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Biotransformation of phenols using immobilised polyphenol oxidase

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Abstract

Polyphenol oxidase (PPO), obtained from *Agaricus bisporus*, can be used in hydroxylating a range of phenolic substrates to yield catechols which are then oxidised by the enzyme to give *o*-quinone products. The objective of this study was to develop systems whereby phenols could be transformed by PPO, and the products of the biotransformation could be isolated and characterised. By comparing the product mixtures obtained using soluble PPO and various forms of immobilised PPO, in aqueous and non-aqueous media, we have found significant differences in reaction rates and in the proportions of catechol and quinone produced. PPO in solution is inactivated by the reaction products, but when it is immobilised, the separation of products from the enzyme reduces this inhibition. Immobilisation also leads to increased stability, and allows continuous use of the enzyme. In bioreactors containing customised novel asymmetric capillary membranes as the enzyme support, high concentrations of phenolic substrates were converted. The addition of a chitosan-containing column downstream from the capillary membrane bioreactor facilitated the removal of the coloured quinone products from the permeate, and recycling of the substrate solution.

Keywords: Polyphenol oxidase; Biotransformation of phenols; Immobilization; Membrane bioreactor

1. Introduction

The insertion of a hydroxyl group into aromatic systems is an unusual and useful reaction. It can be achieved biocatalytically, using the enzyme polyphenol oxidase, (PPO, E.C. 1.14.18.1, also called tyrosinase) which is obtained from various sources, including the common mushroom, *Agaricus bisporus* and the bread mould *Neurospora crassa* (Fig. 1). We have used PPO from these sources as an immobilised biocatalyst in phenol conversion reactions, under various conditions, and have found that a number of factors, including the enzyme source, the method of purification, the nature of immobilisation and the medium, can alter the reaction products and affect the reaction efficiency. A range of phenolic compounds can be converted in high yield by PPO, depending on the conditions of the reaction, and the proportions of catechol and quinone can be changed by manipulating these conditions. A major drawback in the application of PPO is the marked product inhibition (suicide inactivation)

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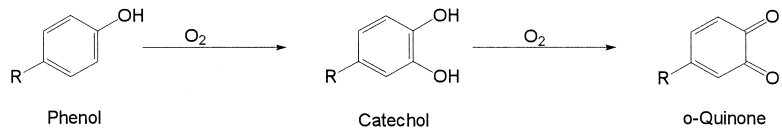

Fig. 1. Hydroxylation of phenols and oxidation of catechols catalysed by polyphenol oxidase.

exhibited by the enzyme. One of our objectives is to develop systems in which reaction conditions minimise this inhibition.

2. Experimental

PPO was extracted and assayed using L-DOPA as described previously [1]. (1 Unit = 1 μmol dopaquinone produced min^{-1}). In measurements of phenol conversion rates using soluble PPO, the enzyme (3000 U) was added to stirred phenolic substrate solutions at concentrations from 1–5 mM (20 ml). Substrates used: phenol; *p*-cresol; *m*-cresol; 4-chlorophenol; 4-methoxyphenol. Products were quantified by HPLC (Beckman System Gold; C-18 RP column; mobile phase acetonitrile/water (4:6); flow rate 1 ml min^{-1}).

For immobilisation of PPO on nylon membranes, the membranes (0.45 μm, 47 mm) were acid-washed (3 M HCl overnight), then treated with glutaraldehyde (3%, overnight), followed by PPO (6000 U, of which 50% was immobilised). Activity was measured using varying concentrations of the same phenolic substrates as above, in batch reactions (20 ml), with continuous stirring.

