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Purification of Extracellular Laccase from *Rhizoctonia praticola*

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Rhizoctonia praticola was found to produce large amounts of extracellular laccase when grown aerobically on the optimized Lindenberg and Holm medium in fermenter culture with automatic pH control. The laccase from this source was purified to homogeneity by a rapid procedure, using ion–exchange chromatography, affinity chromatography and chromatofocussing. The enzymes isoforms were recovered with a 38– to 115–fold increase in specific activity and a yield for lac1 = 38.93%; lac2 = 14.71% and lac3 = 7.65%. The molecular weight of the purified enzymes proved to be 215, 175 and 68 kDa respectively as determined by size–exclusion HPLC. The isoelectric points were between 6.6 and 7.8, and the carbohydrate content in the purified enzymes was between 6.4–9.7%.

INTRODUCTION

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases (PPO) that catalyze the oxidation of various substituted phenolic compounds by using molecular oxygen as the electron acceptor (Thurston, 1994). Phylogenetically, laccases are members of the multi–copper protein family including ascorbate oxidase, ceruloplasmin, ferredoxin, phenoxazinone synthase and bilirubin oxidase (Mayer and Staples, 2002; Nakamura and Go, 2005). Until recently, laccases were only found in eukaryotes (fungi, higher plants, insects) (Mayer and Staples, 2002), but now there is strong evidence for their widespread distribution in prokaryotes and the first crystal structure of a bacterial laccase is already available (Enquita *et al*., 2003). Fungal laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is an enzyme secreted into the medium by mycelia of *Basidiomycetes, Ascomycetes* and *Deuteromycetes* (Leonowicz *et al*., 2001).

The enzymes are involved in the pathogenicity, immunity and morphogenesis of organisms and in the metabolic turnover of complex organic substances such as lignin or humic matter. The ability of laccases to act on a wide range of substrates makes them highly useful biocatalysts for various biotechnological applications (Claus, 2004; Sharma *et al*., 2007). Such applications include the detoxification of industrial effluents as well as use as a tool for medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil. Laccases are also used as cleaning agents for certain water purification systems, as catalysts for the manufacture of anti–cancer drugs, and even as ingredients in cosmetics (Couto and Herrera, 2006). A fascinating character of the direct four–electron reduction of oxygen to water is another important application of laccase in the modified electrode of dioxygen sensing system (Rowiński *et al*., 2004) and in the cathode compartment of biofuel cells (Shleev *et al*., 2005; Stoica *et al*., 2009). In 1979 a fungal source of extracellular laccase was found: *Rhizoctonia praticola*, which produces this enzyme showing an alkali pH optimum for several substrates (Bollag *et al*. 1979) as well the laccase from *Melanocarpus albomyces* (Kiiskinen *et al*., 2002) in contrast to other laccase producers. This characteristic makes *Rhizoctonia praticola* laccase a potentially very useful tool in many applications mentioned above.

The goal of the presented work was to isolate and purify the extracellular laccase from the basidium fungus *Rhizoctonia praticola* and to characterize the enzyme including determination of physicochemical and kinetic properties.

MATERIALS AND METHODS

Strain and culture conditions

*Rhizoctonia praticol*a 93a was obtained from the culture collection of the Pennsylvania University, USA. The fungus was maintained in 2% (w/v) malt agar slants. As an inoculum, pieces of agar were grown in the Lindenberg and Holm (Lindeberg and Holm, 1952) medium in non–agitated conical flasks for 7 days at 28 ˚C. The mycelial mats were subsequently collected and homogenized in a Waring blender. After inoculation with 2.5% (v/v) crumbled fungal mats, the cultures were run at 26 ˚C in 100 ml wide–mouth Erlenmeyer flask (each of them containing 40 ml of culture medium) placed on a rotary shaker (180 rpm).

