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Improved activity and stability of an immobilized recombinant laccase in organic solvents

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Abstract

Laccase (E.C. 1.10.3.2) from *Trametes versicolor* was immobilized (adsorbed) by drying on various supports (glass, glass powder, silica gel, and Nylon 66 membrane). The enzyme activity and stability were determined in diethyl ether, ethyl acetate, and methylene chloride. The initial rate for the oxidation of syringaldazine varied up to 245-fold depending on the solvent and support, the best results being obtained with Nylon 66 membrane. No inactivation of immobilized laccase over 72 h was observed in diethyl ether and ethyl acetate, while exposure to methylene chloride resulted in significant activity decreases regardless of the support material.

Introduction

Various fungi can efficiently degrade polycyclic aromatic hydrocarbons (PAH) (Field *et al.* 1992) and polychlorinated biphenyls (PCB) (Yadav *et al.* 1995). This degradation capacity involves the so-called ligninolytic system consisting of Mn-dependent and Mn-independent peroxidase, lignin peroxidase, and the copper containing enzyme laccase (Kirk & Farrell 1987).

The potential of ligninolytic enzymes to degrade xenobiotics under non-aqueous conditions has not been explored systematically thus far. If they could be used directly in organic solvents this could be of considerable value, because many problematic pollutants, viz., PAH and PCB, are poorly soluble in aqueous systems or are generated in organic solvents. For example, PAH occur in used motor oils and the PAH content of recycled motor oils is frequently higher than that of the original used oils (Clonfero *et al.* 1996) causing health impacts on populations living in proximity to reprocessing plants (Kilburn & Warshaw 1995).

Herein we present a first step in employing ligninolytic enzymes under non-aqueous conditions to enable the aforementioned applications and investigate the activity and stability of recombinant laccase (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) from *Trametes versicolor* in some model organic solvents. Laccases are enzymes which are capable of oxidizing complex polyphenolic substrates, such as lignin (Gianfreda *et al.* 1998) and PAH (Rama *et al.* 1999). The redox process involves the participation of multiple copper centers. Four electrons are subsequently removed from the substrate(s) and transferred to O_2.

Many methods are now available allowing to use enzymes in organic solvents under nearly non-aqueous conditions (Klibanov 1989, 1997) and some have been used to obtain catalytic data employing laccases (Milstein *et al.* 1989, van Erp *et al.* 1991). However, thus far there have been no systematic investigations on establishing enhanced preparation procedures for obtaining active and stable laccase in organic solvents. Herein, we report on the effect of different supports on the activity and stability of immobilized recombinant laccase in various organic solvents.

Materials and methods

Chemicals

Glass powder (106 μ particle size) and syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) were from Sigma, silica gel (70–230 mesh) and solvents (HPLC grade) were from Aldrich, and Nylon 66 membrane (0.45 μm pore size) was from Supelco.

Preparation of laccase

Recombinant laccase from *Trametes versicolor* was produced in *Aspergillus* sp. and was a kind gift by Novo Nordisk, Denmark. The enzyme displays optimum activity from pH 4.5 to 6.5. The enzyme preparation was purified on a Sephadex G25 column (50×1.5 cm). Water adjusted to a pH of 5.5 was used to equilibrate and develop the column. Fractions of about 5 ml were collected. The fraction containing laccase was dark-blue and UV-Vis spectra were obtained to qualitatively evaluate its purity. The protein concentration was obtained using the Bradford method from a bovine serum albumin (BSA) standard calibration curve.

Laccase lyophilization

Aliquots of 1 ml of an aqueous solution of the enzyme (0.5 mg ml^{-1}) at pH 5.5 in 20-ml vials were rapidly frozen in liquid N_2 and then lyophilized for 48 h at a chamber pressure of <100 μm of Hg and at -42 °C.

Laccase immobilization

Nylon: 50 μl of the enzyme solution (pH 5.5) was immobilized by drying on approximately 1×1 cm squares of Nylon 66 membrane under dry N_2 gas. *Silica gel and glass powder:* 0.5 ml of enzyme solution plus 0.5 ml of water (pH 5.5) were added to 100 mg of support and allowed to evaporate for more than 14 h until free-flowing particles were obtained. Portions of 10 mg of the resulting powders were used in kinetic studies. *Films:* 150 μl of enzyme solution and 50 μl of water (pH 5.5) were immobilized by drying with N_2 gas under rotation on the wall of 20 ml glass vials. The films were dried under a vacuum of <100 μm of Hg for more than 14 h prior to measurements.

