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Immobilization as a Tool for the Stabilization of Lignin Peroxidase Produced by *Phanerochaete chrysosporium* INA-12

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ABSTRACT

Lignin peroxidase immobilization was achieved by covalent coupling on CNBr-Sepharose 4B. Protein immobilization yield was around 80%. For veratryl alcohol oxidation, in the presence of hydrogen peroxide, both soluble and bound enzymes exhibited the same pH profile with an optimum near 2.5. Catalytic parameters (kc and K_m) were seriously affected by immobilization. On the other hand, immobilization provided a noticeable stabilization of the enzyme against acidic pH and high temperatures. A 15–20 increase in the half-inactivation times at pH 2.2 and 2.7, respectively, could be observed. Bound enzyme was also much more thermostable than soluble.

Index Entries: Lignin peroxidase; immobilization; stabilization; *Phanerochaete chrysosporium*.

INTRODUCTION

Lignin peroxidase plays a key role in the breakdown of lignin (*1–4*) and has been the subject of an extensive research effort because of its potential applications in several biotechnological processes, including wood pulp biobleaching (*5,6*) and organopollutants degradation (*6–10*).

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LiP is secreted by the white rot fungus *Phanerochaete chrysosporium* and exists as a family of multiple isoenzymes (*11–13*). Each consists of a single polypeptide chain, with an iron protoporphyrin IX prosthetic group. Their molecular weights range from 38–42 kDa, and isoelectric points are found to be between pH 3.0 and 5.0. Characterization of the formation and the reactivity of the oxidized intermediates LiP compounds I, II, and III indicates that these oxidized states and the catalytic cycle of LiP are comparable to other peroxidases.

Recently, we developed an improved purification procedure for major LiP isoenzyme (H_2) that avoids critical time-consuming and enzyme-inactivating steps (*14*). Moreover, we demonstrated that *Phanerochaete chrysosporium* strain INA-12 secretes the same major isoforms than strain BKM-1767 generally used by the authors (*11–14*).

Difficulties linked particularly to the definition of optimal conditions for the in vitro enzymatic depolymerization of lignin are increased by the relative instability of the enzyme. Indeed, ligninase is rapidly inactivated at low pH (*15,16*), which conflicts with the fact that ligninase activity increases with decreasing pH (*17*). These problems greatly limit potential applications.

It is usually accepted that one of the powerful tools for enzyme stabilization is immobilization (*18,19*). Inactivation of the enzyme owing to unfolding of the molecule under denaturing conditions may be sharply delayed if the protein is rigidified by being attached to a complementary surface of a relatively rigid support in a multipoint fashion (*18*). Moreover, immobilization allows investigations concerning catalytic mechanisms of some enzymes acting on complex substrates (*20,21*). It could be shown that the nature of the support and the immobilization method influence not only the product reaction yield, but products distribution as well (*22*). Sometimes, immobilization offers the possibility of reusing enzymes so that increasing industrial applications imply immobilized enzymes. In this context, immobilization of lignin peroxidase was considered. Attempts to immobilize ligninase have already been made, but the purpose was to use the enzyme in analysis (*23*). Kinetic properties of the enzyme have been studied through a flow injection analysis system. Here, we report a simple method for LiP immobilization. Catalytic properties and stability of soluble and immobilized enzyme are compared.

MATERIALS AND METHODS

Microorganism

The microorganism used in this study was *Phanerochaete chrysosporium* INA-12 (Collection Nationale de Culture de Microorganismes I398, Institut Pasteur, France).

Culture Conditions

Cultures were grown under conditions previously described (*24*) and kindly provided by M. Asther and M. Delattre, Laboratoire de Biotechnologie des Champignons Filamenteux, INRA, Thiverval-Grignon, France.

Purification

Purification of LiP major isoform (H_2) was performed according to Asther et al. (*14*).

Enzyme Assay

Lignin peroxidase activity was determined spectrophotometrically at 30°C by the method of Tien and Kirk (*25*) using veratryl alcohol as substrate. The reaction mixture contained 100 m*M* sodium-tartrate buffer, pH 3.0, 2 m*M* veratryl alcohol, 0.3 m*M* hydrogen peroxide, and the enzyme preparation in a final vol of 1 mL. The oxidation rate of veratryl alcohol to veratraldehyde was monitored at 310 nm. Activities were expressed in nkatal (nmol of substrate oxidized/s) using $9300M^{-1}.cm^{-1}$ as molecular extinction coefficient for veratraldehyde. Control was made in the absence of hydrogen peroxide in order to detect contaminant veratryl alcohol oxidase activity (*26*).

