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Immobilization of laccase from *Phlebia radiata* on controlled porosity glass

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Abstract

Laccase from the white-rot fungus *Phlebia radiata* was immobilized on glass beads which were activated by γ-aminopropyltriethoxysilane. 98% of the protein and 96% of the laccase activity were coupled to the support. The final preparation contained ca. 1 mg of protein per gram of glass beads. The activity of the immobilized enzyme retained after two weeks preservation at +4°C was 100% and at +25°C over 90%. The activity in the presence of organic solvents was rather similar irrespective of the form of the enzyme, free or bound. However, the catalytic activity of the immobilized laccase was less vulnerable against inhibitors such as Cu-chelators and 2,6-dimethoxy-1,4-benzoquinone.

Keywords: Controlled porosity glass; Enzymes; Immobilization; Inhibitors; Kinetics; Laccase; Organic solvents; *Phlebia radiata*; Protein purification

1. Introduction

Fungal laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is usually secreted by various wood or litter-decomposing or soil-inhabiting fungi, mainly by those fungi which belong to the class *Basidiomycetes*. In this group the white-rot fungus *Phlebia radiata* is an efficient lignin degrader [1,2], and it readily produces extracellular ligninolytic enzymes [1,3]. Laccase shows a broad specificity, oxidizing many, mainly phenolic compounds which allows it to detoxify a number of aquatic and terrestrial xenobiotics [4] and industrial waste waters [5].

Lignins are natural polymeric compounds arising from an enzyme-initiated dehydrogenative polymerization of hydroxyphenyl alcohols precursor [6]. They are annually produced in tremendous amounts by plants, together with cellulose and hemicellulose. Lignin in plant cell walls protects cellulose and hemicellulose from enzymatic hydrolysis. In soil, it is the main substrate for humus formation. Lignin in wood cell walls may only be destroyed by drastic physicochemical treatment of lignocellulose materials [4–6], or in nature most efficiently by fungi [7–9] producing ligninolytic enzymes. Decomposition by microorganisms yields phenolic derivatives which may be toxic to the environment. Several enzymes such as lignin peroxidases, manganese peroxidases and laccase may cause structural modifications in lignin [7]. Among these enzymes, laccase apparently plays a role both in

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lignin polymerization and degradation [10,11]. This enzyme is commonly produced by basidiomycetous white-rot fungi [7,12,13]. It has been assumed that the role of laccase in lignin transformation is no less important than that of lignin peroxidase [14]. However, efficient lignin-degrading white-rot fungi typically produce laccase [7,12,14].

Laccase from *P. radiata* has been purified and characterized [3,15,16], the sequence of the gene encoding it has been determined [17], and it has successfully been produced in eukaryotic heterologous system [18]. Two copper atoms have been reported to be present in this laccase [19]. In contrast, the classical *Coriolus* (*Trametes*, *Polyporus*) *versicolor* laccase contains four copper atoms [20,21]. Laccase with two copper atoms has already been isolated for example from *Schizophyllum commune* [22].

The aim of the present work was to produce *P. radiata* laccase stimulated by veratryl alcohol, to immobilize the enzyme on controlled porosity glass. We also compared the activity and some kinetic and stability properties of the immobilized enzyme with those of the free enzyme.

2. Materials and methods

2.1. Fungus and culture conditions

Phlebia radiata Fr. 79 (ATCC 64658) was isolated at the Department of Microbiology, University of Helsinki, Helsinki, Finland [2]. The fungus was maintained on 2% (w/v) malt agar slants. For inoculum, pieces of agar were grown in asparagine–dimethylsuccinate (ADMS) medium [2] containing 2.0 mM of nitrogen (i.e., nitrogen limited conditions) and 56 mM glucose in non-agitated conical flasks for 6 days at 28°C. Then the mycelial mats were collected and homogenized in a Waring blender. After inoculation with 4% (v/v) of the homogenate, the stationary cultivations were carried out at 28°C in 100 ml conical flasks which contained 10 ml of ADMS medium. Veratryl (3,4-dimethoxybenzyl) alcohol (Janssen Chimica, Beerse, Belgium) was added to stimulate the production of lignin-olytic enzymes [1,3,16] on the third day after the inoculation to make the final concentration of 1 mM. Every 24 h three flasks were harvested and separately analyzed.

