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Properties of the Glycoprotein Laccase Immobilized by Two Methods

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Laccase (*p*-diphenol:oxygen oxidoreductase; EC 1.10.3.2) from *Neurospora crassa* has been immobilized by two different procedures: (1) Covalent attachment to Sepharose 4B activated with cyanogen bromide, and (2) Adsorption to Concanavalin A-Sepharose *via* the carbohydrate moiety. Except for small changes in the Michaelis-Menten constants, no differences were noted in the enzymological properties of the immobilized enzymes when compared to free enzyme.

The carbohydrate moiety of laccase involved in the interaction with Concanavalin A does not appear to be closely associated with the active center since binding to the lectin has no effect on the enzymological parameters investigated.

The immobilization of enzymes has been accomplished by several procedures, including covalent attachment to various matrices, adsorption to insoluble substances, or entrapment within a matrix. Such insoluble preparations have been useful not only for industrial applications but also in the study of multienzyme systems and as tools in biological research.[1–3] We report here the immobilization of laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) from *Neurospora crassa* by two different procedures. The first, covalent attachment to CNBr-activated Sepharose, has been widely used.[4] The second takes advantage of the ability of *Neurospora* laccase to bind to Concanavalin A.[5] This plant lectin, which is commercially available attached to CNBr-activated Sepharose, adsorbs α-D-mannopyranosyl, α-D-glucopyranosyl and sterically similar residues in a manner reminiscent of the antigen-antibody reaction. *Neurospora* laccase is a glycoprotein of the type which binds Concanavalin A and thus can be immobilized by adsorption to Concanavalin A chemically bound to Sepharose.

MATERIALS AND METHODS

Insoluble matrices. CNBr-activated Sepharose 4B and Concanavalin A-Sepharose (Con A-Sepharose) were both purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Preparation of pure laccase. Laccase was prepared from cycloheximide-induced cultures of *Neurospora crassa* [5] as described by Froehner and Eriksson.[6] The enzyme was homogeneous according to polyacrylamide gel electrophoresis and had a specific activity of 28 U/mg (see below for definition of U). $E(1\ \%,\ 1\ \text{cm}) = 14.0$ at 280 nm was used to calculate protein concentrations.

Assay for laccase activity. Oxygen consumption was measured with an oxygen electrode as previously described.[6] One unit (U) of activity is defined as the amount of enzyme required to consume 1.0 μmol/min of oxygen at 25 °C at pH 6.0 with 10 mM guaiacol as the reducing substrate. For the substrate specificity studies, each compound was used at a concentration of 10 mM in 0.10 M phosphate buffer, pH 6.0.

Preparation of immobilized laccase with CNBr-activated Sepharose. The procedure described in the Pharmacia brochure ("CNBr-activated Sepharose 4B for immobilization of biopolymers") was used with minor modifications. 1.5 mg of enzyme in 100 ml of 0.50 M sodium phosphate buffer, pH 7.2, was incubated with CNBr-activated Sepharose (previously washed

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and swelled in 10^{-3} M HCl) with constant stirring at 22 °C. The reaction was monitored by measuring laccase activity in the supernatant at various times. After 4 h, the gel was collected by filtration and washed once with 20 ml of 0.50 M sodium phosphate buffer, pH 7.2. The preparation was then incubated for 45 min at 22 °C with 10 ml of 1.0 M ethanolamine, pH 8, with constant stirring. After filtration, the gel was washed with 25 ml of 0.50 M sodium phosphate buffer, pH 7.2, followed by 25 ml of 1.0 M sodium acetate, pH 4.5, on a sintered glass funnel. This washing procedure was repeated three times. The gel was suspended in 0.10 M sodium phosphate buffer, pH 6.0, containing 0.01 % merthiolate (total volume = 5.0 ml) and stored at 5 °C. 20 µl was used for each assay.

Preparation of immobilized laccase with Concanavalin A-Sepharose. 0.75 mg of enzyme in 10.0 ml of 0.10 M sodium phosphate buffer, pH 6.0, was stirred with 1.8 ml (packed volume) Concanavalin A-Sepharose for 1 h. The volume was reduced to 5.0 ml by removing part of the supernatant, merthiolate added to 0.01 %, and the preparation stored at 5 °C. 20 µl was used for each assay.

