sometimes lichens contained both laccases with prevalence of large form over the monomeric one, but in most cases only large laccase was present. Thus, the nature of monomeric laccases in peltigerous lichens remains unknown, as do the physico-chemical properties of the enzyme. The ability of oligomeric and monomeric lichen laccases to perform transformation of natural organic matter has not been investigated so far.

Lichens represent a permanent and abundant component of soil cover in climax communities in oligotrophic environments. The possible involvement of enzymes leached from lichens in organic matter turnover represents a problem of large scale and is important for evaluation of carbon dynamics in extreme soils. The aims of the present paper were to clarify the nature of monomeric laccase in peltigerous lichens and to study potential ability of lichen's laccases to transform recalcitrant components of soil organic matter. We have purified homodimeric (large) laccase of soil-stabilizing lichen *S. crocea*, and were able to transform it into a monomeric form. We could then compare the physico-chemical and catalytic properties of both laccases and studied their interactions with soil humic acids. The lichen *S. crocea* was chosen for the study because it is abundant on the soil surface in northern ecosystems, it is most active laccase producer among the species studied and grows tightly attached to soil particles facilitating the contact of leached laccase with soil components.

2. Experimental

2.1. Materials

2.1.1. Lichen

The lichen *S. crocea* was collected in Khibiny mountains (the Kola peninsula, Murmansk region, Russia). Lichen thalli were stored for 24 months at room temperature in air-dried form until use.

2.1.2. Humic acids (HA)

Humic acids from soddy-podzolic soil (HA_s), chernozem (HA_{ch}) and peat (HA_p, Merck preparation) were used. Extraction, purification, characterization and transformation of HA into their water-soluble NH_4-form was as described by (Zavarzina et al., 2002, 2004).

2.2. Methods

2.2.1. Laccase extraction

Lichen thalli (60 g) were homogenized with a mortar and a pestle. Enzyme extraction was performed on a rotary shaker (200 r.p.m., 29 °C, 30 min) using 20 mM Na-acetic buffer, pH 5.0 (buffer A) with 0.1% Tween 80 and 1% ascorbic acid as extracting solution. A volume/mass ratio of extracting solution/homogenized lichen thalli was 100:1. The extract was separated from the solid phase by centrifugation (5000 g, 30 min).

2.2.2. Laccase purification

Crude preparation of laccase was obtained from the extract by precipitation with $(NH_4)_2SO_4$ at a 90% of saturation. The precipitate was separated from the liquid by centrifugation of the mixture (12,000 g, 1 h). After the precipitate was re-dissolved in buffer A, solution was passed through a filter with pore size 20 μm and loaded on HiLoad 26/60 Superdex 200 gel-filtration column (GE Healthcare, Sweden) with 0.1 M NaCl in buffer A as an eluent. Fractions containing laccase activity were combined and further purification was carried out by two different ways. (1) *Ion-exchange chromatography*. Laccase-containing solution was dialyzed against 20 mM Na-acetic buffer (pH 5.8) and loaded on UNOQ column (BioRad, USA) equilibrated with the same buffer. Elution was carried out at linear gradient of NaCl (from 0 M to 1 M). (2) *Hydrophobic interaction chromatography*. Laccase solution was mixed with $(NH_4)_2SO_4$ to adjust the salt concentration to 1 M and then loaded on Phenyl Sepharose column (40 cm × 2 cm) equilibrated with 1 M $(NH_4)_2SO_4$ in buffer A. Elution was carried out using linear gradient of $(NH_4)_2SO_4$ (from 1 M to 0 M).

After ion-exchange or hydrophobic chromatography the second gel-filtration on HiLoad 26/60 Superdex 200 was carried out. The conditions of the second gel filtration were the same as of the first one. Laccase preparations were dialyzed against buffer A and concentrated by ultrafiltration on a micro-concentrator Vivaspin 2 (exclusion limit 10 kDa, Sartorius group, Germany). The protein concentration in final preparations was measured according to Bradford (1976).

