Biol Metal (1990) 3:98-102

BIOLOGY OF METALS



# Specific immobilization of laccase on *p*-benzoquinone-activated supports

**Mircea-Alexandru Mateescu[1], Enzo Agostinelli[2], Grazyna Weltrowska[1], Marek Weltrowski[1], and Bruno Mondovi[2]**

[1] Département de chimie et GREFA, Université du Québec à Montréal, C.P. 8888, succursale A, Montréal (Québec), H3C 3P8, Canada
[2] Department of Biochemical Sciences and CNR - Center of Molecular Biology, Rome University "La Sapienza", I-00185 Rome, Italy

**Summary.** A specific immobilization of laccase (EC 1.10.3.2) onto a ready-to-use *p*-benzoquinone-activated agarose support is described. The single-step procedure leads to a laccase protein coupling of 18% and an enzyme activity immobilization yield of 27%, while the retained specific activity of the immobilized enzyme was 150% of the specific activity of the free laccase. This peculiar result is thought to be related to the fact that during the process of support activation by *p*-benzoquinone, a significant amount of the hydroquinone by-product of the activation process is coupled to the support. These coupled derivatives constitute substrate (hydroquinone) analogues for which laccase exhibits a high affinity. Therefore, simultaneous affinity retention on the hydroquinone groups and covalent coupling on the *p*-benzoquinone groups allow the binding of the enzyme in an advantageous conformation which can generate this increase specific activity by immobilization. The entire process can be considered as an affinity immobilization. The immobilized enzyme is much more stable to the inhibitory action of chloride and azide ions, with a recovery of 100% of the activity, than the free laccase, with a recovery of 67% and 32%, respectively, after removal of the inhibitors by dialysis. The stability was 95% after storage for 14 months at 4° C.

**Key words:** Laccase - Immobilization - Agarose - *p*-Benzoquinone - Activated supports

## Introduction

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a copper enzyme catalysing the oxidation of various types of phenols, cresols, chlorophenols, aryldiamines and derivatives to the corresponding quinones (Peisach and Levine 1965; Shuttleworth and Bollag 1986). The enzyme is becoming more popular for use in various biotechnological fields due to its capacity to oxidize phenols. For instance, laccase has been proposed to be used in detoxification and treatment of phenolic wastes to prevent the phenols from contaminating natural resources (Bollag et al. 1988). Some other interesting potential applications of laccase are related to the degradation of lignin in the context of efforts to introduce new ways to transform ligno-cellulosic materials (Ander and Eriksson 1976).

In order to improve the storage and operational stability of the enzyme and to obtain a re-usable continuous solid-liquid substrate oxidation system, several procedures were developed for laccase immobilization. Thus, the enzyme was immobilized by adsorption on Concanavalin-A-Sepharose and by covalent binding to BrCN-activated Sepharose 4B (Froehner and Ericksson 1975) and by azo linkages (Shuttleworth and Bollag 1986) to a variant of celite (a diatomaceus silica derivative).

Usually, a covalent immobilization procedure implies two distinct steps: the activation of the support and the coupling of the enzyme. The whole procedure involves several operations and is often longer than 1-2 days.

Within the last decade, several ready-to-use supports in bead or granular form, for example BrCN-Sepharose 4B, vinyl-sulfone-activated supports (Porath and Axen 1976) and epoxy(oxiran)-activated supports such as Eupergit® (Krämer 1988), and in membrane form, for example Immobylon™ (Millipore), Immunodyne™ (Pall Corp.), etc. have been introduced.

The advantage of these ready-to-use activated supports for the immobilization is that the support only needs to be swollen for a few minutes, washed with a proper buffer and immediately treated with the product to be coupled, or alternatively, directly swollen in the medium containing the product to be coupled.

Recently, new types of ready-to-use *p*-benzoqui-

Part of the work was presented at the Satellite FEBS 1989 Symposium on *Biochemical and biophysical approaches to the study of copper proteins*, Camerino, Italy.