Kinetic measurements

Kinetics of the oxidation reaction of syringaldazine were followed by determining the product concentration from its absorbance at 525 nm (van Erp *et al.* 1991). For the supports silica gel, glass powder, and Nylon 66 membrane, 60 μl of the substrate stock solution [9 mM syringaldazine in a 1:1 (v:v) mixture of ethanol:acetonitrile] (van Erp *et al.* 1991) was added to 3 ml of organic solvent containing the immobilized enzyme followed by agitation with a magnetic stirrer at 600 rpm at 25 °C. For the films and the lyophilized laccase preparation, 200 μl syringaldazine stock solution was added to 10 ml organic solvent and placed in an orbital shaker at 250 rpm and 25 °C. The final syringaldazine concentration was 0.176 mM in all tests. The initial rates V_0 of the enzymatic reactions were obtained from plots of product formation *vs.* time. Supports did not have any catalytic activity in the solvents in the absence of immobilized enzyme.

Results and discussion

Lyophilized laccase powder was suspended in three buffer-saturated model solvents, diethyl ether, ethyl acetate, and methylene chloride. No activity of laccase was detectable under these conditions. Laccase was and remained active in two other solvents, butanol and hexane, as indicated by the substrate, syringaldazine, turning red-violet. However, any quantitative work was impossible because laccase precipitated and formed large particles, which stuck to the vessel walls. In order to investigate and optimize the activity and stability of laccase in organic solvents, development of alternative methods to prepare the enzyme was necessary.

Activity of laccase immobilized on various supports

Four different support materials were investigated (glass, glass powder, silica gel, and Nylon 66 membrane) and laccase immobilized (adsorbed) on those by simple drying procedures. One of the advantages of working under non-aqueous conditions is that simple drying procedures are sufficient to immobilize enzymes because of their insignificant solubility in most organic solvents (Chin *et al.* 1994). The kinetic data obtained with these preparations are presented in Table 1 for three organic solvents. Laccase simply immobilized as films on glass had the by far lowest activity. To overcome these limitations with enzyme

Table 1. Initial rates for the oxidation of syringaldazine by laccase immobilized on various support materials in buffer-saturated organic solvents[a].

Support	Initial rate (nmol syringaldazine oxidized min^{-1} mg $enzyme^{-1}$)[b]		
	Diethyl ether (log $P = 0.85$)[c]	Ethyl acetate (log $P = 0.68$)[c]	Methylene chloride (log $P = 1.2$)[c]
Glass (films)[d]	0.35	0.37	0.02
Glass powder[e]	4.3 ± 1.5	14.4 ± 3.4	4.9 ± 3.4
Silica gel[e]	42.9 ± 5.5	2.1 ± 0.3	0.6 ± 0.6
Nylon 66 membrane[e]	52.0 ± 12.9	89.0 ± 8.3	4.9 ± 1.2

[a]All organic solvents were saturated with 10 mM KH_2PO_4 (pH 5.5) on an orbital shaker at 250 rpm overnight. Laccase activity proved insignificant in the neat solvents.
[b]Reaction conditions: buffer-saturated solvents, 25 °C, syringaldazine at 0.176 mM.
[c]The values for log P (P is the partition coefficient of the solvent between octanol and water) were obtained from Laane *et al.* (1987) and Chin *et al.* (1994).
[d]Laccase concentration 65 μg of protein per ml of solvent.
[e]Laccase concentration 11 μg of protein per ml of solvent.