2.2.3. Enzyme activity assay

The laccase activity was determined by the rate of 2,2-azino-bis-(3-ethylbenzthiazolin-6-sulfonate) (ABTS) oxidation. The reaction mixture contained 1 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 Shimadzu UV-1650PC spectrophotometer. Laccase activity unit (U) was expressed as the amount of the enzyme oxidizing 1 μM of a substrate per 1 min. The substrates used to determine the kinetic constants were: guaiacol (2-methoxyphenol) – $\varepsilon_{470} = 12\,100\ M^{-1}\ cm^{-1}$ (Munoz et al., 1997); 2,6-dimethoxyphenol – $\varepsilon_{469} = 49\,600\ M^{-1}\ cm^{-1}$ (Wariishi et al., 1992); hydroquinone (1,4-dihydroxybenzen) $\varepsilon_{254} = 21\,000\ M^{-1}\ cm^{-1}$ (Camarero et al., 1999).

2.2.4. Enzyme characterization

The molecular weights of purified laccases were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel according to Laemmly (1970). The standard proteins were as follows: phosphorylase A (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 native PAGE electrophoresis of acidic proteins was performed as described above but in the absence of SDS and dithiothreitol in solutions and without boiling the samples. Laccase was stained in the gel by 0.5 mM ABTS in buffer A. The apparent MW of native enzymes were 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, USA).

The pH optimum of laccase activity was determined using 0.1 M Britton-Robinson buffer within the pH range from 2.7 to 7.3. ABTS and 2.6-dimethoxyphenol were used as substrates. The 0.1 M Britton-Robinson buffer was prepared 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 M NaOH. Kinetic constants were measured at 30 °C in buffer A. The calculations were done in inverse coordinates, 1/V:1/S. The enzyme thermostability was determined by an ABTS assay after 1 h incubation of the enzyme in buffer A at 50 or 60 °C.

2.2.5. Inhibitory effects of humic acids on laccases

The inhibitory effect of humic acids on laccase activities was studied using HA from soddy-podzolic soil (HA_s), chernozem (HA_{ch}) and peat (HA_p). Reaction mixtures contained 0.05 mg ml^{-1} of a HA and 2 nmol of laccases in 1 ml of 20 mM Na–acetate buffer (pH 4.5).

2.2.6. Transformation of humic acids

The effect of lichen laccases on molecular weight and optical characteristics of humic acids was studied using HA_s and HA_{ch}. Incubation mixtures contained 1 mg of a HA and 0.11 U of laccase in 1 ml of 20 mM Na-acetic buffer (pH 4.5). Mixtures with 2 mM NaN_3 were used as controls. After 24 and 72 h of incubation at room temperature, aliquots were taken and diluted 1:1 in 0.1 M Na-phosphate buffer (pH 7.0) with 0.1% SDS and 0.05% NaN_3 for HPLC analysis or 1:50 in 0.1 M NaOH for recording absorption spectra. Absorption spectra of HA were recorded on T70 UV–Vis Spectrometer (PG Instruments). HPLC was carried out at 280 nm on Agilent 1100 system equipped with diode array detector. TSK 2000 SW column (7.5 × 60 mm) was used, the eluent was 0.1 M Na-phosphate buffer (pH 7.0) with 0.1% SDS and 0.05% NaN_3. The aliquot for analysis was 100 μl, flow rate 0.75 ml min^{-1}. The standards were as follows: cytochrome C (12.5 kDa), ribonuclease A (13.7 kDa), chymotripsinogen A (25 kDa), albumin from hen egg (45 kDa), albumin from bovine serum (67 kDa), Blue Dextran 2000.

3. Results

3.1. Laccase extraction and purification

The extract from the homogenized lichen thalli had dark brown color due to presence of phenolic compounds in *S. crocea* tissue (Huneck and Yoshimura, 1996). To prevent oxidation of phenolics by laccase, 1% of ascorbic acid was added to the extracting solution (Pierpoint, 2004). First gel-filtration step allowed removal of most of the pigment present. The remaining pigmentation was eliminated during the next purification stages (ion-exchange or hydrophobic chromatography).

