Process Biochemistry 44 (2009) 191–198

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Laccase immobilization on mesostructured cellular foams affords preparations with ultra high activity

Adriana Rekuć [a], Jolanta Bryjak [a,*], Katarzyna Szymańska [b], Andrzej B. Jarzębski [b,c]

[a] Wrocław University of Technology, Faculty of Chemistry, Department of Bioorganic Chemistry, Norwida 4/6, 50-373 Wrocław, Poland
[b] Department of Chemical Engineering, Silesian University of Technology, M. Strzody 7, 44-100 Gliwice, Poland
[c] Institute of Chemical Engineering, Polish Academy of Sciences, Bałtycka 5, 44-100 Gliwice, Poland

ARTICLE INFO

Article history:
Received 25 June 2008
Received in revised form 18 September 2008
Accepted 12 October 2008

Keywords:
Laccase
Immobilization
Mesostructured cellular foams
Kinetics
Decolourisation
Indigo carmine

ABSTRACT

Extracellular laccase produced by the wood-rotting fungus *Cerrena unicolor* was immobilized covalently on the mesostructured siliceous cellular foams (MCFs) functionalised using various organosilanes with amine and glycidyl groups. The experiments indicated that laccase bound via glutaraldehyde to MCFs modified using 2-aminoethyl-3-aminopropyltrimethoxysilane remains very active. In the best biocatalyst activity was about 42,700 U mL^{-1} carrier (66,800 U mg^{-1} bound protein), and hence significantly higher than ever reported before. Optimisation of the immobilization procedure with respect to protein concentration, pH of coupling mixture and the enzyme purity afforded the biocatalyst with activity of about 90,980 U mL^{-1}. For the best preparation, thermal- and pH-stability, and activity profiles were determined. Experiments carried out in a batch reactor showed that k_{cat}/K_m for immobilized enzyme (0.88 min^{-1} μM^{-1}) was acceptable lower than the value obtained for the native enzyme (2.19 min^{-1} μM^{-1}). Finally, potentials of the catalysts were tested in the decolourisation of indigo carmine without redox-mediators. Seven consecutive runs with the catalysts separated by microfiltration proved that adsorption of the dye onto the carrier and enzymatic oxidation contribute to the efficient decolourisation without loss of immobilized enzyme activity.

1. Introduction

Phenol oxidases are enzymes that catalyse reaction of dioxygen reduction to water with production of substrate radicals, which are non-enzymatically converted to dimers, oligomers and polymers [1]. Increasing numbers of oxidative biotransformations of phenol oxidases, especially laccases (EC 1.10.3.2), have been extensively reviewed [1–5]. They show fairly wide specificity to substrates such as alkenes, methoxyphenols, phenols, *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines and lignin-related molecules, and demonstrate the exceptional abilities to oxidize substrates with very high potential in the presence of redox-mediators. Laccases are widely distributed in plants, bacteria and fungi [6] and this plus their attractive properties make them very interesting catalysts for applications in textile or stain bleaching, decolourisation, bio-bleaching of pulp, detoxification of xenobiotics, synthesis of polymers and biopolymers modification, chemical synthesis, bioremediation and in biosensors production [1–5,7,8]. However, operational stability of native enzymes is rather limited. Therefore, immobilization is very often applied in order to ease this limitation, as was summarized by Duran et al. [2,9].

Our previous experiments with *Cerrena unicolor* laccase immobilized on cellulose-based carriers [10] showed that the enzyme bound to a large pore (35 nm) carrier exhibited much higher activities (total and specific) than attached to a conventional acrylic carrier with smaller pores [11]. However, the extensive comparative studies of activities of glucoamylase, trypsin and invertase immobilized on the same cellulose carrier [12–14] and on the siliceous mesostructured cellular foams (MCFs) [15,16] revealed significant advantages of mesoporous silica supports. Particles of MCF are significantly smaller (28–30 μm) then those of cellulose-based supports (150–300 μm) and have much more open structure, and this relaxes considerable diffusional constrains. We can thus expect that the nanostructured silicates with very large mesopores can also be the supports of choice for laccase immobilization. To date, there are no reports on laccase immobilization in/on sol–gel materials, except for *C. unicolor* laccase immobilized in mesostructured thin films, used in biosensors construction [17–19].

Mesoporous materials, especially mesoporous silicas, possess quite unique, highly ordered structure with pore sizes even over 20 nm, thus similar or larger than most enzyme molecules.

* Corresponding author. Tel.: +48 71 3202677; fax: +48 71 3280475.
E-mail address: jolanta.bryjak@pwr.wroc.pl (J. Bryjak).