*Abbreviations.* HQ, hydroquinone; *p*-BQ, *p*-benzoquinone; U, enzyme units

*Offprint requests to:* M. A. Mateescu

none(*p*-BQ)-activated supports (agarose, polyvinylalcohol, chitosan) have been introduced (Mateescu et al. 1989). The protein coupling on supports activated with *p*BQ has been described by Brandt et al. (1975). The procedure consists of two steps support activation by treatment with an excess of *p*BQ and, after washings, coupling of the protein on the activated support.

Our ready-to-use *p*BQ-activated support can be obtained in a dried form and stored for more than a year (Mateescu et al. 1989). For protein coupling, the support should be simply swollen in the coupling (e.g. phosphate) buffer for a few minutes, filtered and then incubated with the protein of interest for several hours. Coupling yields of 10–95% have been obtained with several immobilized materials (propylamine, bovine serum albumin, etc.).

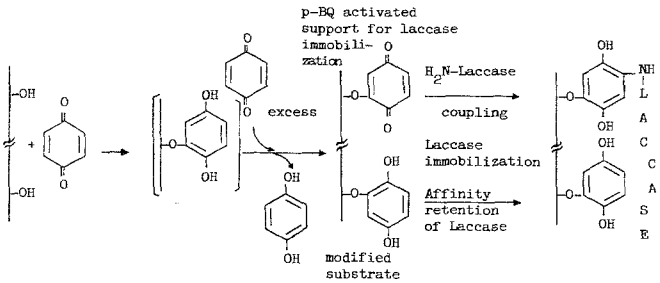
The coupling reaction involves addition of the compound to be immobilized to the activated vinyl-keto function of the *p*-BQ residues of the support (Scheme 1). This reaction may involve the free amino hydroxyl or thiol groups of the compound to be immobilized. In addition to the *p*-BQ groups, the support also exhibits HQ groups obtained as by-products of the activation process. As HQ constitutes one of the main substrates of laccase, it was interesting to see the behaviour of this enzyme when coupled to our *p*-BQ-activated supports which also contain HQ groups which can be recognized by laccase as substrate analogues.

In this paper, the immobilization and some properties of *Rhus vernicifera* laccase on *p*-BQ-activated agarose are described. This enzyme obtained from the lacquer tree (*Rhus vernicifera*) contains four copper atoms/molecule classified into three different kinds of copper which have been indicated as type-1 (blue) copper(II), type-2 copper(II) and type-3 (two atoms) copper(II).

## Materials and methods

*Materials.* Commercial grade reagents were used in this work without purification. Cross-linked agarose bead gels (Sepharose-CL 4B) and BrCN-activated Sepharose 4B, supplied by Pharmacia Fine Chemicals, Uppsala, Sweden) were used.

*Synthesis of the activated p-BQ supports.* The *p*-BQ-activated agarose support was obtained by a procedure described previously (Mateescu et al. 1989). Practically, 100 ml agarose (Sepharose-CL 4B) gel bed was washed on a Büchner funel with 0.1 M phosphate pH 8, filtered and then incubated with the 160 ml of the same buffer solution containing 10.4 g *p*-BQ for 6 h with continuous agitation. The gel was then filtered and intensively washed with water, phosphate buffer and 30% (by vol.) acetone in water, until the excess of non-coupled *p*-BQ and HQ were eliminated. As a final step, the gel was washed, then filtered, dried with acetone and, after complete drying, stored in dark flasks. The amount of *p*-BQ active groups on the support was determined by titration of a known activated gel-volume (e.g. the volume generated by swelling 100 mg dried activated support) with KI in $H_2SO_4$ medium and measuring the amount of $I_2$ released due to the action of *p*-BQ; a 2% starch solution was used, as indicator.




**Scheme 1.** Hypothetical representation of laccase 'affinity immobilization' on *p*-BQ-activated supports

*The immobilization of laccase on p-BQ-activated agarose.* Samples of 1 g dried *p*-BQ-activated agarose were swollen in the coupling buffer (0.01 M phosphate pH 6), washed with the same buffer on a Büchner funel, filtered and then treated with 25 ml purified laccase preparation (3.5 mg/ml) and then incubated (with agitation) for 24 h. The coupling reaction was blocked by addition of 9 ml 2 M Tris/$H_2SO_4$ pH 7 for another 6–10 h and then the gel with the coupled laccase was alternatively washed with the same stopping Tris/$H_2SO_4$ buffer and water, until no absorbance at 280 nm and no protein in the washings was detected by the method of Bradford (1979).