2.2. Laccase assays

Laccase activity in culture liquids and after purification was measured spectrophotometrically with syringaldazine as a substrate [23]. The activity is expressed as nanokatals, i.e., nanomoles of substrate oxidized in one second. The activity of immobilized laccase was assayed by polarographic analysis with a Clark-type oxygen electrode (Rank, Cambridge, Great Britain) equipped with a linear TZ 4100 recorder [24]. The K_m values were calculated from double reciprocal plots [25] using the ENZFITTER (1987, Biosoft) software in an IBM-AT computer (Siemens, PCD-2M).

2.3. Purification of Phlebia radiata laccase

Phlebia radiata culture filtrates were pooled and concentrated to ca. one tenth of the volume by ultrafiltration using a membrane with 10 kD cut off (YM-10, Amicon). The concentrated crude preparate was applied onto a DEAE–Sephadex column for ion exchange chromatography as described earlier [26]. Absorbances at 280 nm and 405 nm (to trace hemoproteins) were measured together with laccase activity. Laccase activity was found in the peak absorbing mostly at 280 nm. The enzyme was eluted from the column by 0.5 M potassium phosphate buffer pH 6.0. The fractions exhibiting laccase activity were pooled and desalted on a Sephadex G-25 column.

2.4. Polyacrylamide gel electrophoresis (PAGE)

To check the homogeneity of the collected enzyme preparation the gel electrophoresis under non-denaturing conditions according to Leonowicz et al. [26] was performed. Electrophoresis was

carried out under Tris/borate buffered conditions at pH 8.45 [26]. Protein bands were visualized with Coomassie Blue, and laccase was identified by the reaction with *para*-phenylenediamine after adjusting the pH in the gel to 4.5 [26].

2.5. Isolation and immobilization of Coriolus versicolor laccase

The cultivation conditions for *Coriolus* (*Trametes*) *versicolor* adopted for laccase production were described earlier [24]. Purification of the inducible form of laccase and the immobilization procedure were performed as described previously [24,27].

2.6. Preparation of controlled porosity glass (CPG)

The controlled porosity glass, obtained from Cormey-Lublin, Poland, was prepared according to the method described earlier [28]. The support ($S_{BET} = 87\ m^2/g$; $D = 71$ nm) was activated by γ-aminotriethoxysilane (APTES obtained from Sigma) according to the method which permits a high density level of amino groups on the glass surface [29]. The activated support (APTES–CPG) was further used for laccase immobilization.

2.7. Immobilization of laccase

The enzyme was coupled to APTES–CPG using glutaraldehyde (Sigma) according to the method reported earlier [30]. In this case 5 mg of laccase protein and 5 g of APTES–CPG were applied.

2.8. Chemicals

2,6-Dimethoxyphenol (2,6-DMP), caffeic acid, ferulic acid, syringic acid, and guaiacol were obtained from Aldrich (Steinham, Germany) and methoxyhydroquinone from EGA-Chemie (Steinheim/Albud, Germany). Organic solvents dimethylsulphoxide (DMSO) (Uvasol), dioxane (LiChrosolv), ethanol (LiChrosolv, 99,5%), acetone (LiChrosolv) and methanol (LiChrosolv) were from Merck (Darmstadt, Germany). Thioglycolic acid was from Merck (Darmstadt, Germany), thiourea from Sigma (St. Louis, USA), NaN_2 and EDTA from Merck (Darmstadt, Germany). 2,6-Dimethoxy-1,4-benzoquinone was cordially supplied by Dr. M. Tomaszewski, the Polish Academy of Science, Department of Dendrology, Kornik-Poznan, Poland.

