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The development and evaluation of β-glucosidase immobilized magnetic nanoparticles as recoverable biocatalysts

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ABSTRACT

Magnetic nanoparticle (MNP) solid core supports bound with β-glucosidase (βG) were evaluated as recyclable biocatalysts. To determine whether the relative positioning of immobilized βG to MNP surfaces impacts enzyme activity, MNPs were conjugated with βG using a glutaraldehyde cross-linker for proximal binding with different polyethylene glycol spacer-linkers (MW: 200, 400, 1000). MNP surface modifications were validated by X-ray photoelectron spectroscopy and the measurement of saturation magnetization for the bioconjugates revealed minor decreases in magnetic moments relative to core MNPs. All four bioconjugates showed similar binding efficiencies for βG, but measured increases in K_m values and decreases in V_{max} values, regardless of the spacer length, indicated that the specific activity of βG was lowered upon enzyme immobilization and spacer lengths were negligible. Despite a reduction in βG activity, immobilization conferred thermal stability under conditions in which the native enzyme is inactivated. To evaluate the recycling properties of the bioconjugates, enzyme activity measurements were conducted for ten consecutive rounds at 45 °C. The bioconjugates hydrolyzed total amounts of *p*-nitrophenyl *β*-D-glucoside comparable to a single application of unmodified βG following the third recycling round along with significant activity after ten rounds. Based on these results, the enhanced stability of immobilized βG on recoverable MNPs provides a means for reducing the amount of βG required for cellulose hydrolysis, thereby reducing costs associated with ethanol production.

1. Introduction

Rising concerns regarding the depletion of fossil fuels and the impact of these fuels on increased greenhouse gas emissions has attracted considerable attention to the development of renewable and sustainable biofuels [1–3]. Currently, the majority of commercially available biofuels consist of ethanol derived from corn and sugar cane, and biodiesel from algae, vegetable oils and animal fats [4]. Despite their promise as viable energy alternatives, however, the allocation of agricultural land resources devoted to biomass supported fuel production effectively competes with food production targeted for human and livestock sustenance. Additionally, the production of ethanol derived from feedstock and other biomass sources entails several cost and energy inefficient steps, one of the most problematic of which is the enzyme-mediated hydrolysis of cellulose [3,5,6]. Despite the natural abundance of cellulose, its hydrolysis to fermentable glucose requires several processing steps that are mediated by different classes of enzymes that need to be replenished during commercial production [7,8].

Cellulose degradation requires the synergistic enzyme activities of endoglucanases, exoglucanases, and β-glucosidases (βG) [5,9,10]. Endoglucanase cleavage of internal β-1,4 linkages disrupts the crystalline structure of cellulose to provide exposed polysaccharide chains, which are then hydrolyzed to the disaccharide cellobiose by exoglucanase. Finally, cellobiose is hydrolyzed by βG into free glucose. The efficient hydrolysis of cellulose to glucose in commercial production is limited, however, by the concentration dependent feedback inhibition of cellobiose and glucose on total cellulase activity [5,11]. To compensate for this effect and enhance the level of glucose production, βG is constantly supplemented into the reaction system. This entails a substantial cost factor that limits the full commercialization potential of cellulosic ethanol [12,13].

In an effort to lower the costs of cellulosic ethanol production, bioengineered improvements in both the catalytic properties and stabilities of cellulose-degrading enzymes have been developed [14,15]. Several different strategies have also been implemented for the recycling of βG, which results in a reduced demand for added enzyme by the removal and recovery of the cellobiose and glucose that accumulates during cellulosic hydrolysis and would otherwise contribute to feedback inhibition [16–20]. As a solid

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support for immobilized enzyme catalysis, magnetic nanoparticles (MNPs) have excellent bioseparation and biocompatibility properties, and have found wide use in diverse applications ranging from drug delivery and biosensor assay development [21,22] to biocatalysis [23] and environmental remediation [24]. Enzymes that have been successfully immobilized on MNPs and shown to retain activity include yeast alcohol dehydrogenase [25], lipase [26], glucose oxidase [27–29], xylanase [30] and numerous others [31,32].

In the present study, we have investigated the stability of βG immobilized on the surface of MNPs and evaluated the effect that tethering βG-MNP linker molecules of different lengths has on cellobiose hydrolysis. To facilitate stable conjugation with βG, the MNP surfaces were first modified using the following schemes: 1) 3-(aminopropyl)triethoxysilane (APTES) + glutaraldehyde for proximal βG surface positioning and 2) 3-(glycidyloxypropyl)trimethoxysilane (GOPMS) + three different polyethylene glycol (PEG) linkers (200, 400, 1000 MW) for more distal βG surface positioning. The four bioconjugates were then thoroughly characterized for the validation of surface modifications and shown to function as recyclable biocatalysts due to the stabilization of enzyme activity by surface immobilization and their rapid separation and recovery from the reaction media by magnetic capture.

2. Materials and methods

2.1. Materials

βG from *Aspergillus niger* was generously donated by Novozyme (Bagsvaerd, Denmark), and APTES, GOPMS, PEG (200, 400, 1000 MW), iron (II) chloride, glutaraldehyde (8%, v/v), cellobiose, cyanogen bromide, and citric acid were purchased from Sigma-Aldrich (St. Louis, MO). Hydrogen peroxide (30%, v/v) and ammonium hydroxide (29.5%, v/v) were obtained from Fisher Scientific (Pittsburgh, PA), and anhydrous methanol (ACS grade) and sodium citrate dihydrate were purchased from EMD Chemicals (Gibbstown, NJ). Phosphate-buffered saline (PBS) pH 7.4 was purchased from Invitrogen (Carlsbad, CA). The βG activity assay kit was purchased from BioAssay Systems (Hayward, CA) and the protein assay kit (BCS assay) was purchased from Thermo Scientific (Rockford, IL). Neodymium magnets (14,800 Gauss) were purchased from K & J Magnetics, Inc. (Jamison, PA). Deionized (DI) water was used in the preparation of aqueous solutions.

