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Tyrosinase immobilized enzyme reactor: Development and evaluation

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ABSTRACT

Immobilized enzyme reactors of tyrosinase (tyr-IMERs) for use on-line in HPLC system were prepared by different procedures and then compared. The enzyme, obtained from *Agaricus bisporus*, was immobilized on epoxy-silica which was prepared using different conditions. Enzyme immobilization was conducted by both *in situ* and *in batch* techniques. The different procedures were compared in terms of protein and activity retention, IMERs activity, kinetics and stability. The influence of immobilization procedure on enzyme activity and the behavior of the IMERs against a standard inhibitor were also investigated. *In situ* immobilization on epoxy-silica, synthesized using microwave assistance, provided the best conditions to prepare tyrosinase IMERs. The tyr-IMERs were successfully tested with known and potential inhibitors of tyrosinase, and the results showed that they can be used for the screening of inhibitors of that enzyme.

1. Introduction

Tyrosinase (Tyr) catalyses the oxidation of tyrosine into dopaquinone, which is converted spontaneously into dopachrome. The enzyme is widely distributed in nature, being responsible for skin, eye, inner ear and hair melanization and browning in fruits and vegetables [1–3]. Melanin plays an important role in the protection of skin from the harmful effects of the UV radiation [4]. However, hyperpigmentation, characterized by abnormal accumulation of melanin, may also occur. This undesirable condition has motivated the search for tyrosinase inhibitors using bioassays mainly with free enzyme [5–8] and also immobilized on magnetic nanobeads for amperometric assay of the enzyme [4].

Immobilized enzymes have many practical applications. When packed into columns, the so-called immobilized enzyme reactors (IMERs) can be used on-line in chromatographic systems for enzyme inhibition screening of drug candidates. This technique has many advantages over procedures using enzymes in solution, such as the reutilization of expensive enzymes, thereby reducing costs [9,10]. There are various procedures for enzyme immobilization for IMER preparation. They depend on the nature of the chosen support and the derivatization chemistry prior to enzyme linkage. These variables may influence enzyme activity and stability and, therefore, must be optimized. Tyrosinase IMERs (tyr-IMERs) have been prepared using aminopropyl-controlled pore glass as support, previously activated with glutaraldehyde [11–14].

In this work we describe the preparation of tyr-IMERs using epoxy-silica as support, which was synthesized by two different methods. The immobilization was performed by both *in situ* and *in batch* techniques and the IMERs obtained were fully evaluated, compared, and tested with known and also with potential tyrosinase inhibitors.

2. Materials and methods

2.1. Reagents and chemicals

Fresh *Agaricus bisporus* was obtained in a local market. 3,4-Dihydroxy-L-phenylalanine (L-DOPA), bovine serum albumin (BSA), glycine, kojic acid and (3-Glycidyloxypropyl) trimethoxysilane (GPTMS) were purchased from Sigma–Aldrich. LiChrosorb Si 100 (10 μm) was purchased from Merck. All solutions were prepared using water purified in a Millipore Simplicity 185 system. All other chemicals and solvents were of analytical or HPLC grade and were used without any further purification.

2.2. Apparatus

HPLC analyses were performed using a Varian ProStar (Varian, Inc., Palo Alto, CA, USA) consisted of a pump (model ProStar 230), a

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photodiode array detector (ProStar 335), and autosampler (model ProStar 400). Spectrophotometric determinations were conducted in a Shimadzu UV-2401 PC instrument, using cuvettes of 1 cm path length. Statistical analyses, kinetics plots and nonlinear curve-fitting regression analysis were carried out using GraphPad Prism 5 software.

2.3. Tyrosinase extraction and purification

Frozen *A. bisporus* (80 g) was ground, and extracted with cold acetone (3 × 100 mL) with sonication, for 10 min in an ice bath. After filtration the residue (acetone powder; AP) was dried at room temperature for 3 h, and stored at −4 °C until use. Extraction of tyrosinase from AP was carried out as previously described [15]. The extract obtained was evaluated for enzymatic activity and protein content. Protein concentration was spectrophotometrically determined by absorbance at 280 nm, using a bovine serum albumin (BSA) analytical curve (0.1–1.0 mg/mL) [16].

2.4. Tyrosinase assay

An enzyme solution (2.5 mL) was mixed with 0.5 mM L-DOPA (2.5 mL), and the absorbance *versus* time (min), at 475 nm, was immediately read at 37 °C, for 3 min. The log phase (linear, $r > 0.99$) of the curve was selected for calculation of the reaction rate using an analytical curve of dopachrome prepared as described below. The enzymatic activity was expressed as μM of dopachrome/min and the assays were done in triplicate.

Dopachrome was obtained by oxidation of L-DOPA with excess $NaIO_4$, in phosphate buffer (pH 6.0; 10 mM). Standard solutions (25–250 μM) were then used to construct the analytical curve.