Laccase preparation obtained after the first gel-filtration step contained only the large laccase form (Figs. 1A and 2 lane 1 on A, B). After subsequent ion-exchange chromatography on an UNOQ

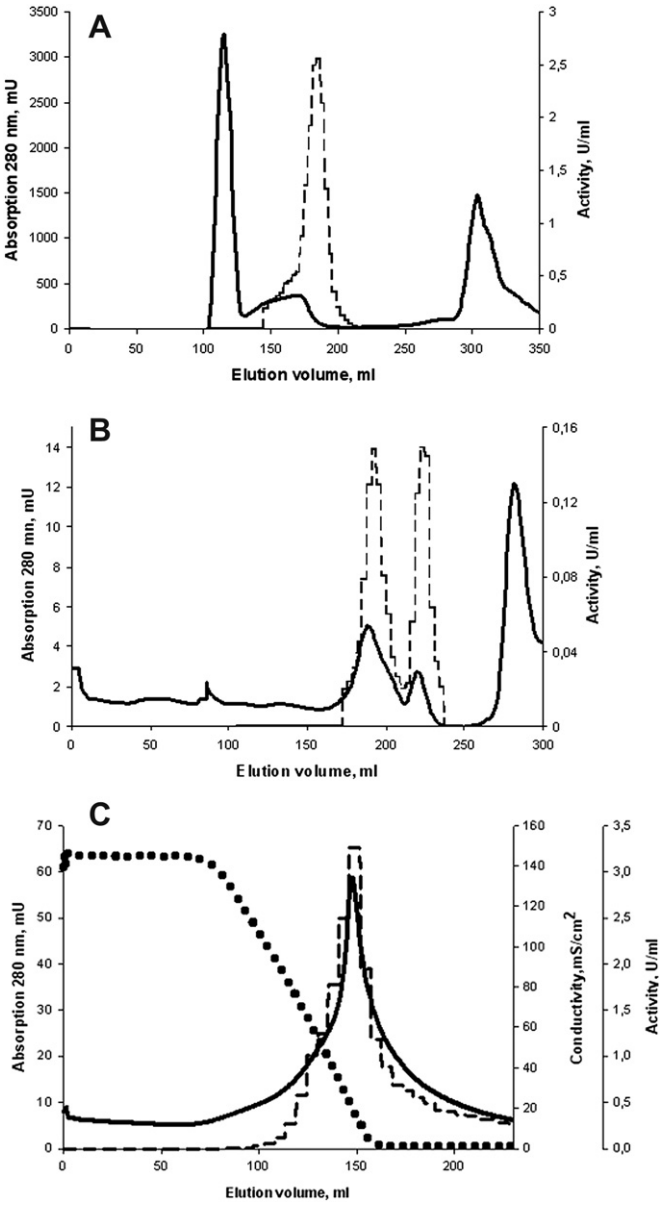

Fig. 1. Purification of laccases from lichen *S. crocea*. A – the first gel filtration after precipitation by $(NH_4)_2SO_4$; B – gel filtration after ion-exchange chromatography on UNOQ column; C – elution profile on Phenyl Sepharose column. The solid line denotes absorption at 280 nm, the short dash line shows laccase activity, the dotted line is $(NH_4)_2SO_4$ gradient.