1359-5113/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2008.10.007

Therefore, large internal surface area of these supports can be accessible to enzymes, and the presence of various active (e.g. silanol) groups on the surface can additionally facilitate protein adsorption resulting in high enzyme loads [20–23]. Moreover, the mesoporous silicates show very high chemical, thermal, mechanical and biological resistance and they are environmentally inert. The advances in the encapsulation/adsorption and covalent attachment of enzymes into mesoporous materials were already been reviewed [21,24–27] and reported in many studies [15,16,28–32].

Our investigations mainly aimed at obtaining very active MCF-bound laccase catalysts. In order to prevent protein leakage from the support, that is often the case when encapsulation or adsorption is used [28,29,31,32], only the covalent attachment of protein was considered. Siliceous MCFs with controlled pore size were modified with organosilanes with amine or glycidyl groups. The immobilization efficiency was optimised and then the properties of carrier-enzyme preparations were determined. Finally, the performance of the catalysts obtained was tested in a model reaction of indigo carmine decolourisation without mediator.

2. Materials and methods

2.1. Materials

Trihydroxymethylaminomethane (tris), glutaraldehyde (GLA), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) sodium salt (ABTS), divinyl sulfone (DVS) and sodium borohydride were purchased from Sigma (USA). Tetraethoxysilane (98%, TEOS), 1,3,5-trimethylbenzene (TMB), 1-chloro-2,3-epoxypropane, chloroacetic acid, 3-glicydoxypropyltriethoxysilane (GPTS), 3-aminopropyltriethoxysilane (APTS), 2-aminoethyl-3-aminopropylmethyldimethoxysilane (AEAPMDS), 2-aminoethyl-3-aminopropyltrimethoxysilane (AEAPTS) were from Aldrich (USA). Pluronic PE 9400 was from BASF. Other reagents, all of the analytical grade, were supplied by POCh (Poland).

2.2. Synthesis of MCFs

The preparation of modified siliceous MCFs was described in detail earlier [15,16]. In brief Pluronic PE 9400 was dissolved in 1.6 M HCl (5.3 mM) and then TMB (0.23 M) and NH_4F (8 mM) were added. Mixture was heated and TEOS was added. Then it was stirred at 60 °C for 2 h, stored at 0 °C for 20 h and after that at 60 °C for 24 h. The precipitate obtained was filtered off, dried at room temperature and calcined at 500 °C for 8 h. Thus obtained silica was labelled MCF0 and before modification it was contacted with water vapour and dried at 200 °C. Modification of MCFs was carried out as given before [15,16]. Direct grafting of organosilanes (AEAPMDS, AEAPTS in toluene, and GPTS, APTS in hexane) to silanols present on silica surface was directed to obtain the load of amino or epoxy groups of about 1.0 or 1.5 mmol/g of silica. The properties of texture (specific surface area, S_{BET}, mesopore volume, V_p, and pore sizes, d_p) of pristine and functionalised MCFs were determined from nitrogen adsorption/desorption isotherms measured at 77 K using a Micromeritics ASAP 2000 apparatus and BJH algorithm.

2.3. Laccase production and purification

The wood-rotting fungus *C. unicolor* (Bull.ex.Fr.) Murr, No. 139, was obtained from the culture collection of the Department of Biochemistry, University of Lublin (Poland). Microorganism cultivation and laccase production was performed according to method described earlier [33]. The laccase containing culture fluid was separated from the mycelium by filtration on a steel sinter, frosted and defrosted and then microfiltered (Amicon cell with MF-Millipore Membrane Filters GSWP 09000). The permeate was used for immobilization (crude preparation) or as a feed for subsequent concentration and diafiltration on the Labscale TFF System with Pellicon XL Device (PLC-10, Millipore). The laccase preparation obtained (retentate) was precipitated with cold acetone (fluid: acetone – 1.0: 1.5 v/v), centrifuged (10,000 rpm, −2 °C, 20 min, Hettich 32R) and the precipitate was dissolved in the buffer. Depending on the culture and purification step, specific activity of the solution obtained ranged from 7000 to 167,300 U per 1 mg of protein.

2.4. Enzyme activity

Laccase activity was determined from the change of optical density in time and calculated from the initial reaction rate region. 207 mM ABTS in 0.1 M citrate–phosphate buffer, pH 5.3, was used as a substrate [34]. The enzyme activity unit (U) was defined as the amount of enzyme required to oxidize 1 μmol of ABTS to coloured products (420 nm, $\varepsilon = 36{,}000\ M^{-1}\ cm^{-1}$ [35]) per min at 30 °C.