The fermentor scale cultivation was performed at 28 ˚C in a 2.5 l Bioflo Ⅲ (New Brunswick; Edison, NJ, USA) fermentor containing 2 l of the sterilized Lindenberg and Holm medium (Lindeberg and Holm, 1952) optimized as in Janusz *et al*. (2006) at 121 ˚C by 30 min. The fermentor was inoculated with crumbled fungal mats

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(10% of total volume), aerated at 1 l air per minute and stirred at 300 rpm. Antifoam B emulsion (Sigma, St. Louis, USA) was occasionally added to the fermentor cultures to break the foam. The pH change was achieved by adding 5% ammonia or phosphoric acid. The stabilization of pH on the level of 8.0 on the second day and adding of 5 μM cooper ions dose before inoculation were the most convenient for maximalisation of laccase activity. The obtained laccase level in culture liquid was (about 4,000 nkat/l) as in Janusz *et al*. (2006).

Enzyme purification

The after culture liquid was centrifuged at 10 000×g on K6 15 (SIGMA, Osterode am Harz, Germany) centrifuge for 10 min. The supernatant was concentrated 10 times on the ultrafiltration system Pellicon 2 Mini holder (Millipore, Bedford, USA) with Ultracel mini cartridge (10 kDa cut off) and used as the source of crude enzyme. Chromatography was performed using a chromatographic Econo–System (Bio–Rad, Richmond, USA). The enzyme solution was purified on a vanillyl–CPG carrier synthesised as in Rogalski *et al*. (1990) and equilibrated on the column (1.5×10 cm) with 0.01 M Mc Ilvaine buffer (pH 8.0). The proteins not bounded to the column were eluted by the same buffer, whereas bounded ones by applying 0.7 M ammonium sulphate. Fractions showing laccase activities were pooled and applied to a DEAE–Sepharose column (2.5×15 cm) pre–equilibrated with 20 mM Tris–HCl buffer (pH 6.5). The proteins adsorbed on the column were eluted with a 0–0.5 M linear gradient of NaCl at the flow rate of 1 ml/min. Active laccase fractions (obtained after DEAE–Sepharose) were next chromatofocused on PBE–94 ion exchanger packed in chromatography column (1×20 cm) equilibrated with 250 ml of a 0.025 M TRIS/acetate buffer (pH 8.35). A sample showing laccase activity (5 ml) was injected onto the column, and the enzyme was desorbed by elution with 200 ml of Polybuffers 96 and 74 (25/75; v/v), stabilizing at pH 4.5 by acetate acid, at the flow rate of 0.5 ml/min. The active fractions were pooled, and the purified enzyme solutions were used for further kinetic experiments.

Laccase and other assay

The activity of the laccase was determined by following the oxidation of 0.025 mM syringaldazine in 0.1 M citrate–phosphate buffer at pH 5.0 (Leonowicz and Grzywnowicz, 1981) by photometry at λ = 525 nm (ε_{525} = $6.5 \cdot 10^4$ $M^{-1} cm^{-1}$ at 25 °C). The activity was expressed in nkat per liter. The protein content was determined with bovine albumin as a standard (Bradford, 1976). The carbohydrate content in the purified laccases were determined by the method of Dubois *et al*. (1956) using glucose as a standard. The mean standard error in protein determination was ± 4.8×10^{-3} mg and ranged from ± 5.7×10^{-5} to ± 9.1×10^{-3}. For the cooper concentration determination in laccase isoforms the homogenic isoforms of laccase was concentrated (110 μg/ml) and dialyzed against deionized water (Milli Q) by ultrafiltration on centricon YM10 filters (Millipore, Billeria, Mass, USA). The copper content was determined by GR atomic absorption spectroscopy on SpectrAA–880Z with the Zeeman background correction system (Varian, Mulgrave, Australia). Optical absorption spectra were recorded at 25 °C on a UV 160A spectrophotometer (Shimadzu; Tokyo, Japan) connected to IBM–PC computer with PC160A software and equipped with a TCC controller thermostat cuvette holder in 1 cm quartz cuvette.