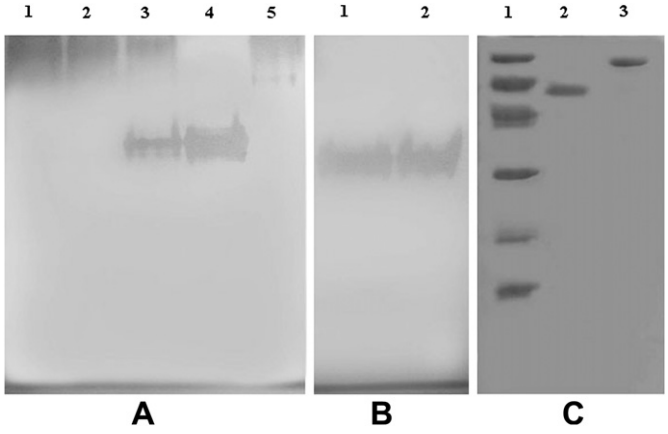

Fig. 2. Laccase specific activity staining in native electrophoresis gel after each stage of purification. A – purification using UNOQ ion-exchange chromatography: lane – initial crude extract from thalli; lane 2 – gel-filtration; lane 3 – laccase preparation after ion-exchange on UNOQ; lanes 4 and 5 – preparations of small (4) and large (5) laccases after the final gel-filtration step. B – purification using Phenyl Sepharose hydrophobic chromatography: lanes 1 – preparation after hydrophobic chromatography on PhenylSepharose column; lane 2 – gel filtration. SDS-PAGE of large and small laccases: lane 1 – protein standards, lane 2 – small laccase, lane 3 – large laccase subunit. Molecular markers – 97, 66, 45, 29, 20, 14 kDa.

column laccase activity eluted as a single peak (data not shown). However, native PAGE electrophoresis of active fractions revealed the appearance of small laccase form in addition to large laccase (Fig. 2A lane 3). The final gel-filtration step allowed separation of small and large forms from each other (Fig. 1B). Since the column UNOQ is packed with anionite which matrix is characterized by some hydrophobicity, we hypothesized that the appearance of small laccase was a result of transformation of large laccase into monomeric form due to hydrophobic interactions on the column. Therefore ion-exchange chromatography step was replaced by hydrophobic interaction chromatography on a Phenyl-Sepharose column (Fig. 1C). After passing active fractions through this column, all large laccases were transformed into the small form (Fig. 2B lanes 1, 2).

SDS-PAGE electrophoresis showed that large and small laccases were present as a single protein bands (Fig. 2C). Concentrated preparations of both laccases had a blue color, characteristic of blue laccases. The apparent MW of large enzyme determined by gel-filtration was 155 kDa. According to SDS-PAGE, the molecular weight of large laccase subunit was 80 kDa, suggesting that it is a homodimeric protein. Apparent MW of small laccase determined by gel-filtration was 50 kDa, but MW determined by SDS-PAGE was 60 kDa. These data confirm that small laccase is a monomeric protein.

3.2. Characterization of laccases

Large and small laccases differed in their physico-chemical properties. The optimum pH of ABTS oxidation for large laccase was around 4.0. After its transformation into small (monomeric) form, the pH optimum became lower than 3.0 (Fig. 3A). The pH optimum of 2,6-dimethoxyphenol oxidation was 4.0 for large laccase and 4.5 for small one (Fig. 3B). Monomeric laccase was more thermostable than large enzyme at 50 °C and 60 °C (Fig. 3C, D). The half-inactivation time for monomeric laccase was 60 min at 50 °C and 30 min at 60 °C. The dimeric laccase was half-inactivated within 25 min at 50 °C and fully inactivated at 60 °C during 35 min (Fig. 3C, D). Large laccase rapidly lost activity during storage, losing 95% during three days of storage at 22 °C and 85% during 10 days of storage at 5 °C. Under the same conditions small laccase has lost 70% and 28% of activity respectively.

Both enzymes did not oxidize the specific tyrosinase substrate L-tyrosine during 1 h of reaction. Kinetic constants of some common laccase substrates are given in the Table 1. Substrates affinities (K_m) of both laccase forms were similar with the exception of hydroquinone, for which the large laccase had a slightly higher affinity. Nevertheless, the maximum velocities (V_{max}) of reactions catalyzed by small laccase were always higher. The reaction rates (V_{max}/K_m) showed that small enzyme oxidized each of the substrates tested more efficiently than did the large enzyme.

3.3. Interaction with humic acids

Both laccases were inhibited by humic acids, but the activity of the large enzyme was suppressed to a greater extent (Table 2). In order of increasing inhibitory effect, the sequence of laccase inhibition was $HA_{ch} < HA_s < HA_p$.