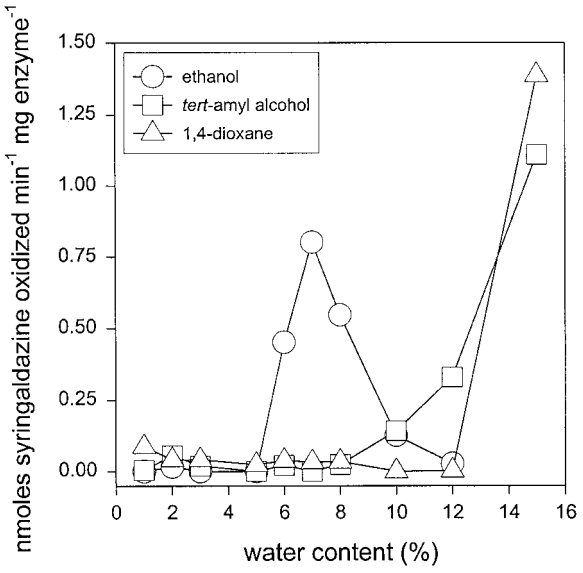

Fig. 1. Initial rates of laccase immobilized as film on glass surface in hydrophilic solvents at varying water contents: (◯) in *tert*-amyl alcohol; (□) in 1,4-dioxane; (△) in ethanol. Reaction conditions: 25 °C, 0.176 mM syringaldazine.

films, the strategy introduced by van Erp *et al.* (1991) was followed and water-miscible organic solvents with varying water contents employed. No activity was detected in ethanol, *tert*-amyl alcohol or 1,4-dioxane. In agreement with literature data (Milstein *et al.* 1989, van Erp *et al.* 1991), laccase was activated in these solvents by the addition of water (Figure 1). However, the activity was still lower than that obtained with the other support materials (Table 1), even if 15% (v:v) water were added. Currently, alternative possibilities are being explored to activate laccase in such enzyme films.

When laccase was immobilized using glass powder, silica gel, or Nylon 66 membrane as supports, a significantly higher activity was found than with the films (Figure 2). For example, the activity of laccase immobilized on Nylon 66 membrane in diethyl ether was 150-fold increased over the activity of laccase immobilized as films (Table 1). Similarly, the activity was significantly increased in this solvent when laccase was immobilized on silica gel (ca. 120-fold) and glass powder (ca. 12-fold). The differences between laccase immobilized on glass and glass powder might be indicative of diffusional limitations, despite the fact that the surface area was ca. 10 cm^2 mg^{-1} of laccase under both conditions. However, inspection of both preparations by surface-scanning electron microscopy qualitatively revealed some morphological differences, in particular concerning the surface distribution of laccase. Laccase formed islands when dried as a 'film' and large areas of the glass surface were not covered. In the case of glass powder, smaller and more islands were formed. This in turn might reduce diffusional limitations in the latter system.

Solvent effects

Enzyme activity in organic solvents displays marked solvent effects (Klibanov 1997). Different solvents can affect, e.g., the substrate-enzyme interactions and also the conformational mobility and structure of the catalyst. For immobilized enzymes activity is also influenced by the properties of the support, e.g., the aquaphilicity (Orsat *et al.* 1994).

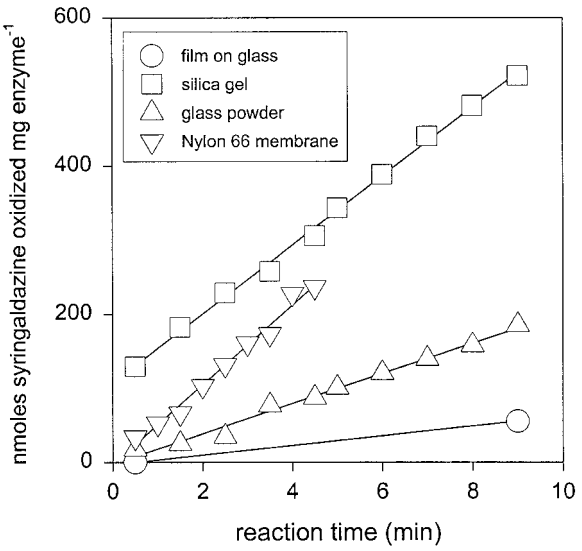

Fig. 2. Activity of laccase immobilized on different supports: (◯) films on glass surface (data was obtained for 60 min); (□) on silica gel; (△) on glass powder; (▽) on Nylon 66 membrane. Reaction conditions: buffer-saturated diethyl ether, 25 °C, 0.176 mM syringaldazine.