Immobilized enzyme activity was measured under the same experimental conditions using an Uvikon 940 (Kontron) spectrophotometer. A magnetic stirrer was used during the reaction.

Protein Determination

Lignin peroxidase concentration was calculated from the absorbance at 409 nm with a molecular extinction coefficient of $168{,}000M^{-1}\cdot cm^{-1}$ (*17*). Protein concentrations were determined by the Bicinchoninic acid (BCA) method (*27*) using bovine serum albumin as standard. This method allowed the direct determination of the amount of protein covalently attached to agarose supports (*28*). Specific activity was expressed in nkatal/mg of protein determined by the BCA method.

Immobilization Procedures

Three different matrices were investigated.

CNBr-Activated Sepharose 4B (Pharmacia-LKB Biotechnology)

Immobilization was performed under the conditions described by Pharmacia, slightly modified. After washing the gel with 1 m*M* HCl, coupling was achieved in 25 m*M* phosphate buffer at different pHs in the presence of 1 m*M* veratryl alcohol and 0.5*M* NaCl.

Incubations were performed at room temperature. In order to block the remaining active groups, the gel suspension was transferred in a blocking solution (0.2*M* glycine, pH 8.0). To remove excess adsorbed protein, the gel was washed alternatively three or four times with coupling buffer and 0.1*M* sodium acetate buffer, pH 4.0, containing 0.5*M* NaCl. This step prevented free ligand to remain noncovalently bound to the gel. The gel was then rinsed with sodium phosphate buffer, pH 7.0, and stored at 4°C until it was used.

Affi-Gel 15 (Bio-Rad)

The gel was washed with 10 m*M* sodium-acetate buffer, pH 4.5, and then was incubated with the enzyme in 10 m*M* sodium phosphate buffer, pH 6.75, at 4°C for 4 h. After coupling, the gel was washed with 0.1*M* sodium phosphate buffer, pH 7.0, and stored at 4°C.

Affi-Gel 102 (Bio-Rad)

The gel was incubated with the enzyme in 25 m*M* sodium phosphate, pH 6.0. After adjusting pH to 5.0 with HCl, EDAC reagent [N'-(3-dimethyl aminopropyl)-*N*-ethylcarbodiimide] was added under continuous stirring at room temperature. pH was readjusted to 5.0 with HCl, and the reaction was led to proceed for 3 h. Then, the gel was washed in the same manner as described earlier.

RESULTS AND DISCUSSION

Immobilization of Lignin Peroxidase

Immobilization was choosen as a tool for the stabilization of lignin peroxidase. Among the four classical methods generally used for immobilization—covalent attachment to insoluble supports, adsorption, crosslinking with bifunctional reagents, or entrapment in polymeric matrices—covalent bonding to activated polymers appeared to be the most convenient method (*29*). The irreversible nature of the chemical bond between the enzyme and the gel allows modifications of the enzyme assay medium (pH, ionic strength, substrates, and so on) without desorption of the LiP. Moreover, diffusional limitations are stronger in the case of inclusion within the matrix of highly crosslinked polymers.

Two matrices (CNBr-activated Sepharose-4B and Affi-Gel 15) allowing spontaneous coupling of the protein by a primary amino group were assayed. Affi-Gel 15 carries a cationic charge on its 15-atom spacer arm, which significantly enhances coupling efficiency for acidic proteins at physiological pH. A third gel (Affi-Gel 102) was used. It was an amino terminal crosslinked agarose gel with a six-atom hydrophilic arm. Attachment of enzyme by carboxyl groups was done with EDAC carbodiimide coupling reagent.

Table 1
Immobilization Yields of Lignin Peroxidase on Various Matrices

Immobilization matrix	Bound protein, %[b]	Specific activity, %	
		Remaining soluble LiP[a]	Immobilized
Affi-Gel 102	1.4	44	n.d.[c]
Affi-Gel 15	0.3	77	n.d.[c]
CNBr-Sepharose 4B	80	100	35

[a]LiP was immobilized as described in Material and Methods.
[b]Protein concentration was determined by the BCA method.
[c]n.d.: not determined.