Single-fibre reactors (SFR), consisting of a single capillary membrane (with internal surface area 3.41×10^{-4} m^2) housed within a glass tube (length = 110 mm, o.d. = 7 mm), were operated by recycling the substrate solution through the lumen of the capillary, and separately collecting the permeate from the shell (outer) side (Fig. 4). The difference between the control (with no PPO) and reaction concentrations measured at equivalent times was used to calculate the conversion of the substrate. Two types of capillary (IPS 748 and IPS 763 [2]) which differ in permeability and hence in transmembrane flux, were compared. For purposes of comparison with capillary membrane experiments, reactions were also run with non-immobilised PPO, using 50-ml batches of phenol solutions, and 40 or 140 Units of PPO.

3. Results

3.1. Effects of the source and purification of enzyme extracts

The activity of PPO samples extracted from various samples of *A. bisporus* by variations of a standard method [1], and from *N. crassa*, were measured in aqueous medium, assaying with (1) L-DOPA and (2) *p*-cresol. In organic medium, the activity of three extracts which had been purified to varying degrees, were compared. The most active extracts were found to be obtained from open brown mushrooms which had been previously frozen (specific activity using L-DOPA: 0.82 U mg^{-1}) or *N. crassa* mycelia (specific activity 1.91 U mg^{-1}).

3.2. Effects of immobilisation on reaction rates and product profile

The conversion of a range of phenolic substrates by PPO, under varying conditions, was monitored by HPLC over time, and the relative proportions of catechol and quinone products were observed. In aqueous solution with non-immobilised PPO, phenols were depleted within a few hours, and appreciable proportions of the catechol intermediates were observed (Fig. 2). However, the reaction was observed to cease entirely after 5–6 h.

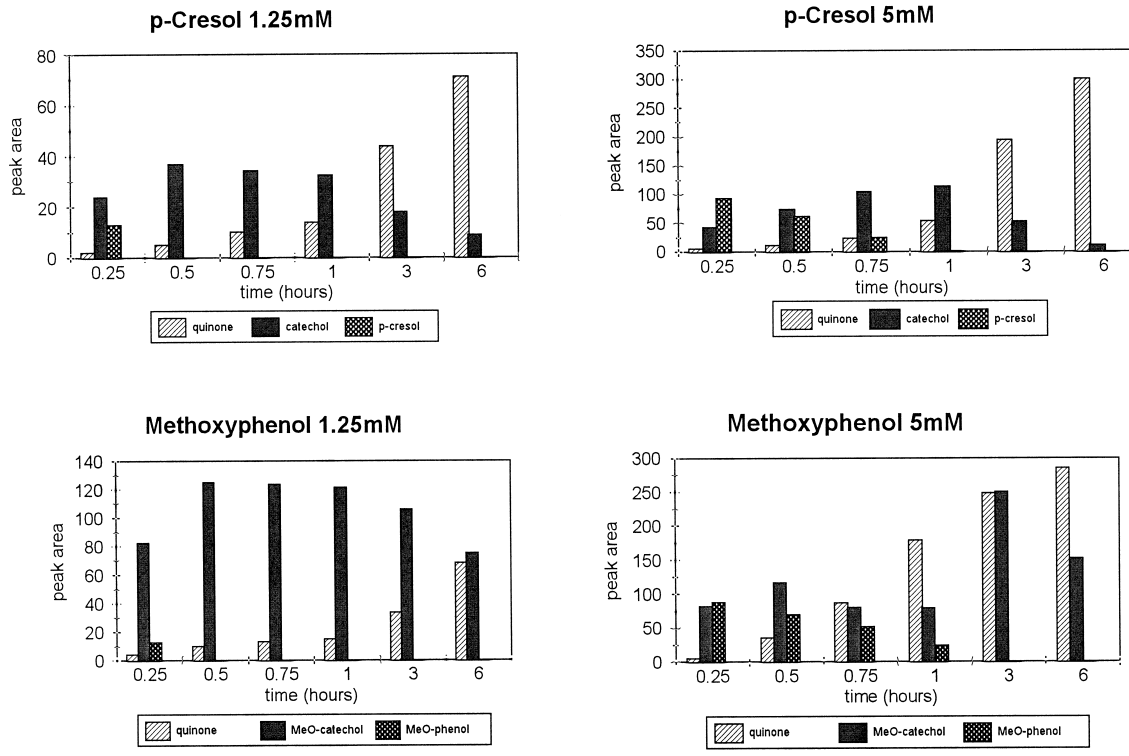

Fig. 2. Examples of product profiles for reaction of phenols with non-immobilised PPO (3000 U) in aqueous medium.

