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Medium engineering to enhance mushroom tyrosinase stability

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ABSTRACT

Tyrosinase could have many applications including biotransformation, bioremediation, and in biosensors. However, its application, especially at industrial scale, is seriously limited by its rapid inactivation. To increase mushroom tyrosinase (MT) stability, the enzyme was entrapped in different matrixes. Then, the structural and operational stability of the entrapped mushroom tyrosinase (EMT) were examined in one-phase-binary solutions (OPBS) obtained by mixing different amounts (0–100%) of water-miscible solvents such as acetonitrile and 2-propanol with PBS. Experiments showed MT lost all of its activity upon entrapment in the classical sol–gels and hybrid silica sol–gels, but could retain up to 25% of its activity in cross-linked polyacrylamide (CLP). These studies revealed while EMT in CLP was losing about 0.06% of its structural stability per hour in PBS, OPBS containing 2-propanol (50 or 75%) did not affect the EMT structural stability and increased its cresolase activity up to 1.5 fold during 50 days storage at 4 °C. The aquaphilicity of CLP helps EMT to function normally in OPBS and using 2-propanol (50% or more) in such media not only stopped EMT loss from the sieve structure of the CLP, but it was also beneficial to the more hydrophobic substrates. Using tyrosine as a substrate in OPBS containing 2-propanol (75%), L-DOPA production reached 4.21 ± 0.23 μM/min^{-1}.

1. Introduction

Due to its mono-oxygenase activity, tyrosinase (EC 1.14.18.1) is considered an efficient reagent for biotransformation of phenolic compounds to their corresponding *ortho*-dihydroxy derivatives [1]. Since tyrosinase is also able to oxidize *ortho*-dihydroxy compounds to their corresponding *ortho*-quinones which readily react with nucleophiles or polymerize to insoluble macromolecules, the enzyme is expected to have diverse applications in biochemical conjugation [2], bioremediation of contaminated water sources and effluents of industrial discharges, biosensing and analysis of phenolic compounds in environment [3,4]. There are numerous and inexpensive sources of tyrosinase such as edible mushroom, but it had added value that there is not a decent chemical substitute for its regio-specific *ortho*-hydroxylation activity called cresolase [5]. Unfortunately, its main drawback is its rather fast inactivation. This limitation has resulted in limited applications such as small applications like biosensors [6], where the inactivation of tyrosinase is not as serious barrier.

In a survey on different reasons for inactivation of tyrosinase, several factors were identified. One consideration is the intricate array of the coordinating ligands in the tyrosinase active site and the accessibility of the site to the solvent molecules make the structure of the enzyme vulnerable to medium components [7]. Similar to some other known examples [8,9], a proper engineering of the medium might increase the enzyme structural stability and prolong its life significantly.

To modify the interactions of the medium with the tyrosinase structure, mushroom tyrosinase (MT) was entrapped in sol–gels, hybrid sol–gels and cross-linked polyacrylamide (CLP). The monophenolase activity of the entrapped mushroom tyrosinase (EMT) was then studied in two different one-phase binary solutions (OPBS); H_2O/2-propanol and H_2O/CH_3CN. Results of these experiments are presented and discussed in terms of stability, activity and practical applications.

2. Experimental

2.1. Materials

Tetramethoxyorthosilane (TMOS, 98% purity), tetraethoxyorthosilane (TEOS, 98% purity), methyltripropoxysilane (MeTPS,

Abbreviations: Ac, acrylamide; APS, ammonium persulfate; bAc, bis-acrylamide; CLP, cross-linked polyacrylamide; DC, denaturation capacity; EMT, entrapped mushroom tyrosinase; MePAPh, 4-[(4-methylphenyl)azo]-phenol; MeTPS, methyltripropoxysilane; MT, mushroom tyrosinase; OPBS, one-phase binary solutions; PBS, phosphate buffer solution; PEG, poly ethylene glycol; Pr-TMS, propyltrimethoxysilane; TEOS, tetraethoxyorthosilane; TMOS, tetramethoxyorthosilane.

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97% purity), propyltrimethoxysilane (Pr-TMS, 97% purity), L-tyrosine and caffeic acid were purchased from Aldrich (Oakville, ON, Canada). Ammonium persulfate (APS), TEMED, acrylamide (Ac), bis-acrylamide (bAc), and Extra Pure Grade solvents were purchased from Merck™. 4-[(4-Methylphenyl)azo]-phenol (MePAPh) was prepared using a reported procedure [10]. Other chemicals used in this work were taken from the authentic samples. MT was extracted and purified from *Agaricus bisporus* [5].

2.2. Entrapment of MT in xerogels and hybrid xerogels

Preparation of xerogels and hybrid xerogels involved a 3-step process. First the silane precursor was hydrolyzed by acid or base in aqueous medium resulting in sol formation. Then to this solution of sol, an equivalent volume of phosphate buffer solution, PBS, (0.1 M, pH 7) was added and mixed thoroughly at room temperature to increase polymerization and form sol–gel. Finally, following gelation the sol–gel(s) were maintained at the desirable temperature to age until no further weight loss was observed. The final xerogels were ground to fine powders and sized through a sieve. In this research, the xerogels of TEOS and TMOS and the hybrid xerogels of TMOS + Me-TPS, TMOS + Pr-TMS, and TEOS + Pr-TMS were made according a previously reported procedure [11] except that the PBS used in the sol–gel formation step contained MT (1 mg/ml).