Coupling yields were determined from activities and protein measurements on both free and immobilized enzyme, and are given in Table 1. Under our experimental conditions, no protein was bound to Affi-Gel 15 and Affi-Gel 102. Moreover, coupling conditions were unfavorable for LiP, since only 44 and 77% of the activity were recovered in the supernatant. However, CNBr-Sepharose 4B gave interesting results. Indeed, 80% of proteins were bound, and immobilized enzyme retained about 40% of the specific activity exhibited by the free enzyme. This apparent loss could not be attributed to diffusional limitations, since substrates used here (veratryl alcohol and H_2O_2) are small molecules and the exclusion limit of Sepharose 4B is close to 20.10^6 daltons. Denaturation on immobilization procedure can be also excluded, since under optimum conditions of coupling, the free enzyme showed a long-term stability. The reduced activity of the bound enzyme could be explained by the decrease in flexibility accompanying chemical crosslinking. Compared to results already published on immobilized lignin peroxidases (*23,30*), our results are noticeably improved. Fawer et al. (*23*) reported immobilization of *P. chrysosporium* LiP on glass beads and activated agarose. If coupling yields were good (between 95 and 53%), effectiveness factors were seriously low (2 to 5%). Recently, lignin peroxidases from *Chrysosporium sitophila* were bound to CNBr-Sepharose 4B, and the percentage of activity retained on immobilization was $< 10\%$ (*30*).

Attempts to improve coupling conditions led us to check the effect of pH and coupling time on immobilization yields. Results indicated that optimal coupling efficiency in relation to specific activity of immobilized enzyme occurred at pH 7.6 after 2 h at room temperature for a ratio enzyme/gel of 100 μg enzyme for 30 mg gel.

Kinetic Characterization of Immobilized Enzyme

Michaelis Constants

Kinetic properties of immobilized LiP were studied and compared to soluble enzyme. Results are given in Table 2. Values between brackets

Table 2
Kinetic Parameters of Immobilized Lignin Peroxidase

pH	kc, s^{-1}	K_m (H_2O_2), μM	K_m (VA),[a] μM	kc/K_m (H_2O_2) $(M^{-1}\times s^{-1})\times 10^{-4}$	kc/K_m (VA)[a] $(M^{-1}\times s^{-1})\times 10^{-4}$
3.0	11	110	1087	6.7	0.67
	(29)[b]	(45)[b]	(235)[b]	(62.8)[b]	(12.4)[b]
3.5	6	272	524	2.0	0.76
	(17)[b]	(28)[b]	(208)[b]	(61)[b]	(8.4)[b]

[a]VA: veratrylalcohol.
[b]Values between brackets refer to soluble enzyme.

were those found for soluble enzyme and were already discussed in a previous paper (*14*).

Evaluation of Michaelis constant values (K_m) for the two substrates (hydrogen peroxide and veratryl alcohol) at pH 3.0 and 3.5 exhibited a noticeable increase in all cases for bound enzyme. Catalytic constants (kc) were decreased almost three times. As for soluble enzyme, immobilized LiP was less active at pH 3.5 than at pH 3.0.

Specificity constants (kc/K_m), which indicate the catalytic efficiency of the enzyme, are seriously affected. A 10–30 decrease can be observed. Fawer et al. (*23*) also reported an increased K_m for H_2O_2 when LiP was immobilized on controlled pore glass beads. A K_m of 3.0 mM was obtained in their case instead of 44 μM for soluble enzyme. No K_m measurements for veratryl alcohol were given.

pH Dependence of Activity

Immobilization has been shown to affect the pH profile of enzymes depending on the nature of the matrice used. When matrice is charged, this effect is not reflecting a real modification of intrinsic properties of the enzyme. In our case, immobilization of LiP has no effect on the pH dependence of the catalytic activity. Both soluble and immobilized enzymes exhibited the same pH profile with an optimum around pH 2.5 (Fig. 1). Similar values for soluble enzyme were previously reported ranging between pH 2.0 and 3.0 depending on the authors (*16,17,23*).

One can note that for free LiP, it was impossible to measure any activity at pHs lower than 2.5 owing to an excessively rapid denaturation of the enzyme during the assay unless using stopped-flow methods. On the other hand, we could measure activities in these conditions with bound enzyme, preliminary indicating an increased stability of the protein at these acidic pHs.