Molecular weight determination of purified laccase isoforms was obtained by the injecting them onto the Protein–Pack 300 SW (10 μm; Millipore–Waters, Milford, MA, USA) column connected with Shimadzu HPLC system composed of LC–9A chromatography pump, the SPD–M6A diode array detector and the model 7125 sampling valve (Rheodyne, Berkeley, CA, USA) with 100 µl loop. The mobile phase 0.1 M acetate buffer (pH 5.0) was run at the flow rate of 0.5 ml/min. The column was calibrated by the linear least square method employing Quattro Pro software (Borland, Qupertino, USA) on IBM PC using the MS II Gel–filtration standard set (Serva, Heidelberg, Germany). The hydrolysis of laccase glycosic compounds was made according to Niku–Paavola *et al*. (1988). The 0.45 ml samples (0.2 mg protein) were mixed with 50 μl 10% SDS in 100 °C by 5 min. Next the 50 μl Triton X–100 and 5 μl N–glucosidase F (Calbiochem, San Diego, CA, USA) was added and the mixture was incubated for 48 hrs at 37 °C. The obtained hydrolysates were analysed by Shimadzu HPLC *vp* system composed of LC 10AD *vp* pump, refractive index detector, SLC–10A *vp* system controller, CTO–10A *vp* column oven and the model 7725i sampling valve (Rheodyne, Berkeley, CA, USA) with 20 μl loop on Kromosil–NH_2 column (0.4×25 cm; 10 μm; Phenomenex) at 25 °C. The mobile phase (a mixture of acetonitrile and water in the ratio 72: 28 v/v) was run at the flow rate of 1 ml/min. For the standardization of this column saccharide standard Sugar and Sugaralcohole for chromatography A and B (Merck, Darmstadt, Germany) was used. The glucoronic acid as well as N–acetylglucosamine was quantified by the same HPLC system on Rezex ROA–org acid column (8 μm, 300×7.8 mm; Phenomenex). The mobile phase in the case of uronic acids was 0.005 M H_2SO_4 (in Milli Q water) run at 0.5 ml/min at 55 °C and for N–acetylglucosamines 1% phosphoric acid (in Milli Q water) run at 0.6 ml/min at 25 °C.

Kinetic constants

The reaction rate was determined at five different substrate (2,6–DMP, syringaldazine, guaiacol, veratric acid and ferulic acid) concentration in the range of 0.01 to 10 mM. All assays were performed in triplicate. The kinetic constants (K_m, and V_{max}) for purified laccase isosymes were calculated on an IBM PC employing Wilman 4 (1985) software (M.S.U., USA) using a Clark oxygen electrode (Rank, Cambridge, Great Britain) provided with a linear TZ 4100 recorder (Rogalski *et al*., 1990).

Chemicals

4–hydroxy–3,5–dimethoxybenzaldehyde azine (syringaldazine) and 2,6–dimethoxyphenol (DMP) were obtained from Aldich (Steinheim, Germeny) whereas

3,4–dimethoxybenzoic acid (veratric acid), 3–methoxy, 4–hydroxy cynammic acid (ferulic acid) and 3–hydroxy, 4–methoxybenzene (guaiacol) from Sigma (St. Louis, USA).

RESULTS AND DISCUSSION

Purification of laccase

The after–culture liquid from the fermentor after centrifugation at 10 000×g was concentrated by ultrafiltration on PELICON 2 unit (10 kDa cut off) and loaded onto a vanillyl–CPG column (Fig. 1). The carrier showed the optimum for sorption of *R. praticola* laccase on vanillyl–CPG at pH 8.0. After eluting the non–adsorbed proteins, further elution was done by applying 0.7 M ammonium sulphate. All the laccase activities were bound to the column, and eluted as one peak. The purification effect of this step was low, as compared to the following ones (Table 1). The enzyme solution obtained in the previous step was applied to a DEAE–Sepharose column (Fig. 2).

