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Efficient decolorization of an anthraquinone dye by recombinant dye-decolorizing peroxidase (rDyP) immobilized in silica-based mesocellular foam

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ABSTRACT

A recombinant dye-decolorizing peroxidase (rDyP) produced from *Aspergillus oryzae* was immobilized in synthesized silica-based mesocellular foam (MCF: average pore size 25 nm) and used for decolorization of the anthraquinone dye, Remazol Brilliant Blue R (RBBR). The adsorption yields of rDyP immobilized in MCF increased as the pH decreased from 6 to 3. However, the activity yields of the immobilized rDyP decreased with decreasing pH. The overall efficiency, defined as adsorption yield × activity yield, reached its maximum of 83% at pH 5. In repeated dye-decolorization tests, 20 batches of RBBR could be decolorized by the MCF-immobilized rDyP. MCF showed significantly better performance for rDyP immobilization in term of retaining enzyme activity and dye-decolorization ability compared to previous studies using other mesoporous materials.

1. Introduction

Dye-contaminated effluents from the textile, paper and leather industries are known to cause environmental problems. Such dyes are likely to be carcinogenic and are known to have potent acute and/or chronic effects on exposed organisms [1], including strong detrimental effects on the growth of microorganisms [2,3]. Moreover, dyes are highly visible to the human eyes, and can cause aesthetic contamination even at very low concentrations. Among the 12 classes of chromogenic dyes, the most common is the azo group, which comprises nearly 70% of all textile dyestuff produced. The next most common is the anthraquinone dye group [4].

Considerable research has focused on the use of enzymes in colored wastewater treatment [5]. Previously, a novel dye-decolorizing peroxidase (DyP) was isolated from *Thanatephorus cucumeris* strain Dec 1 (former name, *Geotrichum candidum* strain Dec 1) [6]. The Dec 1 was deposited at National Institute of Technology and Evaluation, Japan with deposition number: FERM P-15348 (http://www.nbrc.nite.go.jp/npmd/e/). The *dyp* gene of strain DeC 1 was transformed to *Aspergillus oryzae* RD005, and rDyP production was enhanced more than 3000-fold [7,8].

Immobilization of this enzyme in appropriate supports that retain its enzyme activity and even enhance catalytic efficiency in repeated-batch mode has been sought because all carriers tested for immobilization of rDyP were not successful. Among the supports tested for enzyme immobilization, mesoporous materials are particularly efficient candidates because they have uniform and adjustable pore sizes, large surface areas and large pore volumes [9]. We previously succeeded in immobilizing rDyP on two mesoporous materials, FSM-16 and AlSBA-15, which have pore sizes in the range of 6–10 nm. However, the small pore size and two-dimensionality of materials resulted in mass transfer limitations and low activity yields [10].

Recently, the synthesis of silica-based mesocellular foam (MCF) and its successful application in enzyme immobilization have opened up a new era in enzymes applications [11,12]. MCFs possess 3D cage-like structures with spherical pores having diameters of 20–40 nm, interconnected by windows around 10 nm in size. The relatively large size of entrance pores and windows allows MCF to host bulky enzymes with molecular masses as high as 200 kDa [2]. Moreover, enzyme adsorption in MCFs having eight different pore sizes, revealed that the larger the pore diameter, the faster the adsorption rates. This showed that the pore diameter should be 4–5-fold larger than the enzyme diameter to allow free access of the enzyme [13]. As the longest dimension of rDyP is 6.6 nm, the pore diameter of a mesoporous host to immobilize rDyP should be around 26 nm.

Here, we report the immobilized rDyP in a synthesized MCF (pore size around 25 nm), followed by examination of its use in decolorizing RBBR as a representative anthraquinone dye.

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Additionally, this activity was compared with that of rDyP immobilized on a commercially available 3-aminopropyl functionalized silica gel (APSG) possessing a positive charge on its surface.

2. Materials and methods

2.1. Chemicals

The swelling agent, 1,3,5-trimethylbenzene (TMB), was purchased from Acros Organic (Belgium). The non-ionic triblock copolymer, poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) (Pluronic 123, molecular weight 5800, $EO_{20}PO_{70}EO_{20}$), tetraethyl orthosilicate (TEOS), ammonium fluoride (NH_4F) and 3-aminopropyl functionalized silica gel (APSG: pore diameter 6–9 nm) were purchased from Sigma–Aldrich (USA). The Remazol Brilliant Blue R (RBBR) and reactive blue 5 (RB5) were purchased from Nippon Kayaku Co. Ltd. (Tokyo, Japan). These chemical reagents were of guaranteed reagent grade.

2.2. MCF synthesis

MCF was synthesized based on the previously described method [12]. Four grams of the triblock copolymer, Pluronic 123 were dissolved in 75 ml of 1.6 M HCl, and then 3.4 ml of TMB was added dropwise. The resulting solution was heated at 40 °C with vigorous stirring for 2 h. Then, 9.2 ml of TEOS was added, the mixture was stirred for 5 min, and the solution was aged at 40 °C for 20 h under static condition. NH_4F (46 mg) in water (5 ml) was added to the solution, which was then transferred to an autoclave for aging at 100 °C for 24 h. The resulting precipitate was filtered through filter paper, washed with ethanol and water, and then air-dried. This material was extracted with alcohol and called MCF-Ex. The MCF-Ex was calcined at 550 °C in air for 6 h to remove the remaining organic fraction, and the prepared material was called MCF.