Activity in the Presence of Solvent

Applications of aqueous-organic cosolvent media in order to carry out enzyme-catalyzed processes have several advantages with respect to enzymes in purely aqueous solutions, the most important being the in-

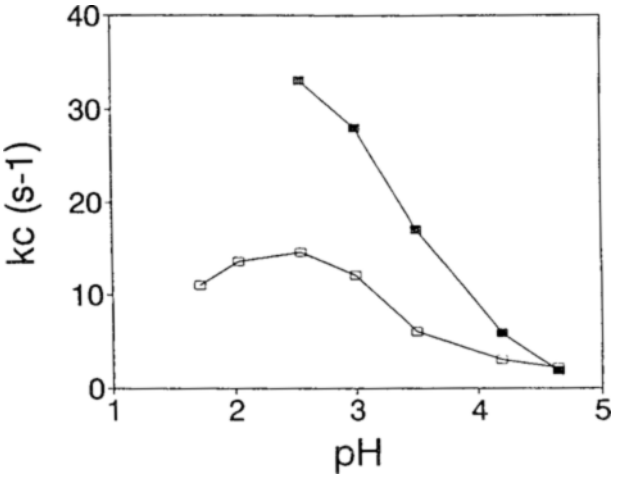

Fig. 1. pH dependence of soluble and immobilized lignin peroxidase activity. Soluble (■) and immobilized (□) lignin peroxidase activity was measured as described in Material and Methods using 0.1*M* Na-tartrate buffer adjusted at different pHs.

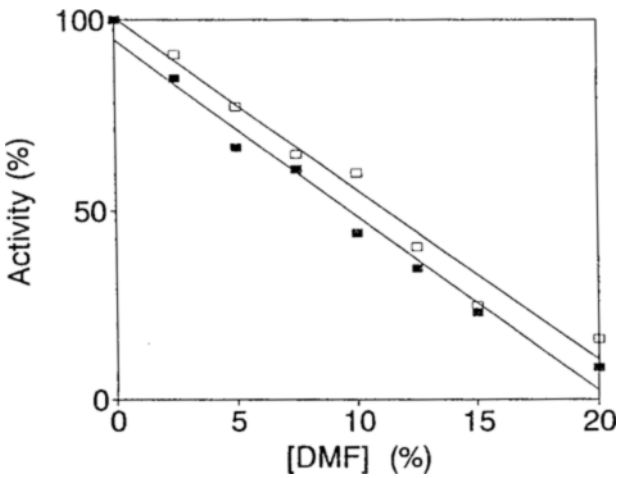

Fig. 2. Lignin peroxidase activity in the presence of dimethyl-formamide. Soluble (■) and immobilized (□) lignin peroxidase activity was measured in standard conditions in the presence of various concentrations of dimethylformamide (DMF). Stability of LiP in such conditions was checked before each measurement. Lines were obtained by linear regression analysis.

creased solubility of poorly water-soluble compounds (*31,32*). This is particularly true in the case of lignin peroxidase, where the physical state of the natural substrate lignin greatly influences the reactivity of the enzyme. Indeed, it was shown that LiP had an increased reactivity toward colloidal lignin in 10% dimethylformamide (*33*). In this context, it was of interest to compare activity of free and immobilized enzyme in a solvent-containing medium. It can be observed in Fig. 2 that LiP activity decreased with

increasing DMF concentrations in the same way for soluble and bound enzyme. This loss of activity appeared to be owing to an effect of the organic solvent on the catalytic properties of the enzyme, since it could be shown that LiP was stable in these media and that no denaturation occurred during incubation on time (results not shown).

Stability of Immobilized LiP

Multipoint covalent attachment of an enzyme to a carrier may result in increased rigidity of the molecule, which is commonly reflected by an increased stability toward unfolding and an increased resistance to inactivation (*19,34*).

Storage Stability

Aqueous suspension of immobilized LiP was stored in 0.1*M* sodium-phosphate buffer, pH 7.0, at 4°C. No significant loss of activity could be observed after several weeks of storage, whereas soluble enzyme was not stable under the same conditions and needed to be stored at −20°C.

Stability Toward pH and Temperature

Native lignin peroxidase has an optimal activity at acidic pH (around pH 3.0). However, stability of the enzyme under these conditions did not exceed a few minutes. pH stability was determined by incubating both free and immobilized enzyme preparations (in 0.1*M* sodium-tartrate buffer at two different pHs [2.2 and 2.75]) at room temperature with respect to time. Activity was then checked under standard conditions at 30°C and pH 3.0. As shown in Fig. 3, immobilized LiP showed an increased stability toward acidic pH. Indeed, half-inactivation times at both pHs were increased by a factor of 15–20 for bound enzyme. Whereas soluble LiP was completely inactivated at pH 2.75 in 4 min, the immobilized form retained almost 80% of its initial activity after the same time.