After eluting the non–adsorbed proteins, further elution was done by linear gradient from 0 to 0.5 M sodium chloride solution in the same buffer. The laccase activities were eluted as two peaks (first 0 M and second at 0.1–0.2 M NaCl).This step resulted in a nearly 29 and 17–fold purification of the enzymes with a recovery 47 and 28% respectively (Table 1). The active fractions of all laccase forms were pooled respectively and further fractionated by chromatofocusing on Polybuffer exchanger PBE 94. The chromatographic pattern are depicted in Fig. 3. Fraction lac I was further purified giving one form lac I with the isoelectric points at 6.75. This isoform was purified 115–fold with the recovery of 39%. Fraction lac II was divided into two laccase forms (lac IIa and lac IIb) with the isoelectric points at 7.85 and 6.60. These isoforms were purified 57 and 38–fold with the recovery of 14.7 and 7.65% respectively. The obtained isoelectric points indicate that they differ from the so–called "acidic laccases" as obtained from *Coriolus hirsutus*, *Coriolisimus fulvocinerea*, *Coriolus zonatus* and *Cerrena maxima* (Smirnov *et al.*, 2001). Bollag *et al.* (1979) obtained only one form of laccase *Rhizoctonia praticola* with similar recovery. Similar pI points were determined for laccase from *Rhizoctonia solani*: 7.5; 7.5; 5.0 (Wahleithner *et al.*, 1996). The isoelectric point of bacterial laccases differs more signifi-

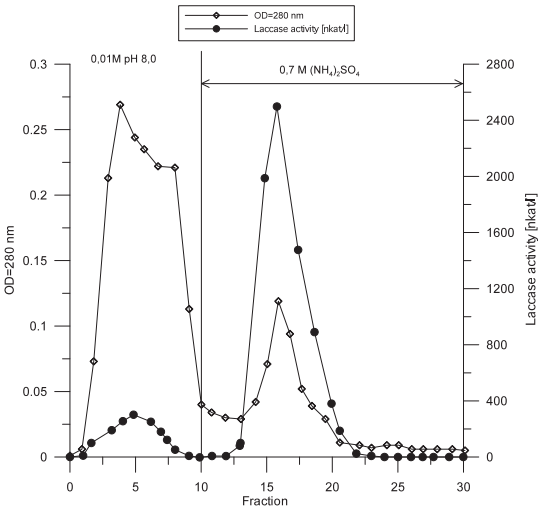

Fig. 1. Chromatography of crude *Rhizoctonia praticola* laccase on vanillyl–CPG.

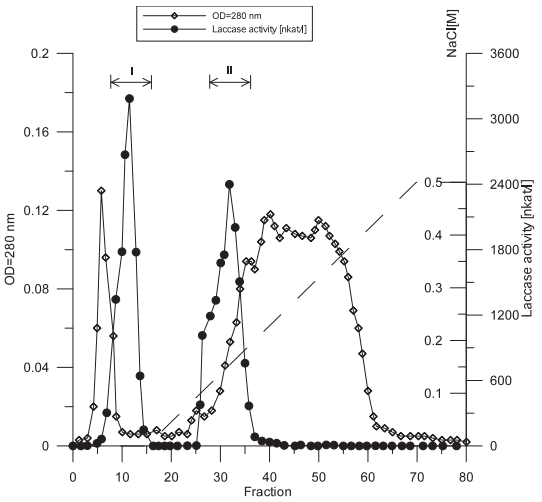

Fig. 2. Chromatography on DEAE–Sepharose of partially purified post vanillyl–CPG laccase.

Table 1. Summary of laccase purification from *Rhizoctonia praticola*

Purification step	Total protein (mg)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude after culture fluid	277.2	18,000	1.00	100
Ultrafiltration YM–10	222.9	20,520	1.14	92
Vanilin–CPG	57.6	69,480	3.86	80.2
DEAE–Sepharose (fast flow) (fraction I)	4.53	516,420	28.7	46.9
PBE–94 fraction–lac I (pI=6.75)	0.94	2,075,940	115.3	38.9
DEAE–Sepharose (fast flow) (fraction II)	1.40	313,920	17.4	28.5
PBE–94 fraction–lacIIa (pI=7.85)	0.72	1,022,040	56.8	14.7
PBE–94 fraction–lacIIb (pI=6.60)	0.55	692,280	38.5	7.7