3. Results and discussion

Low molecular-weight aromatic compounds, structurally related to lignin, increase the production of laccase, lignin peroxidase and manganese peroxidase in cultures of *Phlebia radiata* [31]. Among the compounds tested veratryl alcohol remarkably stimulates the production of laccase in stationary [31] as well as in semi-continuous cultivations [16]. Veratric acid also enhances the production of laccase by *P. radiata* [32]. In order to select the optimum concentration of glucose in the medium and the most suitable time for veratryl alcohol (1 mM) addition, *P. radiata* was stationarily cultivated for 9 days. The results presented in Fig. 1 show that when veratryl alcohol was added at the moment of inoculation (Fig. 1a) the maximum activity of laccase appeared between days 4 and 6. When veratryl alcohol was added on day 3 (Fig. 1b), the maximum activity was detected between days 5 and 6. In the former case the activities were slightly lower and showed more variation depending of the glucose concentrations than in the latter cultivations. Based on these results, for the production of laccase, glucose was supplemented at the concentration level of 0.5% (w/v), and veratryl alcohol was added three days from inoculation.

Purification of extracellular enzymes was made on DEAE–Sephadex as in [24]. Laccase eluted as one peak, constituting a single protein band which exhibited laccase activity. The results of the purification steps are given in Table 1. Laccase was purified about 7-fold.

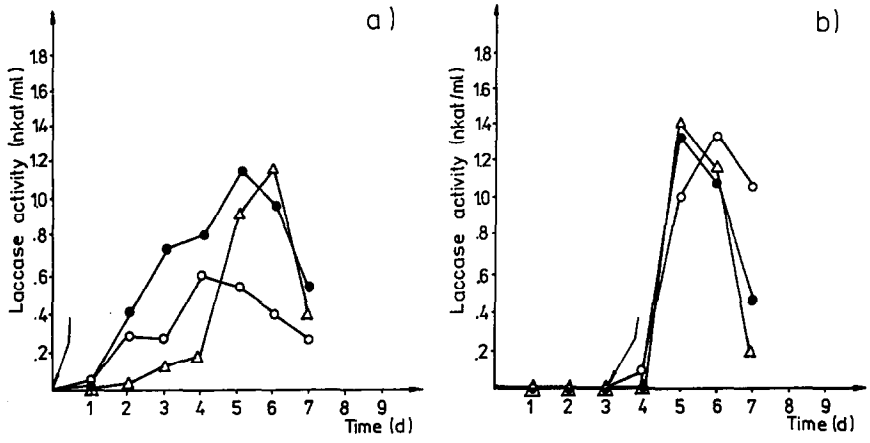

Fig. 1. Production of laccase by *Phlebia radiata* in ADMS–LN medium with (●) 1.0% (w/v) glucose; (△) 0.5% (w/v) glucose and (○) 0.2% (w/v) glucose as a carbon source. Veratryl alcohol, to make a final concentration of 1 mM, was added (a) in the beginning or (b) on the third day of growth (arrow).

Table 1
Purification of laccase from *Phlebia radiata*

Purification degree	Total protein amount (mg)	Specific laccase activity (nkat/mg prot.)	Purification degree	Activity yield (%)
Culture fluid	46.0	2,600	1.00	100.00
Ultrafiltration	43.6	2,418	0.93	88.14
DEAE–Sephadex A-50 anion exchange chromatography and desalting on Sephadex G-25	0.1	17,800	6.85	1.49