RESULTS

The immobilization procedure, utilizing CNBr-activated Sepharose yields about 60 % of the input laccase activity. As shown in Fig. 1, the immobilization reaction is about 75 % complete after 1 h as measured by the amount of activity left in the solution. This, however, usually gives an overestimate of the amount bound to the matrix. By the Concanavalin A-Sepharose procedure, yields of nearly 100 % were obtained as determined by the amount of activity bound. Furthermore, the reaction is complete in less than 1 h. Both immobilized preparations could be stored for at least several months without detectable loss of activity. No activity could be detected in the supernatant

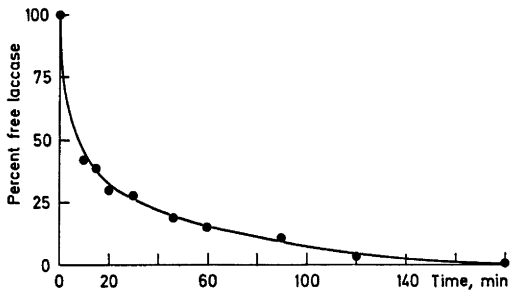

Fig. 1. Binding rate of laccase to CNBr-activated Sepharose.

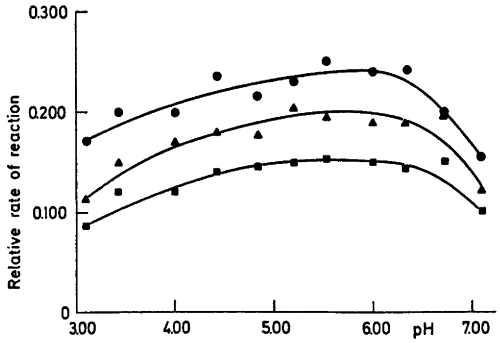

Fig. 2. pH-profiles of rates of reaction with 10 mM guaiacol. Free laccase, ●; laccase-Con A-Sepharose, ■; laccase-CNBr-Sepharose, ▲.

above the gel, indicating that leakage of the enzyme off of the matrix did not occur. Immobilization of an enzyme often changes some of its properties.[2] We have carried out investigations to compare both enzymological and structural properties of free laccase, laccase immobilized with CNBr-activated Sepharose (laccase-CNBr-Sepharose), and laccase immobilized with Concanavalin A-Sepharose (laccase-Con A-Sepharose). First, all three preparations are active over a wide pH range and no major differences in the profiles are apparent (Fig. 2).

A comparison of the Michaelis-Menten constants is shown in Fig. 3. For free laccase, the K_m for guaiacol is 0.36 mM, while this value is larger for the immobilized enzymes. Laccase-CNBr-Sepharose exhibits a K_m of 0.74 mM, while that for laccase-Con A-Sepharose is 0.67 mM. Although significant, the differences in these values are not very large since immobilized enzymes often have a K_m which is 10 – 20 times larger than for the free enzyme. The rate of oxygen consumption with different substrates did not show any significant differences among free laccase, laccase-CNBr-Sepharose, and laccase-Con A-Sepharose (Table 1). As expected from previous results,[6] laccase is most active with *ortho* and *para* diphenols, whether free or immobilized.

A comparison of the rate of oxygen consumption as a function of temperature failed to show any significant differences among free laccase, laccase-CNBr-Sepharose, and laccase-Con A-Sepharose. However, these experiments were complicated by the change in oxygen