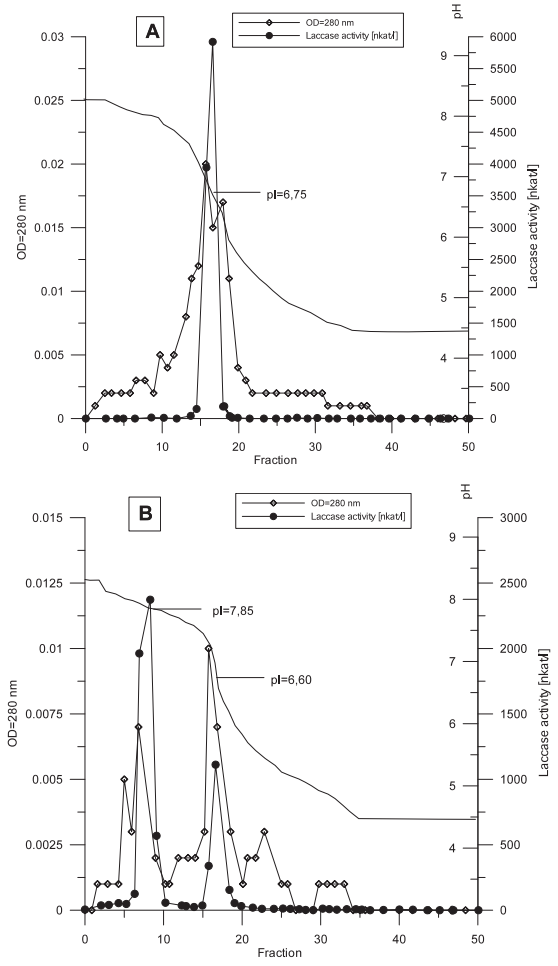

Fig. 3. Chromatofocusing pattern for fraction I (A), fraction Ⅱ (B) of DEAE–Sepharose purified laccases.

cantly in ranges pI=5.6 for laccase from Streptomyces cyaneus (Arias *et al*., 2003) to pI=7.7 for the one from *Bacillus subtilis* (Martins *et al*., 2002).

Physico–chemical and kinetic characterization of laccases

The purified laccase isoforms showed a high level of specific activity (from 300 to over 2000 μkat/mg protein), which were about 2 times as previously reported in (Bollag *et al*., 1979) although the condition of determining activity was in above paper different (DMP as a substrate). Homogeneity of the laccase fractions were examined and confirmed by size exclusion HPLC, where the final purified enzymatic preparations appeared as a single protein peak. The molecular weight was determined to be (for lac I = 215; lac Ⅱa = 175; lac Ⅱb = 68 kDa) presented in Table 2. It was proved several times that laccase may appear as monomer but as dimer, trimer or even oligomer as well (D'Annibale *et al*., 1996; Wahleithner *et al*., 1996; Min *et al*., 2001).

Wahleithner *et al*., (1996) characterized three of four laccases from *Rhizoctonia solani* as dimeric. These results may suggest that probably lac I is trimeric and lac Ⅱ is dimeric in *Rhizoctonia praticola*. Bollag *et al*. (1979) determined molecular weight of single form of laccase from *Rhizoctonia praticola* as 78 kDa, which would be similar to our monomer form of lac IIb. Therefore, the obtained molecular weights of purified laccases forms were very similar to the molecular weights of most other fungal laccases, which have been found to be between 60 kDa and 90 kDa (Yaropolov *et al*., 1994; Smiths and Thurston, 1997; Xu *et al*., 1999).

The carbohydrate content in the purified enzymes varied from 6.4 to 9.7%, indicating that they are a typical

Table 2. Characteristics of the purified laccases from *Rhizoctonia praticola*

			LacI	LacIIa	LacIIb
Isoelectric point (pI)			6.75±0.14	7.85±0.20	6.60±0.18
Molecular weight (kDa)[a]			215±7.9	175±9.8	68.7±2.3
Carbohydrate content (%)			6.40±0.54	9.70±0.70	6.93±0.60
Kinetic constants:	(for 2,6–DMP)	K_m (mM)	0.017±0,004	0.012±0.004	0.013±0.003
		V_{max} (μM O_2/min)	2.055±0.061	3.183±0.150	2.859±0.073
	(for guaiacol)	K_m (mM)	2.861±0.064	0.181±0.015	0.479±0.063
		V_{max} (μM O_2/min)	1.956±0.165	1.136±0.016	1.797±0.064
	(for ferulic acid)	K_m (mM)	0.385±0.041	3.396±0.088	7.399±0.095
		V_{max} (μM O_2/min)	1.754±0.037	6.664±0.748	7.202±0.539
	(for veratric acid)	K_m (mM)	–[b]	–[b]	–[b]
		V_{max} (μM O_2/min)	0.00±0.00	0.00±0.00	0.00±0.00
	(for syringaldazine)	K_m (mM)	0.673±0.024	0.235±0.042	1.159±0.081
		V_{max} (μM O_2/min)	4.660±0.094	5.598±0.337	14.975±0.787
Cooper ions content (mol/mol)			3.23±0.28	3.35±0.31	3.26±0.30
Optimum pH			7.4	7.4	7.4
Optimum temperature (°C)			60.0	60.0	60.0