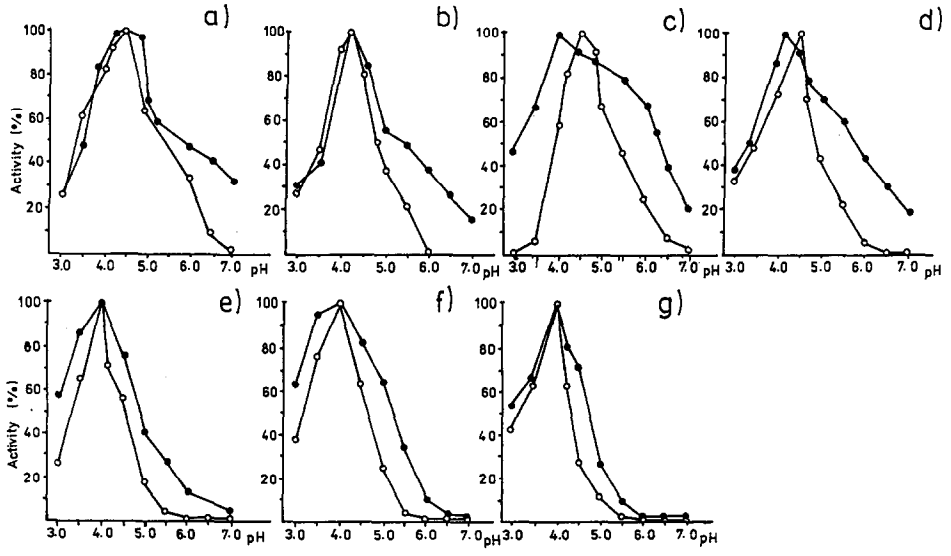

Fig. 2. The effect of pH on the activity of (○) native and (●) immobilized laccase from *Phlebia radiata* using (a) 2,6-dimethoxyphenol (2,6-DMP); (b) veratric acid; (c) syringic acid; (d) ferulic acid; (e) caffeic acid; (f) guaiacol and (g) methoxyhydroquinone as a substrate.

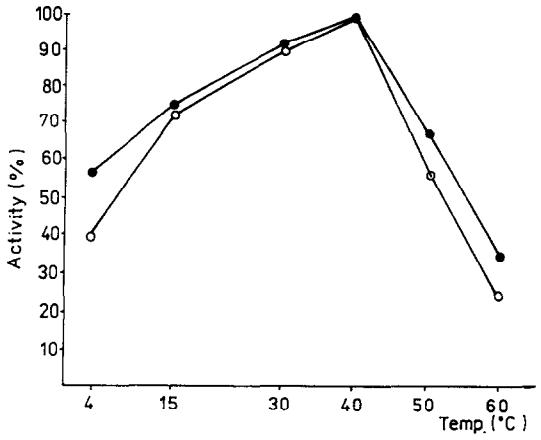

Fig. 3. The effect of temperature on the activity of (○) native and (●) immobilized laccase from *Phlebia radiata* using 2,6-dimethoxyphenol (2,6-DMP) as a substrate.

The purified laccase protein was immobilized on γ-APTES–CPG support. As a result of the bonding procedure 98% of protein and 96% of laccase activity were coupled to the support. The final preparation contained ca. 1 mg protein per gram of glass beads determined from the differences of protein concentration and laccase activity before and after immobilization procedure.

The effect of pH on the free and immobilized laccase acting on various substrates is shown in Fig. 2. Compared with the free enzyme, the optimum pH for the immobilized enzyme was either the same, as in the case of caffeic acid, guaiacol or methoxyhydroquinone (MHQ) used as a substrate (Figs. 2e, 2f and 2g, respectively), or slightly shifted towards a more acidic pH as in the case of syringic or ferulic acids (Figs. 2c and 2d). The optimum pH showed a little broader region with a shift to less acidic pH when 2,6-dimethoxyphenol (2,6-DMP) was used (Fig. 2a). Such shifts have previously been detected for various immobilized enzymes [28,33,34]. The laccase of *Coriolus versicolor* showed a similar shift of pH optimum towards a less acidic region [24] as *P. radiata* in the present work when the substrate was 2,6-DMP. The observed shifts can be explained by the partitioning of protons at the active site of an enzyme when affected by ionized active groups of the support [35].

Fig. 2 further shows that, compared with the soluble laccase, the immobilized laccase displayed a more stable activity profile between pH 3.0 and 7.0. In the case of 2,6-DMP, veratric, syringic or ferulic acids at pH 7 the loss of activity of the immobilized enzyme was 70–80%, while free laccase completely lost its activity. Similar changes in the pH vs. activity profiles for free and immobilized *C. versicolor* laccases on porous glass have been reported [36,37].