Thermal stability of LiP was assayed as follows. Samples of soluble and bound enzyme were incubated in 25 m*M* sodium-phosphate buffer, pH 7.0, for 15 min at various temperatures (between 30 and 65°C). After cooling on ice (15 min), residual activity was assayed under standard conditions at 30°C. Immobilized LiP was more thermostable than soluble one (Fig. 4). The temperature leading to 50% inactivation after 15 min incubation is shifted upward by 5°C for the bound enzyme. Previous studies on heat denaturation kinetics of soluble LiP indicated a higher stability than that described in this article (*35*). Since these studies were performed on crude concentrated culture medium and not on purified enzyme, one cannot exclude the presence of stabilizing agents in this medium.

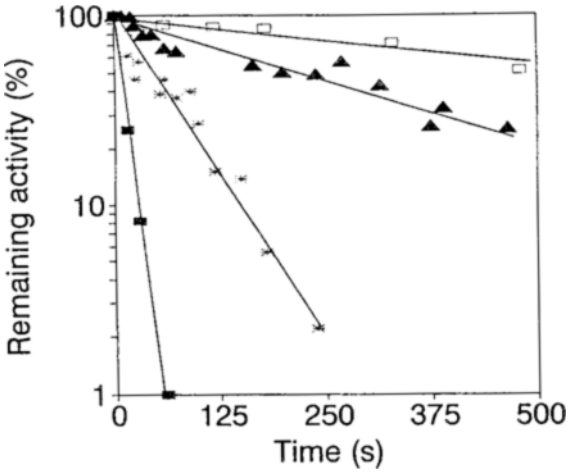

Fig. 3. pH stability of lignin peroxidase. pH stability of soluble (*, ■) and immobilized (□, ▲) lignin peroxidase was assayed by incubating the enzyme at 4°C in 0.1*M* Na-tartrate buffer at pH 2.2 (▲, ■) and 2.75 (□, *). At specified times, aliquots were measured for residual activity in standard conditions at pH 3.0. Results were plotted in semilogarithmic coordinates, and lines obtained by linear regression analysis.

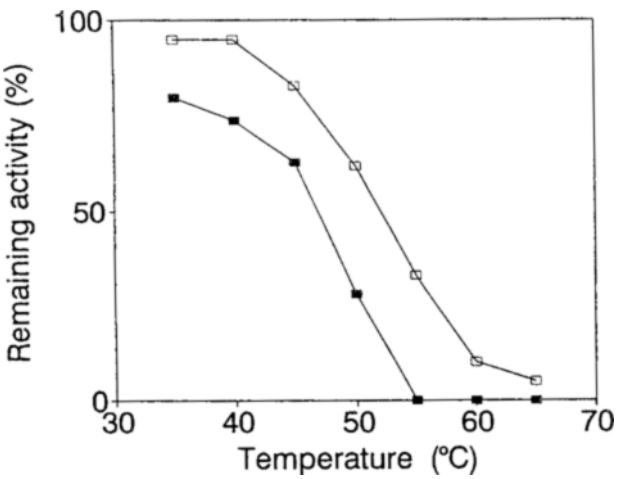

Fig. 4. Thermal stability of lignin peroxidase. Soluble (■) and immobilized (□) LiP was incubated in 0.1*M* Na-phosphate buffer, pH 6.75, for 15 min at various temperatures. After cooling (15 min), residual activity was measured in standard conditions at 30°C.

CONCLUSION

Lignin peroxidase H_2 was immobilized by covalent attachment on insoluble supports. Among the three different matrices assayed, only CNBr-Sepharose 4B gave interesting results. Around 80% of the proteins were efficiently bound, and the immobilized enzyme retained 40% of its initial specific activity. These yields were improved compared to already published results.

Characterization of immobilized LiP indicated that bound enzyme was less active than soluble one, but pH activity profile and solvent behavior were unchanged. In addition, immobilization appeared to be an efficient tool for LiP stabilization. One could note an increased stability of the bound enzyme toward acidic pHs and elevated temperatures as referred to soluble one. The stabilization of LiP may allow us to define reactional conditions more favorable to lignin degradation.

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