[a] – estimated by high–performance gel filtration
[b] – activity was detected only at the highest substrate concentration, and therefore, a K_m could not be determined

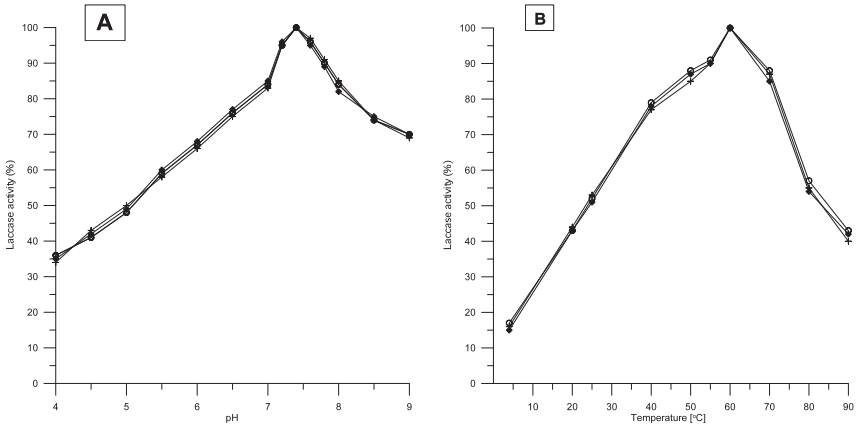

Fig. 4. Effect of pH (A) and temperature (B) on activity of *Rhizoctonia praticola* laccases. (○ lac I; + lac IIa; ◆ lac IIb)

glycoprotein. Waihleithner *et al.* (1996) determined glycosylation content in *Rhizoctonia solani* laccase from 10 to 20%, but these forms were expressed in *Aspergillus oryzeae*, which may possess tendency to hyperglycosylate heterogically expressed laccases. Biochemically, laccase is a glycosylated protein monomers or homodimers generally with a lesser count of saccharide compounds (10–25%) in fungi than plant enzymes (Rogalski and Leonowicz, 2004). In the case of white rot fungi such as *Coriolus hirsutus*, *Coriolus zonatus* and *Cerrena maxima*, these value varied from 10–13%. Laccase from *Coriolisimus fulvocinerea* showed atypical carbohydrate contents (32%) for fungal enzymes (Smirnov *et al.*, 2001). In some species (*Schinus molle* and *Botrytis cinerea*) glycosylation of laccase may reach even from 60 to 80% (Zouari *et al.*, 1985; Bar–Nun *et al.*, 1985).

Thus the *Rhizoctonia praticola* enzymes were glycoproteins comprising monosaccharides such as mannose, arabinose, fucose, xylose, glucose, and N–acetylglycosamine, glucuronic and sialic acid residues (Table 3). Mannose is a predominant sugar in *R. praticola* laccase isoforms as well as in other ones (Koroljova–Skorobogat'ko *et al.*, 1998; Giardina *et al.*, 1999; Dedeyan *et al.*, 2000). Glycosylation of laccase is presumed to protect enzyme from proteolysis and thermal degradation (Li *et al.*, 1999). The effect of pH and temperature on the activity and stability of *R. praticola* laccase isoforms are illustrated in Fig. 4. The optimum pH for all above forms was 7.4. The effect of temperature on laccasse forms activities were investigated over the range of 4–90 °C. As it is evident from the curves, the optimal temperature for them were 60 °C.

