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An immobilized bifunctional xylanase on carbon-coated chitosan nanoparticles with a potential application in xylan-rich biomass bioconversion

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ABSTRACT

Immobilization technology offers many enzymatic advantages and overcomes the limitations of free enzymes. Bi- or multifunctional enzymes for industrial use have elicited much interest in recent years. The present work reported that a novel carbon nanoparticle-based supports was prepared by layer-by-layer self-assemble approach. The constructed bifunctional enzyme (ATXX) was successfully immobilized on the supports by covalent bonds. The prepared carbon-coated chitosan nanoparticles showed high binding capacity of about 289.9 mg g^{-1}-particles for ATXX. The Michaelis–Menten constants (K_m) and maximal activity (V_{max}) of immobilized ATXX were 4.83 mg ml^{-1} and 67.42 μmol min^{-1} mg^{-1}-particles (xylanase activity), as well as 6.13 mg ml^{-1} and 17.92 μmol min^{-1} mg^{-1}-particles (cellulase activity), respectively. The immobilized ATXX showed improved thermostability and storage stability compared with the free enzyme. The immobilized ATXX retained 82.6% xylanase activity after seven successive reactions. High-performance liquid chromatography (HPLC) analysis revealed that xylobiose (X2) was the main hydrolysis product released from beechwood xylan, birchwood xylan, and oat spelt xylan by immobilized ATXX. Wheat bran and wheat bran insoluble xylan could be directly hydrolyzed by immobilized ATXX, which demonstrated a potential use for xylan bioconversion to xylooligosaccharides by the immobilized ATXX.

1. Introduction

Enzymes serving as biocatalysts play key roles in many industrial fields. Endo-β-1, 4-xylanase (EC 3.2.1.8, further referred to as xylanase) is one of the important enzymes involved in the degradation of xylan, which is the most abundant natural cell wall polysaccharide after cellulose [1–3]. In past 20 years, xylanase has drawn considerable research interest mainly because of its known and potential application, such as production of xylooligosaccharides (XOs) in the food industry and conversion of agricultural by-product into reducing sugar [4–7]. These applications need enzymes to retain high activity after being used several times at drastic process conditions (high temperature, high ion concentration, acidic, or alkaline environment) and to easily recover from reaction mixtures for reuse. Immobilization technology offers enzymes many advantages and overcomes the limitations of free enzymes. Immobilized enzymes are achieved by fixing enzymes to or within solid water-insoluble supports. The immobilization methods of enzymes may be broadly classified as physical and chemical. For the latter, covalent or ionic bond is formed between the support and the enzyme. Thus, it is effective and durable [8–10].

The water-insoluble supports suitable for technical applications in immobilized enzyme should not only possess a high level of bound protein but also prevent possible separation during the reaction. Carbon nanoparticles (CNP) have received considerable attention because of their enormous potential application in nanoadditives, biotechnology, and energy storage [11,12]. Carbon

Abbreviations: ATXX, chimeric and bifunctional xylanase whose parents were ATX and TfxA; TfxA, *Thermomonospora fusca* xylanase A; AnxA, *Aspergillus niger* xylanase A; ATX, hybrid xylanase whose parents were AnxA and TfxA; CNP, carbon nanoparticles; X, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; HPLC, high performance liquid chromatography; XOs, xylooligosaccharides; K_m, Michaelis–Menten constants; V_{max}, maximal activity.

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materials have good mechanical stability and do not show cytotoxicity and genotoxicity. Therefore, they possess a distinguishing feature of carriers for enzyme immobilization. Being chemically inert, CNP cannot directly combine with an enzyme by covalent bond. To obtain reactive functional groups, CNP can be coated or modified by natural macromolecular means before being used as support for an immobilized enzyme.

The layer-by-layer (LBL) self-assemble approach has been applied to utilize nanometer- and sub micrometer-sized charged particles as core to produce supports for enzyme immobilization. The technique makes use of the alternate adsorption of oppositely charged macromolecules to build up multilayered structures. The brief procedure is as follows. First, the positive solid support is immersed into a solution of an anionic polyelectrolyte for the adsorption of a monolayer, and then it is washed. Second, the treated support is immersed into a solution of cationic polyelectrolyte for the adsorption of a monolayer, and then it is rinsed [13,14]. Chitosan has reactive hydroxyl and amino groups, and the latter makes chitosan a cationic polyelectrolyte (p*Ka* = 6.5). Acidic chitosan solution mixed with a crosslinking agent, such as glutaraldehyde, can easily form a gel that has controlled stability and durability. Chitosan, which can spontaneously combine with anionic polyelectrolyte by LBL self-assemble technique, is a superior support for enzyme immobilization compared with other polysaccharides [15–21].

In our previous study, the chimeric xylanase ATXX was constructed by fusing linker sequences (LS) and xylan binding domain (XBD) to a family 11 hybrid xylanase ATX [22]. The engineered ATXX was a bifunctional enzyme with xylanase and cellulase activity and it could bind to microcrystal cellulose. In the present study, the ATXX was immobilized on carbon-coated chitosan nanoparticles by the C=N covalent bonds. The immobilized enzymes were characterized in detail. Furthermore, the XOs released from soluble xylans (birchwood xylan, beechwood xylan, oat spelt xylan), wheat bran insoluble xylan and wheat bran by the immobilized ATXX were determined and quantified.