2.3. Characterization of the MCFs

Nitrogen adsorption–desorption isotherm measurements of MCF were conducted at 77 K using a Belsorp 28 SA sorptometer (Bell Japan). The specific surface area was calculated with the Brunauer–Emmett–Teller (BET) method using adsorption data in the BET region (P/P_0 = 0.0 to 0.3). The total pore volume was determined from the adsorbed amount of nitrogen at the relative pressure of P/P_0 0.95. The pore sizes and morphology of MCF were obtained using field-emission scanning transmission electron microscopy (FE-SEM) (Hitachi S-5200, Japan). Elemental analysis was conducted using CHN analyzer (YANACO, CHN Corder MT-5, Japan) to calculate the percentages of carbon and hydrogen in MCF and MCF-Ex.

2.4. Production of rDyP

The utilized rDyP was produced by a 5-day cultivation of *A. oryzae* RD005 carrying *dyp* gene isolated from strain Dec 1, in a 10-l jar fermentor using wheat bran powder as the carbon source. The rDyP was purified using the previously reported method [6]. The culture supernatant (4000 ml) was passed through gauze to remove *A. oryzae* pellets. Then, the filtered supernatant, which contained 14 U/ml of rDyP, was centrifuged at 4 °C for 30 min, and then concentrated to 85 ml by ultrafiltration with a 10-kDa membrane. The concentrated enzyme was precipitated in ammonium sulfate (70% saturation) at 4 °C, centrifuged at 4 °C for 30 min, and then dissolved in 25 mM piperazine buffer (pH 5.5). Ammonium sulfate (1.5 M) was added to 40 ml of the enzyme solution and the mixture was applied to a butyl-toyopearl column. Gradient elution was carried out from 25 mM piperazine buffer (pH 5.5) containing 1.5 M ammonium sulfate to ammonium sulfate-free 25 mM piperazine buffer (pH 5.5). The rDyP was eluted at around 0.8 M ammonium sulfate. The collected fractions were used for determination of the activity and specific activity of the produced rDyP, which had a molecular mass of 58 kDa [6], and monomer dimensions of approximately 6.2 nm × 6.6 nm × 4.8 nm [18].

2.5. Immobilization of rDyP in mesoporous materials and measurement of activity

Solutions of 10 mM citrate-buffered rDyP (initial activity of approximately 300 U/ml) at pH values of 3–6 were used for immobilization. The immobilization material (MCF-Ex, MCF or APSG: 30 mg each) was placed in a 1.5-ml microtube containing 1 ml of citrate-buffered rDyP solution (equivalent to 10,000 U rDyP per gram of each mesoporous material), and the mixture was stirred overnight at 4 °C. The mesoporous materials containing immobilized rDyP were recovered by centrifugation and the supernatant was used for determination of rDyP activity. The remaining mesoporous materials were washed twice with 10 mM citrate buffer at the same pH as used for immobilization, and resuspended in the same buffer for determination of rDyP activity. Adsorption yield (%) was defined as $((A_i - A_r)/A_i) \times 100$, where A_i is the initial rDyP activity in the buffered rDyP solution before immobilization and A_r is the residual rDyP activity in the supernatant after immobilization.

The activity of the immobilized rDyP is of critical interest for its use in dyes decolorization. Therefore, we defined activity yield as (measured rDyP activity/expected rDyP activity) × 100 (%). The expected rDyP activity was defined as $(A_i - A_r)$/concentration of the carrier. Overall efficiency (%) was defined as adsorption yield (%) × activity yield (%), thereby reflecting the overall activity performance of the immobilized rDyP.

Leaching of the immobilized rDyP from MCF was assessed by storing it in 10 mM citrate buffer at 4 °C for 10 days, with sequential daily changes of buffer. At each buffer exchange, the activities of the immobilized rDyP and the free rDyP in the supernatant were measured.

2.6. Assay of rDyP activity

To determine the activity of immobilized and free rDyP, RB5 decolorization was monitored at its maximum absorbance of 600 nm by spectrophotometer (UV-2400PC; Shimadzu, Kyoto, Japan) [6]. Citrate buffer (2920 μl, 25 mM pH 3.2), RB5 (15 μl, 25 mM) and immobilized rDyP (50 μl; containing 0.1–0.3 mg mesoporous material with immobilized rDyP) or free rDyP (50 μl) were added to a 3 ml cuvette. The reaction was initiated by the addition of 15 μl of 40 mM H_2O_2. One unit (U) of rDyP activity was defined as the amount of enzyme required to decolorize 1 μmol of RB5 in 1 min.

2.7. Repeated-batch RBBR decolorization by rDyP immobilized in MCF

Decolorization of RBBR was carried out in 1.5-ml vials containing 1 ml of 10 mM citrate-buffered solution (pHs 3 and 4), 150 mg/l (0.24 mM) RBBR and 600 U/l rDyP immobilized in MCF. The mixture was incubated at 30 °C with shaking at 100 strokes per minute (spm), and the decolorization reaction was initiated by addition of 0.24 mM of H_2O_2. When the decolorization ratio (%) reached more than 90%, the reaction mixture was centrifuged at 4 °C at 15,000 rpm for 10 min, the decolorized dye solution was withdrawn, and new dye solution was added for the next round of decolorization.