Laccases share the arrangement of the catalytic sites and the catalytic mechanisms with other blue multicopper oxidases. The enzyme contains four copper ions classified as a T1 (one copper), T2 (one copper), and T3 (two coppers) sites in accordance with their spectroscopic characteristics (Solomon *et al.*, 1996). An electron from the substrate is transferred to the T1 site, the primary electron acceptor of the enzyme, which is then transferred through an intramolecular electron transfer (IET) mechanism *via* a Cys–2His bridge to the T2/T3 cluster, where O_2 is reduced to H_2O (Solomon *et al.*, 1996). It is common to determine cooper content for the newly purified laccase isosymes. The cooper content of the purified laccase was determined by AAS method,

Table3. Sugar content in the purified laccases from *Rhizoctonia praticola*

Laccase	Lac I	Lac IIa	Lac IIb
Mannose [mol%]	61.12	81.56	76.37
N–acetyloglucosamine [mol%]	9.43	11.61	9.80
Glucose [mol%]	–	6.27	–
Glucuronic acid [mol%]	0.08	0.56	0.27
Sialic acid [mol%]	–	–	5.20
Fucose [mol%]	–	–	8.36
Arabinose [mol%]	29.37	–	–

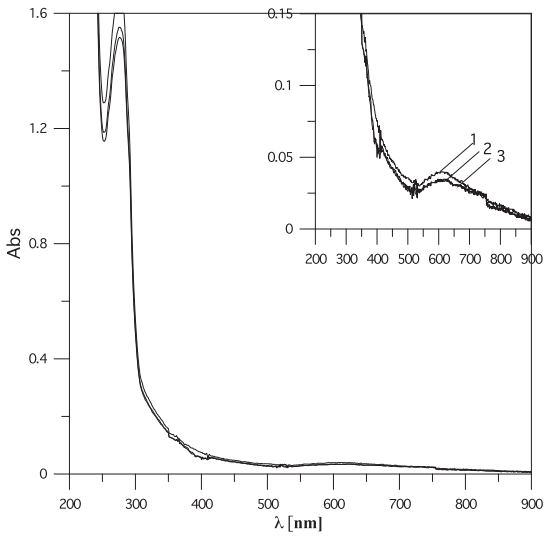

Fig. 5. Absorption spectrum of laccase isoforms from *Rhizoctonia praticola* (0.9 mg/ml in 0.1 M McIlvaine buffer, pH 5.5) in room temperature (1– lac I; 2– lac IIa; 3– lac IIb).

which measured in all cases about 3.26–3.35 ± 0.28–0.31 cooper ions per laccase molecule (Table 2). The absorption of homogenic laccases isoforms solutions showed no heme band around 400 nm. There were a small feature around 300 nm, which is known as the ligand to cooper charge transfer type 3 site (Fig. 5). The blue band of these laccases shows an adsorption maximum at 610 nm with the extinction coefficient of 3,540 $M^{-1}cm^{-1}$. These results are very good correlated with those obtained for other blue laccases (Kim *et al*., 2002).

The K_m and V_{max} values for five various substrates are presented in Table 2. In the case of veratric acid the purified isosymes did not show affinity to this substrate. It is clear that these enzymes can not belong to the class of yellow laccases because only these kinds of the enzymes can oxidase the veratric acid (Leontievsky *et al*., 1997). The affinities of laccase forms are different for each substrate. The best substrate for all isoforms was 2,6–DMP. Lac IIa had the highest affinity to this substrate, in the case of lac IIb the affinity was slightly lower and in the case of lac I – about 50% lower. The affinities to syringaldazine for lac IIa were also highest in relation to the lac IIb and lac I where this value was about 6 and 3 times lower respectively. Lac IIa had the highest affinity to guaiacol the affinity of forms lac IIb was about 50% lower, and lac I – over 10–times lower. The ferulic acid was the best substrate for lac I, in the case of lac IIa the affinity was about 10 times and for lac IIb 20 times lower.

In summary, the laccase isoforms derived from *R. praticola* had some interesting properties which can be used in some technical application *e.g*. in construction of biofuel cell or biosensors.

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