The effect of temperature on the activity of free and immobilized laccase is shown in Fig. 3. Generally the immobilized enzyme was a little more stable in the temperature range from 4° to 60°C, which agrees with the results obtained with *C. versicolor* and *Fomes fomentarius* laccases [27,36,37]. This observation confirms that immobilization generally broadens the temperature range of enzyme activity.

Table 2
Stability of purified laccase preparations at various temperatures

Storage time [a] (days)	Free laccase (% of activity after storage at °C)			Immobilized laccase (% of activity after storage at °C)		
	−25	+4	+25	−25	+4	+25
0	100.0	100.0	100.0	100.0	100.0	100.0
3	91.4	98.6	60.2	90.2	100.0	99.9
6	90.9	96.1	33.9	90.1	100.0	96.4
14	90.6	82.4	14.6	89.8	100.0	90.6
27	90.6	62.2	2.4	89.6	98.9	65.7
58	90.1	40.6	0.1	89.6	98.9	41.2
150	89.4	16.3	0.0	89.1	97.4	29.9
180	89.2	3.7	0.0	88.8	97.2	22.1

[a] The enzymes were stored in 0.1 M citrate–phosphate buffer pH 4.5.

Table 3
Kinetic data of native and immobilized laccase from *Phlebia radiata* [a]

Substrate	K_m (mM)	
	Native laccase	Immobilized laccase
syringic acid	0.21 ± 0.01	1.38 ± 0.01
caffeic acid	0.34 ± 0.01	1.05 ± 0.01
vanillic acid	0.68 ± 0.06	1.63 ± 0.02
guaiacol	1.76 ± 0.07	4.78 ± 0.68
MHQ	7.10 ± 0.14	6.82 ± 0.14

[a] 2,6-DMP was used as substrate four parallel determinations ± s.d.

The effect of storage time at various temperatures on the catalytic activity of free and immobilized forms of laccase are presented in Table 2. The results indicated considerably higher stability of the immobilized preparation. Even at +25°C ca. 30% of the initial enzyme activity was retained after 150 days' storage. However, the most convenient storage temperature for the immobilized preparation was +4°C. Under these conditions the immobilized form preserved about 97% of its activity after half a year. The influence of the enzyme storage at −25°C was almost the same for the immobilized and native forms. Similar results were obtained for the inducible form of the laccase from *C. versicolor* [27]. The only difference was the higher stability of *C. versicolor* laccase since it preserved ca. 100% of initial activity after 2 years storage at +4°C [27].

The kinetic properties presented in Table 3 indicate that free laccase oxidized phenolic substrates in the decreasing order syringic acid > caffeic acid > vanillic acid > guaiacol > MHQ. The immobilized enzyme showed higher oxidizing capacity towards these substrates than the free enzyme. The immobilized enzyme most rapidly oxidized caffeic acid whereas the free enzyme most efficiently oxidized syringic acid. The kinetic properties obtained for *C. versicolor* by Rogalski et al. [27] showed that *Coriolus*-type laccase, both the native and the immobilized forms, had a much higher oxidation capacity for the same substrates, which indicates differences in the specific activities of *P. radiata* and *C. versicolor*-type laccases.

Recently, Milstein and coworkers [38–40] have shown that *C. versicolor* laccase immobilized on DEAE–Sephadex can express its activity in the reaction medium in which most of water is replaced by an organic solvent, including solvents which completely solubilize lignins. Earlier, Dordick and coworkers [41] showed that lignin can be depolymerized with horseradish peroxidase in an organic solvent, dioxane. Zaks and Klibanov [42] further reported that enzymes, including laccase, suspended in hydrophobic solvents require substantially less water for maximum activity than those suspended in hydrophilic ones. The authors concluded that enzymatic activity in medium containing an organic solvent is primarily determined by interactions between water and the enzyme and not by those between solvent and the enzyme [42].