2. Materials and methods

2.1. Microorganism and chemicals

For ATXX production, the recombinant *Pichia pastoris* pPATXX1 strain was cultured in BMMY medium and the enzyme was purified to homogeneity [22]. Birchwood xylan, beechwood xylan, oat spelt xylan, and chitosan with a deacetyl degree of 85% were obtained from Sigma–Aldrich Chemical Company. Sodium dodecanesulfonate was from Sangon. Xylose (X) was from Merck. The standard XOs (xylobiose, X2; xylotriose, X3; xylotetraose, X4; xylopentaose, X5; xylohexaose, X6) were from Megazyme. The wheat bran insoluble xylan was provided by Dr. Shang-Wei Chen (Southern Yangtze University). All other chemicals were of analytical grade.

2.2. Immobilization of ATXX on carbon-coated chitosan nanoparticles

The carbon nanoparticles were synthesized according to chemical vapor deposition method [23]. Up to 5.0 g CNP was soaked in 50% ethanol for 6 h, and then the precipitate was washed three times by 30.0 ml deionized water each time. The CNP was added to 100 ml of sodium dodecanesulfonate solution (0.05%, w/v) and then subjected to ultrasonication at 25 °C for 20.0 min. After being placed at 4 °C for 12 h, the supernatant was removed. The precipitate was added to 30.0 ml of 0.5% chitosan in 1.0% acetic acid solution and stirred at 80 rpm at 25 °C for 12 h. The mixture was centrifuged and the free chitosan was removed. The carbon-coated chitosan nanoparticles were cross linked by 10.0 ml of 2.5% glutaraldehyde at 4 °C for 4 h. To remove excess crosslinking agent, the precipitate was washed by deionized water three times by 30.0 ml deionized water each time.

The carbon-coated chitosan nanoparticles were added to purified ATXX solution (sodium acetate–acetic acid buffer, 0.1 M, pH 5.5) and incubated in a shaking water bath at 27 °C for 4 h. The physically absorbed parts of ATXX were removed by ultrasonication. The particles were separated by centrifugation and washed by acetate buffer (pH 5.5) three times. The obtained immobilized ATXX was stored at 4 °C.

The prepared CNP and immobilized enzyme were characterized by transmission electron microscopy (TEM, JEM 2100), Fourier transform infrared spectroscopy (FTIR, Bruker Tensor 27), and thermogravimetric analysis (TGA, TA Q5000).

2.3. Activity assay of ATXX

The constructed ATXX was a bifunctional enzyme possessing xylanase and cellulose activity. The xylanase and cellulase activity were assayed with 1% birchwood xylan (w/v) and 1% CMC-Na (w/v) as substrate according to the previous study [22]. The liberation of reducing sugars was estimated by the dinitrosalicylic acid (DNS) procedure [24]. One unit of xylanase activity was defined as the amount of the enzyme that catalyzed the formation of 1.0 μmol of reducing sugar (D-xylose) from birchwood xylan in 1 min under its optimal conditions. One unit of cellulase activity was defined as the amount of the enzyme that catalyzed the formation of 1.0 μmol of reducing sugar (D-glucose) from CMC-Na in 1 min under its optimal conditions. The kinetic parameters (K_m and V_{max}) for immobilized and free ATXX were calculated from initial velocities by Lineweaver–Burk plots using birchwood xylan and CMC-Na as substrate. The concentrations of substrate were 2, 3, 4, 5, 6, 7, 8, and 10 mg ml^{-1} and the reactions were performed under the optimal conditions. Protein concentration was measured by the dye-binding method of Bradford, and bovine serum albumin (BSA) was used as the standard [25]. The activity recovery of immobilized enzyme was defined as the ratio of immobilized enzyme activity to the total enzyme activity added to the immobilization system. Triplicate measurements were performed for each assay to obtain the mean value and standard deviation.

2.4. Optimum temperature and thermostability of immobilized and free ATXX

Effect of temperature on activity of immobilized and free ATXX was measured from 30 °C to 90 °C at pH 6.0 (Mc Ilvaine's buffer). For thermal stability assay of immobilized enzyme, the immobilized ATXX was placed in Mc Ilvaine's buffer system (pH 6.0) and then underwent heat treatment from 40 °C to 90 °C for 2 min. The residual activities of xylanase and cellulase activity were determined by assaying under the optimal conditions, respectively.

2.5. Optimum pH and pH stability of immobilized and free ATXX

Effect of pH on activity of immobilized and free ATXX was measured over a range of pH 3.0–7.0 (Mc Ilvaine's buffer system) and 8.0–9.0 (0.2 M glycine, 0.2 M NaOH buffer system) at 60 °C. To determine pH stability, the immobilized ATXX was placed in various pH buffers at 25 °C for 1 h, and then the residual activities were measured at the optimal conditions.

2.6. Storage stability of immobilized and free ATXX

In order to measure storage stability, the immobilized and free ATXX were stored at 20 °C. The samples were obtained at 5 d