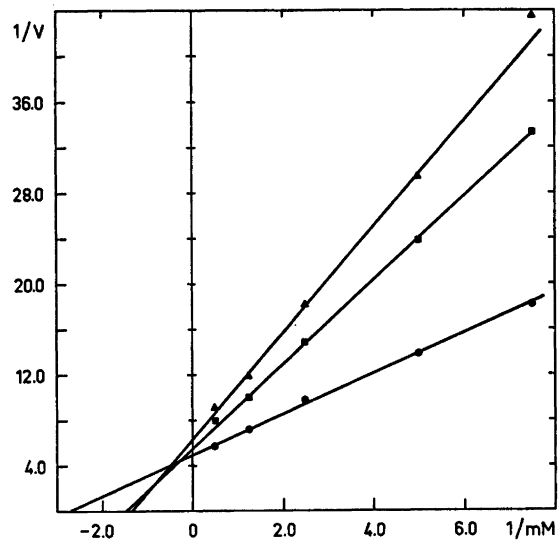

Fig. 3. A comparison of the Michaelis-Menten constants with guaiacol in phosphate buffer, pH 6.0. Free laccase, ●; laccase-Con A-Sepharose, ■; laccase-CNBr-Sepharose, ▲.

concentration in solution as a function of temperature. A plot of the log of activity *versus* the reciprocal of the temperature was not linear.

With the hope of detecting structural changes induced by the immobilization, the stabilities of the enzymes were investigated. Since laccase is released from Con A-Sepharose in the presence of moderate concentrations of urea or at temperatures above about 55 °C, comparisons of heat stability and resistance to urea denaturation could be carried out only on free laccase and laccase-Sepharose. Again, no significant differences were noted. Both have a half-life of about 25 min at 70 °C and are approximately 50 % active in 7 M urea. Similarly, no differences in sensitivity to trypsin were noted among the three preparations.

Table 1. Relative reaction rates with different substrates for free laccase, laccase-Con A-Sepharose, and laccase-CNBr-Sepharose.

Substrates	Free laccase	Laccase bound to Con A-Sepharose	Laccase bound to CNBr-Sepharose
Hydroquinone	1.11	1.03	1.02
Gallic acid	1.35	1.19	1.31
DOPA	0.70	0.48	0.58
Protocatechuic acid	0.70	0.61	0.86
Guaiacol	1.00	1.00	1.00
Pyrogallol	1.80	1.29	0.78
Pyrocatechol	0.76	0.90	1.10
Resorcinol	0.07	0.13	0.10
m-Cresol	0	0.14	0.17
Phloroglucinol	0.48	0.50	0.61
Orcinol	0	0.13	0.14
Tyrosine	0	0.05	0
Potassium ferrocyanide	0	0	0

DISCUSSION

Immobilization of laccase by these two procedures seems to change the enzymatic properties of this enzyme very little. In fact, the only significant difference noted, an increase in the K_m for the two immobilized preparations, is usually attributed to reduced diffusion of the substrate caused by the proximity of the matrix to the enzyme.[2] In view of a recent observation that lactoperoxidase is attached almost exclusively to the surface of Sepharose beads,[11] other factors may be important also. Since the free enzyme is quite stable to heating and to moderate concentrations of urea, it is not surprising that covalent attachment to Sepharose does not increase the stability. Similarly, the free enzyme is active over a wide

pH range, making any changes in pH optimum due to immobilization difficult to detect.

Covalent binding of an enzyme to a matrix can lead to a decrease in enzyme activity due to shielding of the active center,[7,8] or modification of the enzyme structure. Full inactivation of an enzyme is obtained when a covalent bond is formed with the residues in the active center.[9,10] Attachment of laccase to Con A-Sepharose did not reduce the activity nor did it affect the substrate specificity of the enzyme, suggesting that the carbohydrate moiety is not involved in the catalytic center. A similar conclusion was reached in investigations of mammalian dopamine-β-hydroxylase bound to Con A-Sepharose.[12]

Immobilization of glycoenzymes with Concanavalin A-Sepharose is a technique which may be useful for several reasons. First, the procedure is very simple and rapidly performed. Also, the extremely mild conditions required and high yields obtained offer advantages over covalent attachment. Since the carbohydrate moiety is not generally involved in the active site of the enzyme, attachment to Con A-Sepharose should not affect the substrate specificity. The commercial availability of immobilized plant lectins with different specificities should make it possible to attach almost any type of glycoprotein to a matrix by this procedure. Finally, the detection of differences between free and Con A-bound enzymes may clarify the function of the carbohydrate moiety of glycoenzymes.

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