*Enzyme.* Laccase was purified according to the method of Reinhammar (1970) starting from a crude preparation extracted with 0.01 M phosphate pH 6 from the acetone powder of *Rhus vernicifera*.

*Enzyme activity.* The activity of free laccase was determined by the method of O'Neil et al. (1984), based on the measurement of the rate of the enzyme-catalysed potassium ferrocyanide oxidation by absorbance at 420 nm. The enzyme activity of the immobilized laccase was determined by a modification of this method (Mateescu et al. 1989), as follows. An exact volume (obtained with an Eppendorf automatic pipette) of immobilized laccase suspension, corresponding to a precise volume of gel bed (e.g. 0.1 ml), was introduced in a microcolumn and incubated for exactly 1 min with 2 ml ferrocyanide substrate reagent, with agitation in the closed microcolumn. Immediately after this interval, the microcolumn was opened and the reaction medium containing the oxidized substrate was collected directly in the spectrophotometer cells. The rate of change of absorbance at 420 nm against the reagent blank was determined.

*Enzyme concentration.* The concentration of immobilized laccase was determined by atomic absorption spectrometry of copper using an Instrumentation Laboratory aa/ae spectrophotometer 257, with hollow cathode lamp type 3 QNY Cu–Zn (Cathodeon Ltd.). The copper was considered as an internal standard for the laccase macromolecule. Samples of swollen gel (0.1–0.5 ml) were treated with 0.5 ml concentrated nitric acid, for 3 min at 100° C, for mineralization. For both free and immobilized enzyme, an operational specific activity was considered (measured as U/mg copper). From the copper content values, it is possible to calculate the laccase protein concentration using the relation: mass laccase = 434.5 × mass copper (where the factor 435.5 is calculated considering four copper atoms/laccase molecule). The copper content of the *p*-BQ support alone (without coupled enzyme) was also determined as a blank. After proper dilution, the absorbance was measured at 324.7 nm. The inhibitory effects of chloride ($Cl^-$) and azide ($N_3^-$) ions were determined by addition of NaCl and $NaN_3$ in the buffer for the assay of the enzyme activity, in several increasing concentrations, up to a final concentration of 0.02 M in the enzymatic activity determination medium.

*Effects of inhibitors.* The free and the immobilized enzyme were preincubated for 3 min with the buffer containing the inhibitor(s) and then the substrate reagent was added and the rate of change of absorbance measured as in case of enzyme activity determina-

tion. The removal of the inhibitor(s) to study the recovery of enzyme activity of the immobilized enzyme was realized by simply washing with the buffer, directly on the microcolumn of the assay. For the free enzyme, the inhibitor was removed from the enzyme-inhibitor mixture by dialysis against the buffer. For each example, the enzyme activity was determined after the removal of the inhibitor.

*Immobilization of crude enzyme.* Immobilization of a crude *Rhus vernicifera* laccase preparation on *p*-BQ-activated agarose and on BrCN-activated Sepharose 4B supports was also carried out. Practically, 0.5 g of each dried activated support was treated with 25 ml crude laccase extracted from *R. vernicifera* acetone powder (the protein concentration of the crude extract was 2 mg/ml). The following indicators were considered to quantify the immobilization procedures: the coupling yield, considered as the percentage of coupled protein compared to the total amount of protein introduced in the coupling reaction; the immobilization yield, considered as the percentage of the immobilized enzyme activity compared to the total activity introduced for the immobilization; the retained (residual) specific activity of the immobilized enzyme as a percentage of the specific activity of the free (initial) enzyme. In the case of the immobilized enzyme, all the enzyme concentrations based on the copper determination were calculated by subtracting the copper content of the agarose (Sepharose) supports as such, which is not negligible and varied between lots (in the detected range 0.005–0.010 mg copper/ml gel).