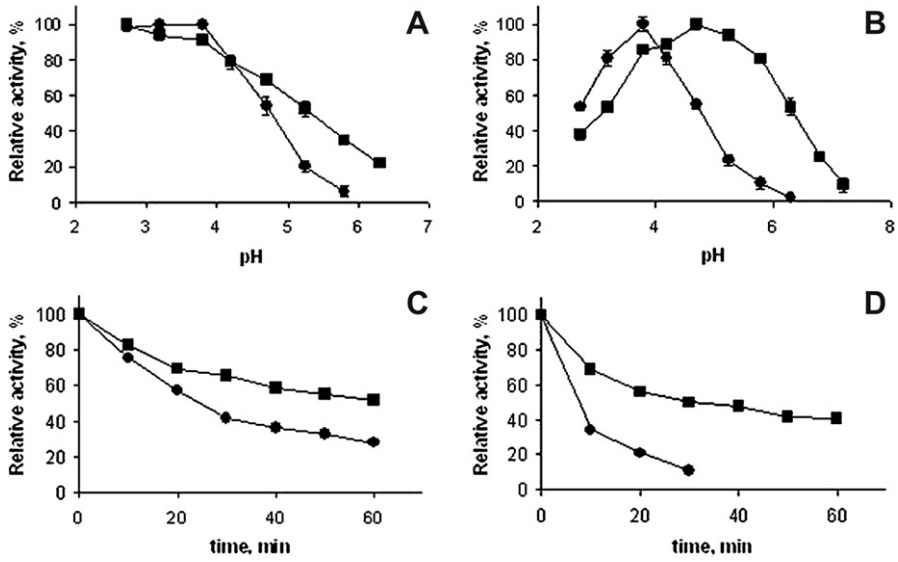

Fig. 3. pH optima (A, B) and thermostability (C, D) of large (●) and small (■) laccases. pH optima: (A) – ABTS oxidation, (B) 2,6 DMP oxidation. Thermostability: (C) 50 °C, (D) 60 °C.

Table 1
Kinetic constants of large and small laccases of *S. crocea*.

Substrate	Large laccase			Small laccase		
	K_m, mM	V_{max}, U mg^{-1}	V_{max}/K_m	K_m, mM	V_{max}, U mg^{-1}	V_{max}/K_m
ABTS	6.3×10^{-2}	25	396.8	8×10^{-2}	63	787.5
2,6-Dimethoxyphenol	0.1	3.8	38	0.17	15	88.2
Guaiacol	0.463	0.63	1.36	0.564	2.96	5.24
Hydroquinone	0.481	3.64	7.56	0.762	7.16	9.4

The interaction of large and small laccases with humic acids resulted in HA depolymerization (Fig. 4). Changes in the humic acids were most evident after 72 h of interaction with laccase. Initial HA_s consisted of two fractions with average molecular weights of >75 kDa (high molecular weight fraction, HMW) and 5.0 kDa (low molecular weight fraction, LMW). After 24 h of the reaction with large laccase the amounts of the HMW and LMW fractions decreased and products with average MWs of 48 kDa and 24 kDa appeared (data not shown). After 72 h of reaction the peak height of HMW fraction decreased and components with MWs of 48 kDa and 24 kDa decreased in the amount; a corresponding increase in the amount of LMW fraction was observed, but its MW decreased to 3.8 kDa (Fig. 4). Changes caused by small laccase in HA_s were similar, except that HMW fraction peak disappeared within 24 h (data not shown). After 72 h of the reaction the components with average MWs of 48 kDa and 24 kDa appeared; the LMW fraction decreased in amount and its average MW became 4.5 kDa. Changes in HA_{ch} caused by large and small laccases were much less pronounced than the changes in HA_s. The initial HA_{ch} consisted of a HMW fraction (MW > 75 kDa) and major LMW peak (5.4 kDa). Incubation with *S. crocea* laccases caused small decrease in the amount of LMW fraction (Fig. 4). Changes in optical properties of HA were insignificant. Both laccases caused slight decolorization of HA_s and HA_{ch}. After 72 h of incubation absorbance at 280 nm of HA_s showed decreased by 15%, while absorbance of HA_{ch} decreased by 10%.