The activity of native and immobilized enzyme was measured in a well-mixed (250 rpm) reactor at 30 °C in batch regime. The immobilized enzyme (0.4 Ml—MCF0 and MCF7-8; 0.1 mL—MCF1-MCF6) was suspended in the buffer (pH 5.3), placed into reactor the temperature of which was maintained at 30 °C. Then, preheated ABTS solution was added (207 μM end substrate concentration) and several samples were taken from the reactor at 1-min intervals. After absorbance (420 nm) measurement the sample was returned to the reactor. The mean analytical error was less than ±2.0% (native enzyme) or ±3.3% (immobilized enzyme).

Protein concentration was determined spectrophotometrically at λ = 280 nm (Helios α, Unicam) and/or by Lowry's method (Sigma procedure P 5656) using bovine serum albumin as a standard. The mean analytical error was less than ±2.5%.

Table 1
Carriers, functionalities, activators of functional groups and pH value of the coupling mixtures.

Carrier	Organosilane	Load [mmol g^{-1}]	Functionality	S_{BET}*	Activator/pH
MCF0			–OH	620	DVS/8.2
MCF1	AEAPMDS	1.0	$(-NH_2)_2$	422	GLA/7.0
MCF2	AEAPMDS	1.5	$(-NH_2)_2$	372	GLA/7.0
MCF3	AEAPTS	1.0	$(-NH_2)_2$	440	GLA/7.0
MCF4	AEAPTS	1.5	$(-NH_2)_2$	353	GLA/7.0
MCF5	APTS	1.0	$-NH_2$	441	GLA/7.0
MCF6	APTS	1.5	$-NH_2$	418	GLA/7.0
MCF7	GPTS	1.0	–Glycidyl	370	–/8.2
MCF8	GPTS	1.5	–Glycidyl	316	–/8.2

* From [15].

2.5. Immobilization of laccase

Functionalised carrier (2–3 mL) was washed by centrifugation (7000 rpm, 10 min) with distilled water and the buffer appropriate to the kind of anchor groups. Activation of carriers and immobilization were carried out as described earlier [15]. Activators and pH value of the enzyme solution used for immobilization are given in Table 1. Briefly, after activation the carrier was suspended in laccase solution (5 mL of enzyme solution per 1 mL of the carrier). The excess protein was removed by washing (microfiltration on MF-Millipore Membrane Filters GSWP 09000) under different pH and ionic strength. All the eluates were collected and analysed for the presence of protein and activity. The amount of bound protein (activity units) was calculated from a difference between the amount used for immobilization and that washed off (protein/activity mass balance). In order to block active groups remaining on the carrier, the filtered preparation was suspended in 0.5 M Tris–HCl buffer, pH 7.8 and stored at 4 °C for 10 h. Finally, the preparations obtained were rinsed with 0.1 M citrate–phosphate buffer, pH 5.3 and activities were measured. The immobilized preparations were stored at 4 °C in the buffer and they were washed several times with the buffer shortly prior to experiments.

To find the conversion factor for expressing the results in U g^{-1} of wet carrier, 0.5 mL of sedimented immobilized preparation was microfiltered and immediately weighted together with the membrane. The averaged mass of the membrane after prefiltration with buffer was 0.315 ± 0.00404 and that of the membrane with 0.5 mL of a carrier was 0.822 ± 0.0345. Thus, the average mass of 0.5 mL of immobilized laccase was 0.507 g and hence the factor of 0.986 was used for recalculations.

Enzyme concentration in the coupling mixture was changed in the range of 0.202–4.056 mg mL^{-1} to determine its effect on immobilization efficiency. For that, the raw enzyme solution was concentrated four times by evaporation at 4 °C, prior to its use. To study the effect of purity of the enzyme used in immobilization on the activity of the immobilized enzyme, the raw laccase solution after microfiltration (specific activity 18,750 U mg^{-1}), purified (see Section 2.3) by ultrafiltration (142,700 U mg^{-1}) and additionally treated with acetone (167,300 U mg^{-1}) were used in comparative studies.

2.6. Properties of immobilized enzyme

In all cases, control experiments using the native enzyme were carried out.

To determine the properties of immobilized preparation they had to be diluted 10^3–10^4 times and thus the amount of the carrier in suspension had to be thoroughly checked in each case. It was done using the working curve for which 0.5 mL of the preparation was suspended in 9.5 mL of the buffer and, as fine particles of the catalysts allowed pipetting them like the solution, several mixtures were prepared by dilution. Absorbencies of the mixtures were measured in the range of 350–1100 nm and the wavelength 1050 nm was selected because of the linear relationship between absorbance and the amount of carrier in the range of 0.01–1.0% (v/v) (Fig. 1).