## Results and discussion

The ready-to-use *p*-BQ-activated agarose support obtained as described above exhibits a degree of derivatization of 80 μmol *p*-BQ/g dried gel. This value was higher than that obtained for other *p*-BQ-activated supports based on polyvinyl alcohol and chitosan (about 50 μmol *p*-BQ/g dried gel) and in the same range of capacities as for other affinity chromatography materials used for ligand immobilization (e.g. 24–55 μmol active groups/g AH- or CH-Sepharose 4B).

The *p*-BQ-activated agarose support obtained contains not only *p*-BQ active groups but also HQ groups introduced as a by-product during the activation procedure (Scheme 1). As HQ is a substrate for laccase, it was thought that a support exhibiting both *p*-BQ and HQ groups could be a convenient material for the immobilization of this enzyme (Mateescu et al. 1989).

The swelling volume of the *p*-BQ-activated agarose obtained is 11.5 ml/g dried gel. The maximal binding capacity of the support in laccase immobilization was of 1.68 mg laccase/ml gel, corresponding to a coupling yield of 18.3% (value in the range of other coupled compounds on various supports). For instance, the coupling yields of different compounds (propylamine, α-amylase, bovine serum albumin) on *p*-BQ-activated supports (agarose, polyvinyl alcohol, chitosan) were in the range 10–95%. Using the same *p*-BQ-activated agarose, the coupling yield of bovine serum albumin was of 95%. The lower value obtained in the case of laccase immobilization can be explained by the fact that laccase is a glycoprotein with a molecular mass of 110 kDa which only 55% represents the protein moiety (Reinhammar 1970; Holwerda and Gray 1974). As a consequence, the relative number of free amino functional groups of the enzyme involved in the covalent coupling to the *p*-BQ active groups is lower.

The immobilization yield was 27%. The specific activity of the immobilized enzyme (10.8 mU/mg coupled protein) when stabilized was higher than that of the free enzyme (7.04 mU/mg protein); thus the retained specific activity of the immobilized enzyme was about 150%. This peculiar result can be explained by the fact that laccase can recognize the HQ functional groups of the support, as a substrate analogue, and, at the same time, react with the active *p*-BQ active groups of the support. Therefore, there are probably two distinct interactions of laccase with the activated support: (a) the enzyme can recognize the substrate analogue HQ groups by affinity and possibly is retained in a favorable conformation which can be maintained during (b) the simultaneous covalent coupling of the enzyme at the level of *p*-BQ active groups.

Since both the retention by affinity and covalent binding occur synergistically, this peculiar type of coupling, and indeed the whole process, can be considered as an affinity immobilization.

The hypothesis of affinity retention prior to and during the coupling is also supported by the fact that a tenfold stronger binding of laccase was observed by Clemmer et al. (1970) for hydroquinone derivatives of the type $H_3C$-O-HQ than for the unmodified HQ. In our *p*-BQ-activated supports, we also have quite similar functional groups, i.e. support-O-HQ. This affinity immobilization can explain the higher specific activity after immobilization. Various controls were executed in order to confirm this behaviour. For instance, no ferrocyanide oxidation was observed in the presence of *p*-BQ-activated support alone (without immobilized laccase).

A similar effect of enhanced enzyme activity of laccase by immobilization was also mentioned by Shuttleworth and Bollag (1986); they obtained a coupling yield of 89% and an immobilization yield of 99%.

On the other hand, it was mentioned that *Rhus* laccase appears to be present in two molecular forms (Andreasson and Reinhammar 1976) one 'active' and another 'inactive'. The equilibrium between the two forms was thought to be controlled by pH. At pH 6, the authors mentioned a higher experimental concentration of te 'active' form; in the presence of the reducing substrate and $O_2$, it was possible to maintain the level of the 'active' form by limiting the transition to the 'inactive' form.

As our coupling procedure occurs at exactly pH 6, it is possible that enzyme in the 'active' form was preferentially immobilized and, in the presence of large amounts of HQ as reducing substrate on the support and of oxygen, the 'active' form was retained. At the same time, transitions to the 'inactive' form are less probable, because immobilization can prevent major modification of the protein. The preservation of the 'active' form of laccase can explain, in the light of this concept, the higher specific activity of the immobilized enzyme as compared with free laccase, where both 'active' and 'inactive' forms can co-exist (and as consequence the specific activity is lower).