In addition to the sol–gel entrapped MT, xerogel adsorbed enzyme was also prepared to complement and support the entrapped MT study. To prepare xerogel adsorbed MT the protocol of Kazandjian and Klibanov was used [12]. This was modified to suit the purpose and blank xerogel of TMOS and hybrid xerogel of TMOS + Pr-TMS were employed as the adsorption matrix instead of glass beads.

2.3. MT entrapment in CLP

Entrapment of MT in CLP was carried out according to the reported procedure [13]. Ac (1.69 M), bAc (20.3 mM), TEMED (6.6 mM), and MT (20 mg) were mixed in 20 ml PBS (0.01 M, pH = 6.8) at room temperature. The mixture was degassed and APS (2.2 mM) was added. The reaction mixture was transferred into a conventional electrophoresis frame to solidify. The resulting gel was then cut into disks with the average weight of 1.265 g containing about 0.32 ± 0.03 mg MT. These disks were transferred into solutions containing PBS and different amounts (0, 25, 50, 100%, v/v) of the desired co-solvent and maintained at 4 °C.

2.4. Simultaneous entrapment of PEG and MT in the matrix

The desired amounts of MT and poly ethylene glycol (PEG) molecules were mixed in PBS on a shaker at 4 °C for 5 or 45 min. This mixture was used as a part of the buffer solution during the synthesis of sol–gels or the above mentioned method. Three different types of PEG, 0.6, 3, and 8 kDa were at various concentrations, up to 10 mg/ml, were used in this study.

2.5. Enzyme activity assays and kinetic studies

All the enzymatic reactions were run in 3 ml of the desired solutions in a conventional quartz cuvette (1 cm path and 4 ml volume) thermostated at 20 ± 0.1 °C. The rates of the reactions were monitored spectrophotometrically using a Jena (UV-210) spectrophotometer through the depletion of the substrates at their λ_{max}(s) as described previously [14]. Cresolase and catecholase activities were studied separately in the presence of MePAPh (λ_{max} = 352 nm) and caffeic acid (λ_{max} = 311 nm), respectively. To run the reactions under identical condition, a constant amount of enzyme, 3 disks of EMT in 3 ml of the reaction mixture, were used in the experiments. Concentrations different from this have been described in the captions. Using this method, the Michaelis–Menten constants for the desired substrates were obtained from the corresponding kinetic data using Lineweaver–Burk method. Routine cresolase assays were carried out in the presence of a constant amount of MePAPh (50 μM) at 20 °C. All the results presented in this article are the averages of at least triplicate measurements.

2.6. Measuring activity of MT in the absence and presence of PEG in OPBS

A stock solution (3 mg/ml) of MT in PBS (0.01 M, pH 6.8) was made and divided into 2 parts. The cresolase activity of 50 μl/ml of the first part was assayed in OPBS containing (0–60% either 2-propanol or CH_3CN). To do this, 50 μl of MT solution was put into a quartz cell followed by addition of the required amount of PBS and finally the desired amount of the co-solvent was also added. The resulting mixture was shaken for 45 min at 4 °C. Finally a fixed amount of substrate was added to this mixture and the reaction was followed spectrophotometrically.

To the second part of the MT stock solution 10 mg/ml of PEG (0.6, 3, and 8 kDa) was added and placed on a shaker (60 rpm) overnight at 4 °C. The cresolase activity of 50 μl of this mixture was assayed in OPBS containing (0–60% either 2-propanol or CH_3CN) following the aforementioned procedure. A similar procedure was employed for studying the effect of glycerin.

2.7. L-DOPA production

The rate of L-tyrosine conversion to L-DOPA by EMT in OPBS containing 75% 2-propanol was measured according to El-Bayoumi and Frieden [15]. An excess amount of ascorbic acid (100 μM) was added to a mixture of L-tyrosine (10 μM) and EMT in the reaction medium at 20 °C. The rate of conversion was measured spectrophotometrically through the depletion of ascorbic acid at 275 nm. A value of 7600 M^{-1} cm^{-1} was determined as the extinction coefficient of ascorbic acid in this medium.

3. Results and discussion

Although water is often considered as the best solvent for enzymatic reactions, it is able to affect enzymes in different ways and cause inactivation [16]. If the electrostatic pressure of the bulk aqueous medium causes a disruption in the specific arrangement of copper ions in the active site of tyrosinase, entrapment in a proper matrix can be the first step of medium engineering to provide the enzyme with a non-destructive micro-environment. This micro-environment is under control of the matrix structure, solvent molecules and additives [17]. As a first step, MT entrapment in silica sol–gels was examined. These materials have promising capabilities and are anticipated be used for more applications in biomolecule encapsulation [9,18].

3.1. MT entrapment in sol–gels

MT was first entrapped in TEOS and TMOS sol–gels and maintained at 4 °C to age. When aging was completed (in 2–3 weeks) the EMT was assayed. Assaying experiments revealed that EMT had no activity. Apparently the entrapped enzyme had lost almost all of its activity during the process while the free MT kept under similar conditions still showed considerable activity.

The alcohols released during the sol formation could not cause the observed inactivation as previous studies have shown reversible inactivation of MT in methanol, ethanol, and 2-propanol (data is not shown). In addition, removing the alcohols before