2.5. Free enzyme kinetics

Tyrosinase solution (0.8 mL of 0.7 mg protein/mL in phosphate buffer (pH 7.0; 15 mM)) was mixed with 0.8 mL of increasing concentrations of L-DOPA (0.0625–25 mM), and the reaction was monitored at 475 nm for 3 min at 37 °C. The reaction rate (μM dopachrome/min) was calculated using a dopachrome analytical curve prepared as previously described (Section 2.4). The results were statistically analyzed and used to construct a Michaelis–Menten plot. Nonlinear regression analysis was applied in order to determine the kinetic parameters K_m and V_{max}.

2.6. Epoxy-silica preparation

2.6.1. Reflux method

Lichrosorb Si 10 μm (1.5 g), previously dried for 15 h at 120 °C, and 10% GPTMS solution in toluene (1 mL) were added to dried toluene (12 mL). The mixture was refluxed for 10 h, filtered, and the residue washed with dry toluene and acetone. The epoxy-silica was then dried and stored at room temperature.

2.6.2. Microwave method

Dry Lichrosorb Si 10 μm (1.5 g) and GPTMS (0.25 mL) were added to methanol (9.75 mL), the mixture was stirred for a few minutes and the solvent was evaporated under vacuum at 60 °C. GPTMS coated silica was then heated in a domestic microwave oven (400 W) for 5 min. The epoxy-silica was then washed with methanol, acetone and ether, filtered, dried and stored at room temperature.

2.7. Tyrosinase immobilization

2.7.1. Batch method

Dry epoxy-silica (2.5 g), prepared by reflux method, was added to 25 mL of tyrosinase solution in phosphate buffer (pH 7.0; 15 mM). The mixture was stirred for 10 h at 4 °C and filtered. The solid (corresponding to 0.5 g of dry epoxy-silica) was slurry packed into an empty stainless steel column (75 mm × 4.6 mm I.D.). The column was then coupled in the HPLC system and flushed with phosphate buffer (pH 7.0; 15 mM) and then with 0.1 M glycine, for 2 h each at 0.5 mL/min. Finally, the IMER was conditioned for 2 h with the same buffer for enzymatic tests.

2.7.2. In situ method

Dry epoxy-silica (0.5 g), synthesized by reflux or by microwave, was packed into an empty stainless steel column (75 mm × 4.6 mm I.D.). The column was connected to the HPLC pump and conditioned with phosphate buffer (pH 7.0; 15 mM) for 1 h at 0.5 mL/min. Tyrosinase solution (25 mL), kept at about 4 °C in an ice-box, was then pumped through the column for 10 h at 0.5 mL/min, followed by phosphate buffer (pH 7.0; 15 mM), 0.1 M glycine solution and the phosphate buffer again (2 h each at 0.5 mL/min).

2.7.3. Determination of immobilized protein and enzymatic activity

The amount of immobilized protein in the column (mg) was calculated from the difference between protein content of the tyrosinase solution before and after immobilization. The protein binding capacity (mg protein/g epoxy-silica) of each type of immobilization procedure was calculated taking into account the amount of epoxy-silica used. Protein concentrations were determined at 280 nm, using a BSA analytical curve.

Retained tyrosinase activity (μM dopachrome/min) by the immobilization process was determined by the difference between the enzymatic activity of the tyrosinase solution prior and after immobilization. The activity removal capacity (μM dopachrome/min/g epoxy-silica) of each immobilization technique was determined taking into account the amount of epoxy-silica that contacted the tyrosinase solution during the immobilization step [17]. The activity was determined at 37 °C by the increase in the absorbance at 475 nm *versus* time, of a solution containing 50% (v/v) 2.0 mM L-DOPA and 50% (v/v) of enzyme solution.

2.7.4. Tyr-IMER kinetics and stability

To determine the kinetics parameters of the IMERs, injections of 20 μL of different L-DOPA concentrations (0.5–25 mM) were made, using phosphate buffer (pH 7.0; 15 mM) as mobile phase at 0.5 mL/min. The amount of dopachrome produced was determined by external calibration using an analytical curve of dopachrome (0.05–1 mM). The activity (μM dopachrome/min) was determined in triplicate for each L-DOPA concentration, and the Michaelis–Menten parameters, K_m and V_{max}, were calculated by nonlinear regression analysis.

All IMERs were stored at 4 °C in a 10 mM sodium azide solution when not in use. To determine the stability of the IMER, injections of 20 μL of 2 mM L-DOPA were made within 20 days. The activity of the columns was used as a stability parameter.

2.8. Tyr-IMER inhibition tests

2.8.1. Kojic acid

Solutions of 10 mM L-DOPA, containing increasing concentrations of kojic acid (0.05–5 mM), were injected in triplicate, into the column. The reduction of the area of dopachrome peak, compared to the control, was calculated as the percentage of inhibition for