In this work we determined the 2,6-DMP-oxidizing activity in the presence of some organic solvents by *P. radiata* laccase, both native and immobilized on CPG matrix (Fig. 4). The strongest denaturing solvents for both forms of laccase were DMSO and dioxane (Fig. 4d and 4e). When 20% of the volume of the buffer was replaced by the solvent the activity of laccase was below 10% of its initial activity towards 2,6-DMP. The highest stability of the native form of enzyme was observed when methanol or ethanol were applied as solvents so that at 20 (v/v)%, 20% units and 40% units higher activities were observed, respectively (Fig. 4a and 4c). Also the stability of immobilized laccase could be increased as shown by Fig. 4b where 20% (v/v) concentration of acetone was applied. In this case the immobilized enzyme showed about four times higher stability than the free one.

The silica carriers (CPG) used for the immobilization of *P. radiata* laccase were also employed earlier for the binding of horseradish peroxidase and polyphenol oxidase (tyrosinase) and also for determination of their activity in mixtures containing organic solvents [43,44]. In these studies [44] the reaction rate closely correlated with the concentration of water in the reaction mixtures. It is known that after a critical

concentration of organic cosolvent (usually 20–50 vol-%) the spectral characteristics of the protein abruptly change [45] because of conformational rearrangements in the protein structure, caused by denaturation of protein.

Table 4 shows oxidation rates of 2,6-DMP caused by both laccase forms and also K_m values in mixtures containing 10 vol-% of water-miscible solvent and 90 vol-% of buffer. Comparison of the kinetic parameters demonstrated that the solvents remarkably increased the K_m values for 2,6-DMP oxidation.

The effect of several enzyme inhibitors on both free and immobilized forms of *Phlebia* laccase was investigated. For comparison the same experiments were carried out with laccase isolated from *C. versicolor*. Cu-chelating agents which totally inhibit extracellular laccase activity affect also either positively or negatively the growth of the mycelium [13]. It has been shown that the response to Cu-chelating agents is very similar for laccases from different fungal strains [46]. Fig. 5 shows the effect of thioglycolic acid (TGA) which is known to be one of the most potent laccase inhibitors [13]. Immobilization made both laccases much less vulnerable for TGA inhibition (Fig. 5) so that the immobilized forms of the enzymes were 1000-fold more stable than the

Table 4
Kinetic properties of the oxidation of 2,6-dimethoxyphenol (2,6-DMP) by the native and immobilized laccases from *Phlebia radiata* in buffer and in organic solvents mixed with buffer (% v/v)

Reaction mixture	K_m (mM)	
	Native laccase	Immobilized laccase
0.1 citrate–phosphate buffer pH 4.5	0.20 ± 0.01	0.15 ± 0.01
10% ethanol	0.35 ± 0.03	0.45 ± 0.05
10% methanol	1.26 ± 0.06	2.34 ± 0.16
10% acetone	1.87 ± 0.07	3.26 ± 0.34
10% DMSO	3.84 ± 0.29	3.53 ± 0.62
10% dioxane	5.10 ± 0.47	2.16 ± 0.57