Marked solvent effects were noted when the activity of laccase immobilized on one support material was determined in the three solvents (Table 1). For example, laccase immobilized on silica gel was 20-fold and 72-fold more active in diethyl ether than in ethyl acetate and methylene chloride, respectively. In general, activity of laccase was much lower in methylene chloride than in the other solvents for all preparations (Table 1). Otherwise, no relation between enzyme activity, solvent, and support was evident. For example, it is generally true that solvents with higher log *P* values (*P* is the partition coefficient of the solvent between water and octanol) are more suitable media for enzymatic catalysis in low water systems (Laane *et al*. 1987). However, it has already been shown that there are many exceptions to this concept and also solvents with a very low log *P* value can be excellent in such applications (Chaudhary *et al*. 1996). In our investigation the solvent with the highest log P value, methylene chloride, is the worst solvent (Table 1).

Furthermore, while laccase immobilized on silica gel was more active in diethyl ether than laccase immobilized on glass powder, the opposite was found in ethyl acetate. This shows that the effect of the support matrix on enzyme activity can be more pronounced than solvent-dependent differences. Overall, Nylon 66 membrane was the support resulting in the best laccase activity in the three solvents.

Stability of laccase immobilized on different supports

In order to be useful in biotechnological applications, an enzyme should be stable for extended periods of time. Laccase stability was evaluated by incubating the immobilized enzyme in the model solvents followed by determination of the enzyme activity under standardized conditions. In general, the stability of the immobilized enzyme was excellent in ethyl acetate and in diethyl ether regardless of the support used. The activity of laccase immobilized on Nylon 66 membrane and silica gel in diethyl ether did not change significantly in the testing period (Table 2). Surprisingly, the activity of laccase immobilized on glass power increased with time by a factor of about two and four for the second and third day, respectively. Since this result was unexpected, the stability experiments were repeated with a batch of independently immobilized samples and the effect was confirmed. However, we still do not have a useful hypothesis to test and possibly understand this observation. The stability of laccase in ethyl acetate remained approximately constant with time regardless of the support used for immobilization. For methylene chloride, the activity of laccase immobilized on glass powder decreased by half in the second day. The activity of laccase in the third day remained constant relative to the second day. Laccase was quite unstable in methylene chloride when Nylon 66 membrane or silica gel were used as support materials.

Conclusions

Two factors are important when optimizing an enzyme for applications under non-aqueous conditions, viz., the activity and the stability of the catalyst. In this work we describe for the first time the effects of different support materials and solvents on both critical parameters for the enzyme laccase. The activity of immobilized laccase depended on both, the support material and the solvent. The activity of laccase immobilized by simple drying methods using silica gel, glass powder, and Nylon 66 membrane did not decrease over a period of 3 days in the solvents diethyl ether and ethyl acetate. However, exposure of immobilized laccase to methylene chloride proved detrimental to its activity and stability. Immobilization on glass powder minimized this effect. Combining the activity and stability results leads to the conclusion, that laccase immobilized by air-drying on glass powder is thus

Table 2. Stability of laccase immobilized on various support materials in different solvents.

Support	Incubation time[a] (h)	Initial rate[b] (nmol syringaldazine oxidized min^{-1} mg $enzyme^{-1}$		
		Diethyl ether[c]	Ethyl acetate[c]	Methylene chloride[c]
Glass powder	24	7.0 ± 1.5	3.1 ± 0.9	3.7 ± 0.2
	48	16.2 ± 0.3	7.0 ± 0.9	2.0 ± 0.3
	72	26.0 ± 4.3	3.7 ± 1.8	2.1 ± 0.3
Silica gel	24	12.5 ± 0.9	2.4 ± 0.3	0.02 ± 0.03
	48	11.6 ± 3.1	3.4 ± 0.6	[d]
	72	9.5 ± 0.6	3.4 ± 0.9	[d]
Nylon 66 membrane	24	9.5 ± 0.9	30.9 ± 11.6	1.4 ± 0.1
	48	15.9 ± 4.3	18.1 ± 0.9	0.5 ± 2.1
	72	12.5 ± 2.8	30.3 ± 18.7	0.2 ± 0.1

[a]Laccase was incubated at room temperature in the organic solvents before kinetic measurements were performed.
[b]Reaction conditions: buffer-saturated solvents, 25 °C, 0.176 mM syringaldazine, protein concentration 37 μg per ml of solvent.
[c]See footnote a in Table 1.
[d]No detectable activity.

far the probably best preparation for biotechnological applications.

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