When the PPO was immobilised covalently on nylon membranes, reactions were observed to proceed at higher rates as shown by the disappearance of the phenol, and greater proportions of the quinone products were produced in substrate solutions of low concentration. However, at high concentrations, appreciable proportions of catechols were obtained (Fig. 3).

PPO was also immobilised, by adsorption, on hydrophobic synthetic (polysulphone) capillary membranes in customised bioreactor modules, to facilitate the conversion of phenols on a semi-continuous basis. Initially experiments were conducted using very small scale single capillary membrane bioreactors. Results are shown in Fig. 4 and Table 1. No catechol products were observed (by HPLC) to be present in reaction mixtures from the membrane bioreactors. Some substrate selectivity was observed in the conversion of a mixture of phenols, in that a greater proportion of phenol and 4-methoxyphenol were removed from the mixed solution than was the case for *p*-cresol and 4-chlorophenol (Fig. 4). This membrane bioreactor, operating in semi-continuous mode, allowed conversion of greater amounts of phenols before enzyme inactivation than was observed in the non-immobilised enzyme reactions (Table 1).

A comparison of total phenol conversions using ‘IPS 748’ (low flux) and ‘IPS 763’ (high flux) membranes and non-immobilised PPO, under varying conditions, suggested that in a situation where products are rapidly removed from the vicinity of the enzyme, greater phenol conversion was achieved, possibly due to decreased product inhibition.

In some experiments, the permeate from the capillary membrane reactor was fed into a chitosan-containing column before collection and