Another peculiar observation is that after immobilization, the enzyme activity seems to be gradually enhanced, at a rate of about 7.8%, within a period as long as a week, and is then stabilized. It is possible that, due to the affinity of the enzyme for the -O-HQ derivative, some post-immobilization re-arrangements can occur. Additional experiments and studies in this direction are now in progress in our laboratories in order to elucidate this behaviour. Moreover, a complementary explanation can complete the understanding of this observation. It is well known that laccase rapidly reduces oxygen to water only when all of its electron acceptors are reduced (Reinhammar 1970). HQ is a good agent to reduce laccase (Holwerda and Gray 1974). However, the re-oxidation of the metallo-enzyme by $O_2$ is much faster than the reduction. As our support carries an important quantity of HQ, the immobilized laccase can be reduced (by the HQ derivative of the support) and then re-oxidized by the $O_2$. Thus, some new *p*-BQ is formed on the support as a result of HQ oxidation induced by the immobilized laccase. These newly formed *p*-BQ residues can be involved in a more advanced covalent binding of the enzyme, and probably not in the enzyme re-oxidation on the basis of the low reduction potential (0.27 V) for the *p*-BQ/HQ couple. The oxygen is probably involved in the enzyme re-oxidation. Much larger amounts of support-HQ derivatives can be converted in situ under the catalytic action of the immobilized laccase in new *p*-BQ groups which, when formed, will be involved in new covalent links with the enzyme, generating post-immobilization re-arrangements probably responsible for the observed post-immobilization enhancement of the enzyme activity.

High retained specific activities, in the range 83–113%, were also obtained when a crude extract from the acetone powder of *R. vernicifera* was immobilized on the *p*-BQ-activated agarose gel. With the immobilization on BrCN-activated Sepharose 4B, carried out under identical conditions and with the same ratio of extract volume/activated support volume, a retained specific activity of about 50% was obtained. As the support matrix was of the same agarose type in both cases and as the protein is bound via the same type of free $NH_2$ groups (obtained from the lysine residues), it is possible that the higher retained activity in the case of *p*-BQ-activated supports is due to this specific binding of the laccase, in which the HQ groups of the support can enhance the activity values.

The storage stability of the immobilized enzyme is sufficiently high. After 14 months of storage at 4° C, the enzymatic activity was 95% of the initial activity.

It is a well known that laccase is particularly sensitive to the inhibitory action of chloride and azide ions (Morpurgo et al. 1974; Peisach and Levine 1965). The behaviour of immobilized laccase in comparison with the free enzyme under the action of the chloride and azide ions has been studied in this work. The immobilized enzyme was found to be more stable to attack by 0.02 M $Cl^-$ and $N_3^-$ ions, maintaining 12–16% of the activity, compared to the free enzyme which was reduced to 10% under the action of $Cl^-$ and totally inhibited by $N_3^-$. The immobilized laccase exhibited total recovery (100%) after removal of the inhibitors, while the recovered activities of the free enzyme were only of 67% and 32% after the removal of $Cl^-$ and $N_3^-$ ions, respectively (Table 1). This total (100%) recovery of the activity of the immobilized enzyme is important, providing more information on the mechanism of inhibition type, which in case of the immobilized enzyme seems to be reversible.

**Table 1.** The influence of $Cl^-$ and $N_3^-$ ions on free and immobilized laccase activity

| Type of sample | Activity (% control) of | |
|---|---|---|
| | Free enzyme | Immobilized enzyme |
| Control (no $Cl^-$ or $N_3^-$) | 100 | 100 |
| +0.02 M $Cl^-$ | 10±1.2 | 16±1.5 |
| +0.02 M $N_3^-$ | 0 | 12±1.1 |
| $Cl^-$ removed | 67±7.5 | 100 |
| $N_3^-$ removed | 32±4 | 100 |

At the same time, this excellent recovery capacity of the immobilized laccase suggests considerable possibilities for application in various biotechnological fields. An interesting possibility is that the high-affinity $N_3^-$ binding to the type-2 Cu(II) which occurs in free laccase (Morpurgo 1982; Spira-Solomon et al. 1986), and which is thought to be due to the presence of a bridge between the type-2 Cu(II) and one of the neighbouring type-3 Cu(I), becomes reversible in the case of the immobilized enzyme. This reversibility is probably due to some conformational modification of the enzyme induced by the coupling with the support, which contributes to the protection of the copper sites under inhibitory attack.

