Phytochemistry, Vol. 27, No. 9, pp. 2731–2733, 1988.
Printed in Great Britain.

0031-9422/88 $3.00 + 0.00

IMMOBILIZATION OF PROTOCATECHUATE 3,4-DIOXYGENASE FROM *PLEUROTUS OSTREATUS* ON ACTIVATED POROUS GLASS BEADS

MARIA WOJTAŚ-WASILEWSKA, JOLANTA LUTEREK and JERZY ROGALSKI

Department of Biochemistry, Maria Curie-Skłodowska University, 20–031 Lublin, Pl. M. Curie-Sklodowska 3, Poland

(*Revised received* 18 *January* 1988)

Key Word Index—*Pleurotus ostreatus*; protocatechuate 3,4-dioxygenase; immobilization; porous glass beads.

Abstract—Protocatechuate 3,4-dioxygenase (protocatechuate: oxygen 3,4-oxidoreductase, EC 1.13.11.3) purified from the wood-degrading fungus *Pleurotus ostreatus* was immobilized on glass beads. The per cent attached protein was 78 with a retained enzymatic activity of 90%. The basic properties of the immobilized enzyme were tested and compared with those of a soluble form. Immobilized dioxygenase was stable over wide ranges of pH and temperature. Moreover, the immobilization brought about a slight increase of its specific activity.

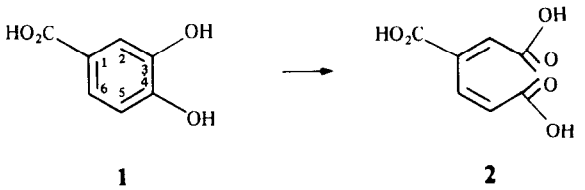
INTRODUCTION

Protocatechaute 3,4-dioxygenase is an enzyme that catalyses the transformation of protocatechuic acid (**1**) to β-carboxy-*cis,cis*-muconic acid (**2**) (Fig.1). It has been purified and characterized from several Gram negative and positive bacteria [1–3] but *Pseudomonas aeruginosa* dioxygenase, obtained in a crystalline form, is the most extensively studied bacterial enzyme [4].

Highly purified preparations of protocatechuate 3,4-dioxygenase from two species of fungi have been obtained in our laboratory [5, 6], and some physicochemical and kinetic properties of the isofunctional enzymes determined.

The objective of the present studies was to prepare a water-insoluble form of the protocatechuate 3,4-dioxygenase purified from *Pleurotus ostreatus*, in order to investigate the properties of an immobilized form of this enzyme and to compare them with those of the soluble unmodified protein. The immobilized preparation would then be used in future model experiments designed to elucidate the mechanism of oxygenation of different lignin model compounds, since it is known that oxygenation process plays a role in biodegradation of lignin in nature [7–9].

The first experiments concerning immobilization of protocatechuate 3,4-dioxygenase from *Pseudomonas aeruginosa* on CNBr-Sepharose were described by Zaborsky and Ogletree in 1972 [10].



1 **2**

Reaction catalysed by protocatechuate 3,4 - dioxygenase

Fig. 1.

This paper describes the immobilization of the fungal protocatechuate 3,4-dioxygenase by attachment to aminoalkylsilyl-porous glass beads activated with glutaraldehyde.

RESULTS AND DISCUSSION

The conditions and results of protocatechuate 3,4-dioxygenase immobilization on silanized porous glass beads which had been activation with glutaraldehyde are presented in Table 1. By this procedure 78% of the enzyme protein was bound to the soild support. Although the total activity of the immobilized dioxygenase was slightly reduced (bound activity 90%), its specific activity was about 16% higher than that of the soluble enzyme. Similar phenomena have been observed for other enzymes immobilized on various solid supports [11–13].

Table 1. Effect of immobilization of protocatechuate 3,4-dioxygenase on porous glass beads

Coupling pH	8.0
Coupling time (hr)	75
Protein added (mg)	1.73
Total activity of the added soluble unmodified enzyme (units)	58
Specific activity before immobilization (units/mg protein)	34
Total activity of the immobilized enzyme (units)	53
Protein uncoupling (mg)	0.38
Specific activity of the enzyme after immobilization (units/mg protein)	39
Attached protein (%)	78
Attached activity (%)	90
Specific activity of the immobilized enzyme after eight-week storage (units/mg protein)	39