4. Discussion

We have shown the occurrence of two laccase forms in the soil-stabilizing lichen *S. crocea*. The monomeric enzyme was purified from homodimeric form using hydrophobic interaction chromatography. Thus, oligomeric nature of large lichen laccases seems to be an artifact. Purified laccases of *S. crocea* depolymerized to some extent soil humic acids. This finding suggests potential involvement of leached lichen laccases in transformation of soil organic matter.

4.1. Occurrence of large and small laccases in S. crocea and their extraction

In our earlier work we found both large and small laccase forms in freshly collected *S. crocea* (Lisov et al., 2007). Small laccases were easily washable from intact tissue, but after homogenization of the thalli and centrifugation were absent in supernatant and appeared only after the treatment of the pellet with a detergent (0.1% Tween-80). In the present work we used dry lichen tissue stored at room temperature for two years. Nevertheless, substantial laccase activity was retained by the thalli. This activity represented enzyme, preserved in lichen during desiccation and storage but not the enzyme synthesized *de novo*, since laccase activity appeared

Table 2
Inhibitory effects of humic acids on laccases of *S. crocea*.

Humic acid	Activity, %	
	Large laccase	Small laccase
Without HA	100	100
HA_p	22	52
HA_s	41	77
HA_{ch}	49	82

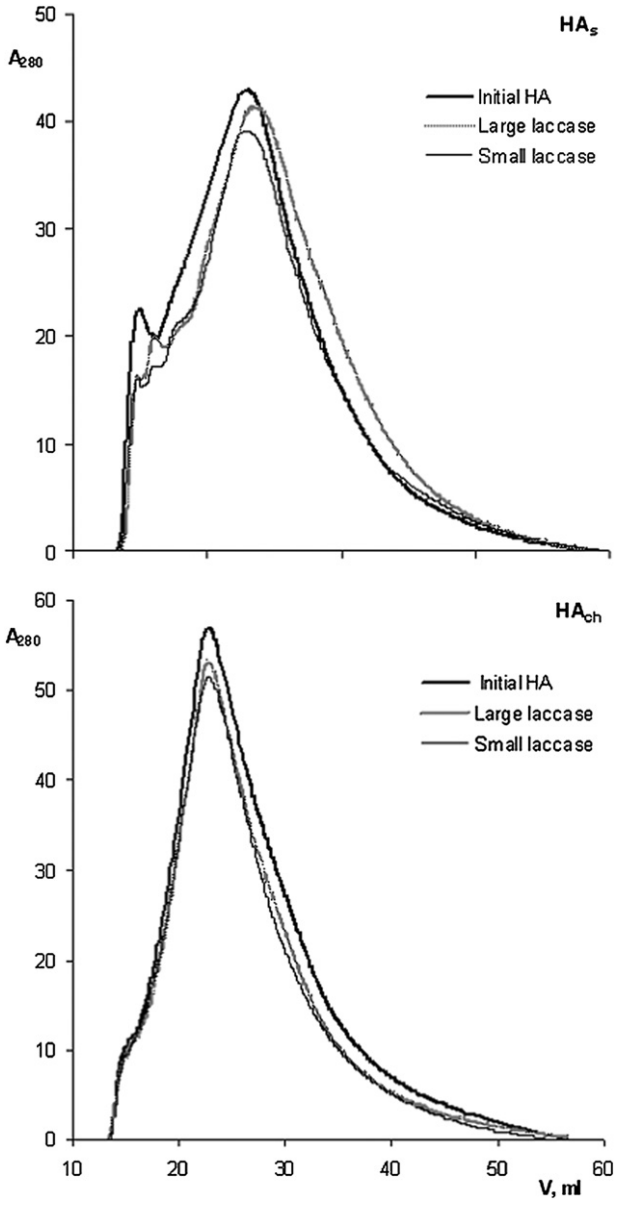

Fig. 4. Molecular weight distributions of humic acids before and after 72 h of interaction with laccases of *S. crocea*. HPLC analysis was carried out on Agilent 1100 system, equipped with diode array detector. TSK 2000 SW column (7.5 × 60 mm) was used, the eluent was 0.1 M Na-phosphate buffer (pH 7.0) with 0.1% SDS and 0.05% NaN_3.

immediately after rehydration. However, only large dimeric laccase was found in crude extract from homogenized thalli (Figs. 1 and 2, line 1on A). It is probable that small laccase was somehow affected by storage.