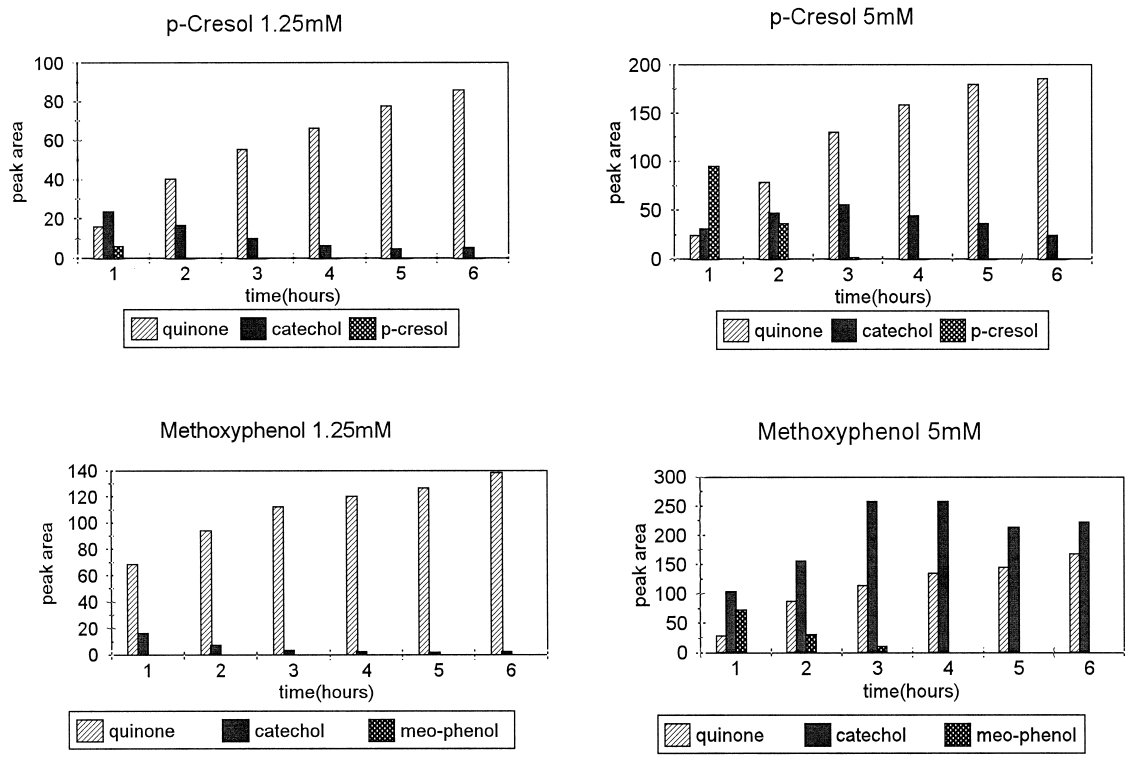

Fig. 3. Examples of reaction product profiles for conversion of phenols by PPO immobilised on nylon membranes.

analysis, so that the quinone products of the reaction were adsorbed, and the permeate was then no longer coloured. The chitosan did not adsorb phenols or catechols, as indicated by the HPLC analysis of these components in the permeate, and thus recycling of the reaction mixture would be feasible.

We have initiated investigations to scale up the conversion of phenols by PPO in aqueous medium, using a larger scale capillary membrane bioreactor comprising 140 capillary membranes, with a total surface area of 198 mm^2. Preliminary results indicate efficiency comparable with the single fibre reactors; a total of 20

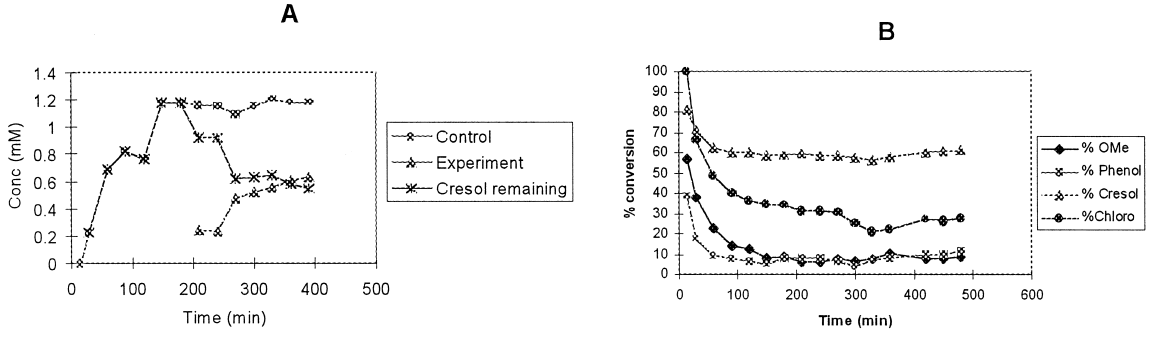

Fig. 4. The conversion of phenols by PPO in a capillary membrane bioreactor operated in semi-continuous mode. (A) shows conversion of cresol relative to a control reaction in which no PPO was present. (B) shows the % conversion of phenolic substrates (each initially at concentration 1 mM) in a mixture fed into the reactor.

Table 1
The total conversion of phenols by PPO in single capillary membrane bioreactor[a]