An extensive kinetic study with both the free and immobilized laccase is also in progress in our laboratories.

*Acknowledgements.* This work has been supported by PAFACC grant (Université du Québec à Montréal), by the Italian CNR Special Project 'Biotechnology and Bioinstrumentation' and MURST. A fellowship at UQAM offered to EA by MRC (Canada)/CNR (Italy) is gratefully acknowledged. Thanks are due to Dr Laura Morpurgo for helpful discussions and Dr Sharon Bennett for the revision of the manuscript.

## References

Ander P, Eriksson KE (1976) The importance of phenoloxidase activity in lignin degradation by the white-rot fungus *Sporothricum Pulverulentum*. Arch Microbiol 109:1–8

Andreasson LE, Reinhammar B (1976) Kinetic studies of *Rhus vernicifere* laccase. Role of the metal center in electron transfer. Biochim Biophys Acta 445:579–597

Bollag JM, Shuttleworth KL, Anderson DH (1988) Laccase-mediated detoxification of phenolic compounds. Environ Microbiol 54:3086–3091

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–254

Brandt J, Andersson LO, Porath J (1975) Covalent attachment of proteins to polysaccharide carriers by means of benzoquinone. Biochim Biophys Acta 386:196–202

Clemmer JD, Gilliland BL, Bartsch RA, Holwerda RA (1979) Substituent effects on the electron transfer reactivity of hydroquinones with laccase blue copper. Biochim Biophys Acta 568:307–320

Froehner SC, Erickson KE (1975) Properties of glycoprotein laccase immobilized by two methods. Acta Chem Scand (B) 29:691–694

Holwerda RA, Gray HB (1974) Mechanistic studies of the reduction of *Thus vernicifera* laccase by hydroquinone. J Am Chem Soc 96:6008–6022

Krämer DM (1988) The development of Eupergit C. Biotechnol focus 1:353–367

Mateescu MA, Weltrowska G, Agostinelli E, Saint-André R, Weltrowski M, Mondovi B (1989) Ready to use *p*-benzoquinone-activated supports for biochemical coupling, with special applications for laccase immobilization. Biotechnol Tech 3:415–420

Morpurgo L, Rotilio G, Finazzi Agro A, Mondovi B (1974) Anion binding to *Rhus vernicifera* laccase. Biochim Biophys Acta 336:324:328

Morpurgo L, Desideri A, Rotilio G (1982) Heterogenity of the type 3 copper in Japanese lacquer-tree (*Rhus vernicifera*) laccase. Biochem J 207:625–627

O'Neil P, Fielden EM, Morpurgo L, Agostinelli E (1984) Pulse-radiolysis studies on the interaction of one-electron reduced species with blue oxidases. Reduction of native and type-2-copper depleted Vietnamese lacquer-tree and Japanese lacquer-tree. Biochem J 222:71–76

Peisach J, Levine WG (1965) A comparison of the enzymatic activities of pig ceruloplasmin and *Rhus vernicifera* laccase. J Biol Chem 240:2284–2289

Porath J, Axen R (1976) Immobilization of enzymes to agar, agarose and Sephadex supports. Methods Enzymol 44:19–45

Reinhammar B (1970) Purification and properties of laccase and stellacyanine from *Rhus vernicifera*. Biochim Biophys Acta 205:35–47

Shuttleworth KL, Bollag JM (1986) Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. Enzyme Microb Technol 8:171–177

Spira-Solomon DJ, Allendorf MD, Solomon EJ (1986) Low temperature magnetic circular dichroism studies of native laccase. Confirmation of a trinuclear copper active site. J Am Chem Soc 108:5318–5328