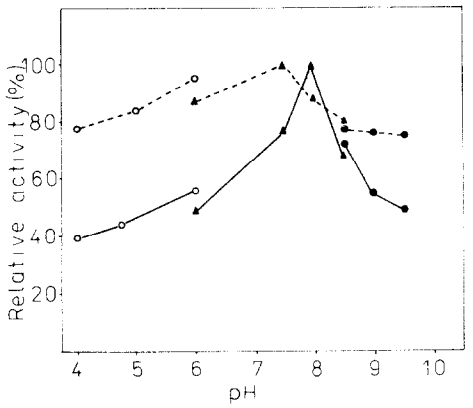


Fig.2. Effect of pH on the activity of soluble (——) and immobilized (----) protocatechuate 3,4-dioxygenase. The activity was measured spectrophotometrically (for details see Experimental) in the following 15 mM buffers; o, acetate; ▲, Tris–HCl and ●, glycinate.

Other basic properties of the dioxygenase were also modified by immobilization. Examination of the effect of incubation temperature on the acitivity of both soluble and immobilized enzyme indicates that the temperature optimum was changed from 37° for the soluble dioxygenase to 45° for its immobilized form. Furthermore, the activities of the immobilized enzyme at 60° and 80° were *ca* 78 and 75% respectively of that at 45°. While the corresponding values for the soluble enzyme at 60 and 80° were 9 and 0.0% of the activity at 37°. The above findings indicate that the immobilized protocatechuate 3,4-dioxygenase was more resistant to inhibition by temperature than the free enzyme.

The effect of pH on the activity of both forms of the enzyme is shown in Fig. 2. The activities of immobilized protocatechuate 3,4-dioxygenase at various pH's in the range 4.0–9.5 were, with one exception, higher than those of the soluble enzyme. Moreover, the optimal pH for immobilized dioxygenase was shifted towards lower pH values. The soluble enzyme had its pH optimum at 8.0 but maximal activity of immobilized dioxygenase was observed at pH 7.5. Very similar results were obtained by Zaborsky and Ogletree [10] for soluble and Sepharose-bound bacterial protocatechuate 3,4-dioxygenase.

The packed column experiment was used to demonstrate aromatic ring cleavage of 3,4-dihydroxybenzoic acid by immobilized protocatechuate 3,4-dioxygenase and to determine if the bound enzyme was re-utilizable. The results showed that the absorption spectrum of the column eluate had a lower A_{290} value (characteristic band of 3,4-dihydoxybenzoic acid) and a higher A_{270} value (characteristic band of the product) than the substrate. These two wavelengths are commonly used to estimate the activity of protocatechuate 3,4-dioxygenase. It was also found that the column, after removal of the reaction product, may be used again with the same result. Moreover, practically no loss of dioxygenase activity was observed after eight weeks storage at 4° (Table 1).

The results clearly show that immobilization of protocatechuate 3,4-dioxygenase on porous glass beads makes it stable over wide ranges of pH and temperature, and that the column can be re-used.

EXPERIMENTAL

Enzyme isolation. Pleurotus ostreatus (Jacqu) Fr. No. 53, obtained from the Department of Plant Anatomy and Physiology, J. E. Purkyne University, Brno, Czechoslovakia, was cultured in Roux flasks on a liquid medium as described previously [6]. Protocatechuate 3,4-dioxygenase was purified from an extract of 4-hydroxybenzoate-induced mycelium by $(NH_4)_2SO_4$ pptn, DEAE-cellulose and Sephadex G-200 chromatography as described previously [6]. Freeze-dried enzyme was used for immobilization.

Activation of glass beads and enzyme coupling. The porous glass beads (CPG) composed of 7 mol% Na_2O, 23 mol% B_2O_3 and 70 mol% SiO_2 were kindly supplied by Dr A. Dawidowicz (Department of Chemical Physics, M. Curie-Skłodowska University). The glass support was silanized with γ-aminopropyltriethoxysilane (APTES) [14] and then bound to glutaraldehyde [15]. The mixture of activated glass beads (5 g) and purified protocatechuate 3,4-dioxygenase (1.73 mg protein in 17.5 ml 15 mM Tris–HCl buffer, pH 8.0) was mechanically shaken in a refrigerator for 12 hr with and then filtered. The solid was washed with 15 mM Tris–HCl buffer, pH 8.0 (2.5 ml). Then the combined origninal filtrate and wash liquid were checked for enzyme activity and protein content. After additional washing with buffer (300 ml), 0.5 M NaCl (50 ml) and again with buffer, the enzyme-glass derivative was suspended in Tris–HCl buffer. The suspension was either transferred to a small column or used in batch experiments.