4.2. Transformation of large laccase of S. crocea into the monomeric form

The fact that large dimeric laccase can be transformed into small monomeric one during hydrophobic interaction chromatography allows us to suggest that both laccase forms in *S. crocea* represent the same enzyme. It is probable that large laccase is homodimeric protein, consisting of two monomeric laccases, each of which is bound with hydrophobic additional component(s) (AC). Thus, large laccase "subunit" with MW of 80 kDa (estimated by SDS-PAGE) actually represents the complex of small laccase with AC. The difference between MW of this complex (80 kDa) and MW of small laccase (50 kDa) allows estimation of approximate MW of AC as 30 kDa. During SDS-PAGE, the ionic detergent SDS breaks the bond between two "small laccase-AC" subunits in the large laccase. But SDS is unable to destroy the bonds between small laccase and its additional component(s). Passage through hydrophobic Phenyl-Sepharose column liberates monomeric enzyme with MW of 50 kDa. We also hypothesize that "small laccase-AC" subunits in the large enzyme are linked together via AC. This suggestion is based on the observation that due to removal of linking agent (AC) no reverse transformation of monomers into homodimer occurred after hydrophobic interaction chromatography.

The role of the AC is unknown at this stage. It might serve as conservation agent preventing laccase in *S. crocea* tissue from inactivation during stress. In other words, the large enzyme (a complеч of small laccase with AC) may represent the form in which the small laccase is preserved in lichen tissue. As indirect confirmation of this suggestion may serve the fact that in many other peltigerous lichens only oligomeric laccases were found (Laufer et al., 2009). We can also speculate that AC might serve as a linkage between laccase and cell wall, since most of laccase in peltigerous lichens was loosely and hydrophobically bound to cell wall (Laufer et al., 2006b). However, future studies are needed to prove if our suggestions are correct.

4.3. Physico-chemical properties of large and small laccases

The large and small laccases differ in their pH optima, thermostability, kinetic properties and oxidation potential. The properties of the small laccase resemble those of the laccases from basidiomycetes (Baldrian, 2006). A less acidic pH optimum of large laccase in metabolizing ABTS and a lower oxidation potential are more common for the laccases of ascomycetes (Rabinovich et al., 2004). If both laccases in *S. crocea* represent the same enzyme (as we hypothesize), then it is most probable that the AC affects substrate binding and catalysis itself.

Purified large laccase was unusually unstable, while small laccase showed stability more typical for laccases from basidiomycetes. Instability of large laccases after their extraction from lichen tissue seems to be common for many peltigerous lichens. According to Laufer et al. (2009) oligomeric laccases have lost 30–90% of activity during dialysis of water extracts (10 kDa cut-off).

4.4. Interaction of lichen laccases with humic acids

In our earlier work (Zavarzina et al., 2004) we found that humic acids were competitive inhibitors of laccase from basidiomycete *Panus tigrinus*. More hydrophobic humic acids and more hydrophobic HA fractions caused the largest inhibitory effect. For comparison we studied the inhibitory effect of the same HA on the large and small laccases of *S. crocea*. The overall tendency was the same as for laccase from *P. tigrinus*: the inhibition of laccase activity by HA decreased in the same order as their hydrophobicity. The activity of the large laccase was suppressed to a greater extent in presence of HA than that of the small enzyme (Table 2). The inhibition of small laccase and laccase of *P. tigrinus* by HA were quantitatively similar (data not shown). The stronger inhibitory effect of humic acids (HA_p in particular) on the large laccase might be explained by its stronger hydrophobic interactions with HA due to presence of the hydrophobic AC in its structure.