Membrane	PPO (Units)	Substrate composition	Concentration (mM)	Time of reaction (h)	Total phenols converted (μmol)	Phenol conversion per PPO Unit (μmol U^{-1})
Low flux	45	Mixed solution of four phenols	Phenols each 1 mM	4	8.87	0.196
			Phenols each 2.5 mM	4	22.85	0.507
			Phenols each 5 mM	4	28.5	0.630
	140	*p*-cresol	1 mM	8	20.6	0.147
			2.5 mM	8	42.2	0.302
		Mixed solution containing four phenols	2.5 mM	4	104.7	0.748
			5 mM	4	136.8	0.977
High flux	45	Mixed solution containing four phenols	Phenols each 1 mM	8	948.9	21.07
	140	*p*-cresol	1 mM	8	828.8	5.92
Non-immobilised	140	Mixed solution containing four phenols	Phenols each 1 mM	8	38.0	0.27
			Phenols each 2.5 mM	8	51.3	0.37
			Phenols each 5 mM	8	73.8	0.53

[a]Based on Edwards et al. [5].

mmoles of mixed phenolic substrates were converted in 4 h, using 9000 U PPO (2.2 μmoles U^{-1}).

3.3. Biocatalysis in organic medium

The activity of PPO in organic media was developed to facilitate the conversion of non-water soluble substrates and to determine the effect of the organic medium on the reaction rates and selectivity [1]. The biocatalyst consisted of PPO extracts immobilised by drying on glass beads. In batch reactions to measure reaction kinetics, portions were added to chloroform solutions of phenolic substrates (in which water content had been optimised). In optimising the product yields, a small column reactor was used in continuous recycle. Reactions were monitored by UV/visible spectroscopy and NMR. Catechols could be obtained from the quinones by shaking the organic phase reaction product mixture with an aqueous solution of ascorbic acid (results not shown).

Only quinone products were observed to be formed in chloroform medium reactions, and the enzyme kinetic constants were altered (Table 2). The substrate range was more limited in the chloroform medium; the steric size of substrates converted in the organic system was limited to substrates with small side chains, and catalytic activity was found to be inversely related to steric size [3]. The water content in the

Table 2
The comparison of amounts of phenol converted per Unit of PPO per hour, for different PPO biocatalysts

PPO Biocatalyst	Productivity (μmol phenol converted U^{-1} h^{-1})
Non-immobilised	0.011
Immobilised on nylon membranes	0.06
Immobilised in single capillary membrane bioreactor, IPS 748	0.24
Immobilised in single capillary membrane bioreactor, IPS 763	2.60
Immobilised in large scale capillary membrane bioreactor	0.56
Immobilised on glass, in chloroform medium	0.96

biocatalyst apparently affects the flexibility of the protein and hence restricting the substrate range. However, product inactivation of the enzyme was decreased in the organic system, as compared with aqueous reactions. Also, the addition of small amounts of SDS has been shown to increase the protein flexibility in organic medium [4].

The rate of phenol conversion per PPO Unit was greater in chloroform than in aqueous systems, with the exception of the high flux membrane reactor aqueous system, indicating the effectiveness of the organic medium in reducing the product inactivation of the enzyme (Table 2).

4. Discussion

Polyphenol oxidase can be used in the conversion of phenolic substrates to catechols and/or quinones. It is readily available as extracts with high activity from inexpensive sources, and does not require extensive purification. The enzyme has potential as a biocatalyst for applications involving biotransformations of phenols or bioremediation of phenol-polluted water [5].

In aqueous media, the enzyme is eventually inhibited by its product quinones, but under conditions where there is high enzyme concentration, and particularly where the product can be removed from the reaction site, high yields of products can be obtained. Thus, the application of polyphenol oxidase immobilised in high flux rate membrane bioreactors was found to facilitate the highest conversion of phenols to quinones.

Under aqueous reaction conditions using PPO immobilised in membrane reactors, the products were found to be quinones, rather than catechols. However, in aqueous solutions where the enzyme was not immobilised, appreciable yields of catechol products can be obtained with high percentage conversion of the phenolic substrates. When nylon membranes were used as the immobilisation support, the reaction product profiles obtained were similar to those of the polysulphone membrane-immobilised reactions, but at high substrate concentrations greater proportions of the catechol products were formed. In organic (chloroform) medium, polyphenol oxidase converts phenols to quinones.

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