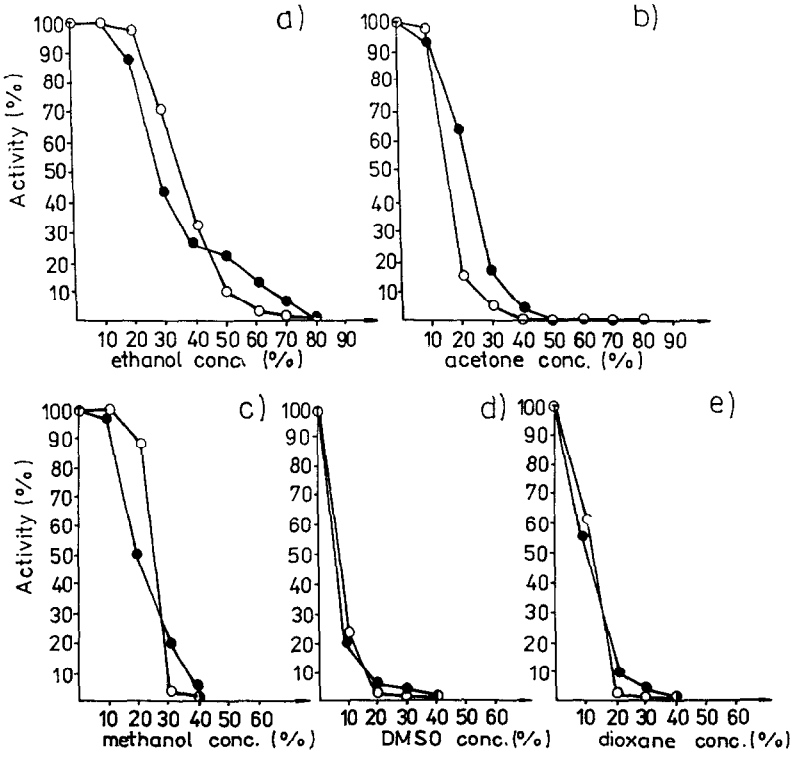

Fig. 4. The effect of (a) ethanol; (b) acetone; (c) methanol; (d) dimethylsulphoxide (DMSO) and (e) dioxane mixed with 0.1 M citrate–phosphate buffer pH 4.5 (% v/v) on the activity of the (○) native and (●) immobilized laccase from *Phlebia radiata*.

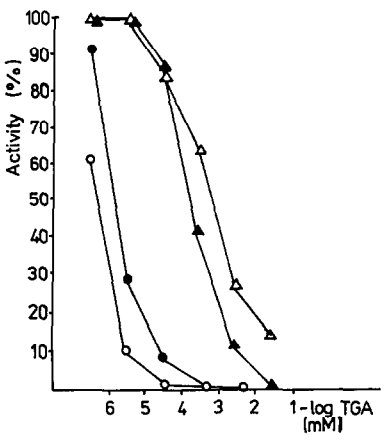

Fig. 5. The effect of thioglycolic acid (TGA) on the activity of native (○) *Coriolus versicolor*; and (●) *Phlebia radiata* laccase, and immobilized (▲) *Coriolus versicolor* and (△) *Phlebia radiata* laccase.

native ones. The laccase from *P. radiata* was less susceptible to the inhibitor than that from *C. versicolor*, and immobilization made it even more stable. For example, 1 μM TGA decreased the activity of the immobilized *P. radiata* laccase by 50% of its initial activity, whereas the same level of inactivation of the immobilized *C. versicolor* laccase was observed with half of the concentration of the inhibitor, i.e. 0.5 μM TGA. *P. radiata* laccase contains less Cu atoms than *C. versicolor* laccase [19] which may explain why it is less susceptible for Cu-chelating agent.

The effect of some other laccase inhibitors on the enzyme isolated from *P. radiata* is presented in Fig. 6. In all cases the catalytic activity of the immobilized form was much less vulnerable for the inhibitors than that of the free enzyme but the differences were not so obvious as with TGA, i.e., the stability of the activity increased by immobilization by only about 10-fold. At the concentration level of 1 μM, thiourea, sodium azide and EDTA did not inhibit the catalytic activity of the immobilized form although the native form of laccase was inhibited by these compounds by 70, 80 and 30%, respectively.

When fungi degrade lignocellulose complex through the action of laccase (or other ligninolytic enzymes), quinoid structures, originating from lignin, will accumulate and make the environment toxic, if they are not reduced or repolymerized [45]. Fig. 7 shows the inhibiting effect of 2,6-dimethoxy-1,4-benzoquinone on free and immobilized laccases from *C. versicolor* (Fig. 7a) and from *P. radiata* (Fig. 7b). This compound is formed, for example, during transformation of syringic acid by laccases of *Rhizoctonia praticola* and *C. versicolor* at the pH region between 3.5 and 8.0 [46]. Also in this case (Fig. 7) the immobilized forms of both laccases were much more resistant to inhibition than the free enzymes. However, immobilization of the laccase from *C. versicolor* (Fig. 7a) only increased the stability of the enzyme activity 10-fold while 1000-fold increase was observed in the case of *P. radiata*