Despite considerable differences in catalytic properties and stability, both laccases of *S. crocea* depolymerized and decolorized HA in comparable rates (Fig. 4). Small laccase was somewhat more effective than large enzyme which is in agreement with its higher oxidation potential (Table 1). The changes caused by lichen laccases in HA_s were somewhat similar to the changes caused by basidiomycete *P. tigrinus*. In both cases the content of high molecular weight fraction in HA_s decreased. However, in the presence of lichen laccases both HA_s fractions reduced their molecular weights, while in presence of *P.tigrinus* laccase the LMW fraction of HA_s polymerized (Zavarzina et al., 2004). The minor effect of lichen laccases on HA_{ch} is somewhat surprising. This HA was effectively depolymerized by laccase of *P. tigrinus*: the relative content of its HMW fraction decreased while the content of LMW fraction correspondingly increased, and at the same time the LMW fraction decomposed to lower MW products (Zavarzina et al., 2004). It should be noted that molecular weight distributions of HA_s and HA_{ch} in this study were somewhat different from gel-filtration data of the same HA in previous work (Zavarzina et al., 2004). The lower pH of the eluent as well as other differences in methods used for MW determination could be responsible.

The data presented here demonstrate that laccases of *S. crocea* can oxidize humic acids *in vitro*. Direct involvement of laccases of this species in organic matter turnover in the environment is also possible. The laccase activities eluted by water in *S. crocea* were extraordinary high in some collections. ABTS immediately turned to dark bluish-green when small piece of fresh lichen thallus was placed into ABTS-containing buffer (our field observations). However, more experiments should be done before generalizations could be made on the role of lichens in organic matter decay, because leached-out laccase activities in other peltigerous lichens were commonly low (Zavarzina and Zavarzin, 2006; Laufer et al., 2006a; Lisov et al., 2007). Drawing analogues with free-living ascomycetes, literature data suggest that they have only limited ability to perform degradation of lignin and humic acids; direct involvement of laccases in these processes is questionable because some species showed bleaching activity in absence of oxidases (reviewed by Zavarzina et al., 2011). Nevertheless, ability for partial lignin degradation is known for wood-colonizing xylariaceaeous ascomycetes; some species perform selective delignification of fallen leaves due to presence of laccase (Liers et al., 2006; Osono, 2007). As for basidiomycetes, it is known that their blue laccases can depolymerize lignin (Maltseva et al., 1991) and soil humic acids (Zavarzina et al., 2004) *in vitro* in absence of mediators or other enzymes. Interestingly, both laccases of *S. crocea* have acidic pH optimum, more close to that of basidiomycetes than that of ascomycetes. According to Rabinovich et al. (2004) neutral laccases of most ascomycetes (pH optimum 6.0–7.0) are predisposed for substrate polymerization, while acidic laccases of the white-rot fungi (pH optimum 4.0–5.0) are predisposed for substrate degradation.

However, future studies need to prove if lichens can metabolize soil organic matter components *in vivo*. Even for white-rot fungi it seems likely that efficient depolymerizing activity of laccases *in vivo* require presence of redox mediators (Eggert et al., 1996;

Temp et al., 1999). Although no studies have been performed so far on lignocellulose degradation by intact lichens, experiments on dye decolorization carried out by Z. Laufer have shown that water leachates from thalli were less effective than intact thalli and that classic laccase mediators speeded up decolorization (personal communication with Prof. R.P. Beckett).

5. Conclusions

Research conducted in this paper have shown the occurrence of two laccase forms (homodimeric/large and monomeric/small) in the soil-stabilizing lichen *S. crocea*. The new finding was that homodimeric laccase of *S. crocea* can be transformed into the monomeric enzyme using hydrophobic interaction chromatography. Thus, oligomeric nature of large laccase of *S. crocea* seems to be artifactual. Based on our purification protocol we hypothesize that large laccase represents a complex of two monomeric enzymes bound together via additional hydrophobic component(s). The physico-chemical and catalytic properties of the small laccase resemble those of the laccases of basidiomycetes and thus have a potential to be involved in similar processes. Purified laccases of *S. crocea* depolymerized to some extent soil humic acids. This finding suggests that in some ecosystems leached lichen enzymes may play an important role in the transformation of natural organic matter, and therefore play a previously unsuspected role in the global carbon cycle.

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