Enzyme assay. The activity of soluble and immobilized protocatechuate 3,4-dioxygenase was estimated spectrophotometrically by measuring the decrease in 3,4-dihydroxybenzoic acid absorption at 290 nm as described previously [5]. The typical assay mixture (4 ml) contained 0.32 μmol substrate 0.1 mg/ml, pH 7.5), soluble enzyme (0.01–0.1 mg protein) or its immobilized form (1 ml suspension contained 180 mg of immobilized enzyme) and 30μmol Tris–HCl buffer, pH 8.0. One unit of enzyme activity was defined as the amount of enzyme which oxidizes 0.1μmol 3,4-dihydoxybenzoic acid/10 min at 37°. The activity is expressed in units/mg of protein.

Protein determination. Protein was determined spectrophotomatrically according to Ehresmann *et al.* [16] by measuring differences in absorption at 228.5 and 234.5 nm. Bovine albumin was used as a standard. The quantity of protein bound on to the glass beads was calculated by subtracting the protein in the combined original filtrate and wash liquid of the immobilized enzyme from the protein used for immobilization.

Packed column experiment. The sample of immobilized protocatechuate 3,4-dioxygenase suspended in 15 mM Tris–HCl buffer, pH 8.0 (*ca* 1 g of immobilized enzyme/6 ml suspension) was packed into a small column (0.5 × 5 cm). After equilibration of the column with the same buffer, the substrate (3,4-dihydroxybenzoic acid 0.5 ml (0.1 mg/ml) previously adjusted to pH 7.5 with NaOH) was applied and elution was started. Fractions of 2 ml were collected. UV absorption spectra (200–350 nm) of the effluent samples were recorded using buffer as the reference. The column was washed with the above mentioned buffer to remove all the reaction products and to obtain equilibration. Then the procedure was repeated.

Effect of pH on enzyme activity. The influence of pH on the activity of soluble and immobilized protocatechuate 3,4-dioxygenase was determined spectrophotmetrically (for details see Enzyme assay) in the following 15 mM buffers: acetate (pH 4.0–6.0), Tris–HCl (pH 6.0–8.5) and glycinate (pH 8.5–9.5).

Effect of temperature on enzyme activity. The influence of incubation temp. on the activity of the soluble and immobilized protocatechuate 3,4-dioxygenase was determined at various

temps from 4 to 80°. All components of the incubation mixture were equilibrated to the required temp. before use (for details see enzyme assay).

Acknowledgement—This work was supported by the Polish Ministry of National Education Projects Nos MR.II.17/2.2.1 and CPBP 04.02/2.2.

REFERENCES

1. Stanier, R. Y. and Ingraham, J. L. (1954) *J. Biol. Chem.* **210**, 799.
2. Ornston, L. N. (1966) *J. Biol. Chem.* **241**, 3787.
3. Cain, R. B. and Cartwright, N. J. (1969) *Biochim. Biophys. Acta* **37**, 197.
4. Fujisawa, H. and Hayaishi, O. (1968) *J. Biol. Chem.* **243**, 2673.
5. Wojtaś-Wasilewska, M. and Trojanowski, J. (1980) *Acta Biochim. Polon.* **27**, 21.
6. Wojtaś-Wasilewska, M., Trojanowski, J. and Luterek, J. (1983) *Acta Biochim. Polon.* **30**, 291.
7. Flaig, W. and Haider, K. (1961) *Arch. Mikrobiol.* **40**, 212.
8. Kirk, T. K. and Chang, H. (1975) *Holzforschung* **29**, 56.
9. Crawford, D. L. and Crawford, R. L. (1980) *Enzyme Microb. Technol.* **2**, 11.
10. Zaborsky, O. R. and Ogletree, J. (1972) *Biochim. Biophys. Acta* **289**, 68.
11. Fadda, M. B., Dessi, M. R., Maurici, R., Rinaldi, A. and Satta, G. (1982) *Twenty-third Natl Biochem. Congr.* Florence.
12. Fadda, M. B., Dessi, M. R., Maurici, R., Rinaldi, A. and Satta, G. (1984) *Appl. Microbiol. Biotechnol.* **19**, 306.
13. Woodward, J. and Zachry, G. S. (1982) *Enzyme Microb. Technol.* **4**, 245.
14. Robinson, P. J., Dunhill, P. and Lilly, M. B. (1971) *Biochim. Biophys. Acta* **242**, 659.
15. Lappi, D. A., Stolzenbach, P. E., Kaplan, N. D. and Kamen, M. D. (1976) *Biochem. Biophys. Res. Commun.* **69**, 878.
16. Ehresmann, B., Imbault, P. and Weil, J. H. (1973) *Anal. Biochem.* **54**, 454.