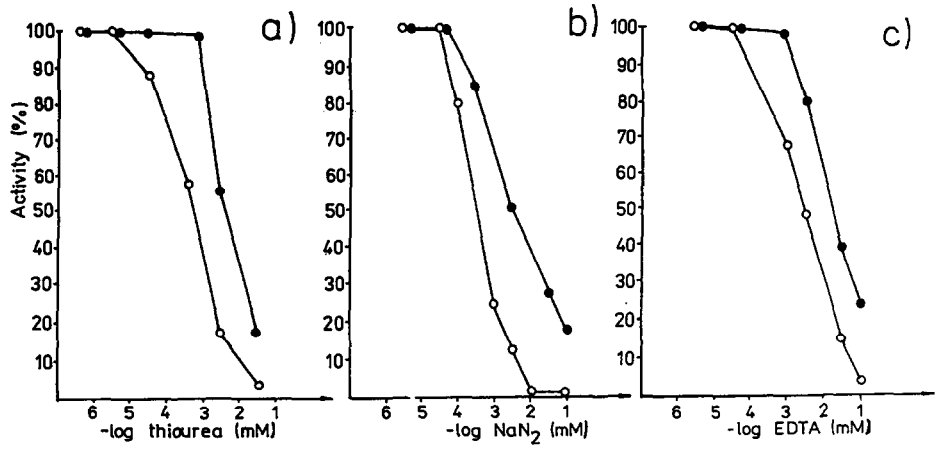

Fig. 6. The effect of (a) thiourea; (b) NaN_2 and (c) EDTA on the activity of native (○) and immobilized (●) *Phlebia radiata* laccase.

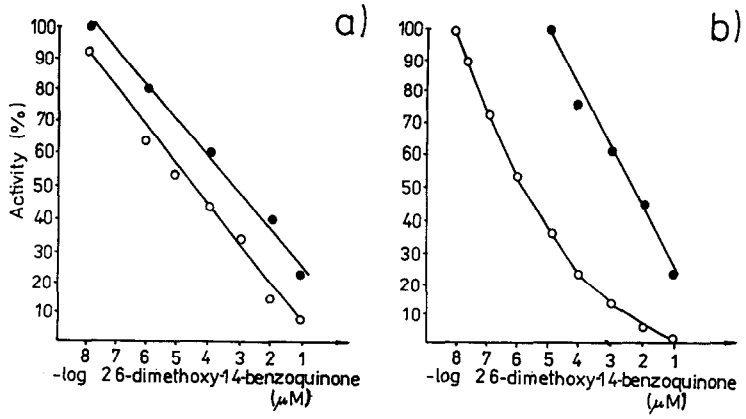

Fig. 7. The effect of 2,6-dimethoxy-1,4-benzoquinone on the activity of (○) native and (●) immobilized (a) *Coriolus versicolor* and (b) *Phlebia radiata* laccase.

laccase (Fig. 7b). The concentration of 10 pM of quinone in the reaction mixtures inhibited the activities of both native laccase-types by about 50%. The similar level of the inhibition for immobilized laccase of *C. versicolor* was achieved at the 100 pM solution of quinone. However, in the case of immobilized *P. radiata* laccase the same effect required much more inhibitor, i.e., 10000 pM (10 nM).

As a conclusion, *P. radiata* produced laccase which was successfully immobilized on controlled porosity glass. The activity of immobilized enzyme showed good stability in a prolonged storage compared with the free enzyme. Catalysis in the presence of organic solvents may open new areas for enzyme applications. Although the activity in the presence of organic solvents was rather similar irrespective of the form of the enzyme, free or bound, the catalytic activity of the immobilized laccase was less vulnerable in the presence of inhibitors such as Cu-chelators and a quinone. This phenomenon may be very useful especially in potential industrial detoxification processes when substrate mixtures to be treated may contain impurities inhibiting enzyme activities.

Acknowledgements

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