2.2. Synthesis of magnetic nanoparticles

MNPs were prepared by the oxidative alkaline hydrolysis of ferrous ions according to Tada et al. [33]. Briefly, an aqueous suspension of $Fe(OH)_2$ particles was prepared by adjusting 0.05 M $FeCl_2$ to pH 7.9–8.0 M with 1 M KOH and allowing precipitation to proceed for 2 h at room temperature. The black MNP precipitate which developed was concentrated by magnetic pull-down, followed by 3 washes with 50 mL of DI water, 2 washes with 50 mL of ethanol and then drying at room temperature.

2.3. Surface modifications of magnetic nanoparticles

2.3.1. APTES/glutaraldehyde modification

10 mg of MNPs were dispersed in 10 mL of 10% (v/v) APTES solution using sonication. The solution was shaken overnight at room temperature and the MNPs recovered by magnetic decantation. The MNPs were then washed 3 times with 10 mL of anhydrous methanol, resuspended in 10 mL of 8% glutaraldehyde pH 7.4, and then briefly sonicated and gently agitated for 1 h. Finally, the glutaraldehyde-modified MNPs were recovered by magnetic decantation, washed 3 times with 10 mL of 50 mM citrate buffer pH 5.0 and stored in DI water at 4 °C for enzyme conjugation. A schematic diagram illustrating MNP surface modification with APTES and glutaraldehyde is shown in Fig. 1A.

2.3.2. GOPMS/PEG modification

10 mg of MNPs were dispersed in 10 mL of 10% (v/v) GOPMS in methanol using sonication. The particles were shaken overnight at room temperature and recovered by magnetic decantation. The GOPMS modified MNPs were washed 3 times with 10 mL of anhydrous methanol and then redispersed in 9 mL of 0.1 mM NaOH pH 9.8 to activate the epoxide ring on GOPMS. 1 mL each of three different molecular weight PEG linkers (MW: 200, 400 1000) were added to the mixture, which was then shaken for 1 h at room temperature. Finally, the particles were washed 3 times with DI water and stored at 4 °C. The schematic structures for these modification are shown in Fig. 1B.

2.4. β-Glucosidase immobilization

2.4.1. APTES/glutaraldehyde modified MNPs

10 mg of the APTES/glutaraldehyde modified MNPs were dispersed in 10 mL of citrate buffer pH 5 containing 0.58 mg/mL βG and gently shaken overnight. The βG-MNP bioconjugate (βG-MNP-I) was then recovered by magnetic decantation, washed 3 times with 10 mL of citrate buffer, dispersed in 10 mL of citrate buffer and stored at 4 °C. βG surface loading was inferred by protein concentration determination of the pooled conjugation reaction supernatant and wash fractions. Protein concentration was determined by absorbance at 280 nm on a spectrophotometer (Molecular Devices).

2.4.2. GOPMS/PEG modified MNPs

10 mg of the 3 GOPMS/PEG modified MNP preparations was individually dispersed in 9 mL of 10 mM cyanogen bromide in DI water, after which 1 mL of 5.8 mg/mL βG in citrate buffer was added and the solution was gently shaken overnight. The 3 bioconjugates (βG-200-MNP-II, βG-400-MNP-II, βG-1000-MNP-II) were then collected by magnetic decantation, washed 3 times with 10 mL of citrate buffer, dispersed in 10 mL citrate buffer solution and stored at 4 °C. The amount of bound protein for each bioconjugate was determined using the same procedure described above.

2.5. Enzyme kinetic and thermal stability measurements for native βG, βG-MNP-I and pegylated βG-MNP-IIs

Native βG and bioconjugate enzyme activities were measured using *p*-nitrophenyl *β*-D-glucoside (*p*NPG) as substrate and determining the rate of *p*-nitrophenol formation for 20 min at 37 °C. For native βG activity measurements, 20 μL of 0.58 mg/mL enzyme in citrate buffer was mixed with *p*NPG reagent (8 μL of *p*NPG and 200 μL of assay buffer pH 7.0), and for the bioconjugates, 1 mg/mL of each βG-MNP preparation in citrate buffer was sonicated prior to adding 20 μL of the suspension to the *p*NPG reagent. The change in the optical density for the reactions was measured at 405 nm using a Molecular Devices Spectramax Plus 884 UV–vis spectrophotometer (Sunnyvale, CA) and activity is expressed as Units (U), where 1U corresponds to the release of 1 μmol of *p*-nitrophenol/min. The Michaelis–Menten kinetic parameters (K_m and V_m) for cellobiose hydrolysis were determined using a range of substrate concentrations (i.e. 0.625, 1.25, 2.5, 5 and 10 mM). For these assay reactions, 2 mL of cellobiose in citrate buffer was incubated in a water bath until the temperature reached 45 °C, after which 100 μL of native βG (0.58 mg/mL enzyme in citrate buffer) and each bioconjugate (1 mg/mL in citrate buffer) was added [34]. Following the initiation of hydrolysis reactions, 25 μL samples were taken at 5 min intervals for glucose measurements using a YSI 2700 biochemistry