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Research paper

DNA-guided assembly of a five-component enzyme cascade for enhanced conversion of cellulose to gluconic acid and H_2O_2

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ABSTRACT

Enzymatic fuel cells have received considerable attention because of their potential for direct conversion of abundant raw materials such as cellulose to electricity. The use of multi-enzyme cascades is particularly attractive as they offer the possibility of achieving a series of complex reactions at higher efficiencies. Here we reported the use of a DNA-guided approach to assemble a five-component enzyme cascade for direct conversion of cellulose to gluconic acid and H_2O_2. Site-specific co-localization of β-glucosidase and glucose oxidase resulted in over 11-fold improvement in H_2O_2 production from cellobiose, highlighting the benefit of substrate channeling. Although a more modest 1.5-fold improvement in H_2O_2 production was observed using a five-enzyme cascade, due to H_2O_2 inhibition on enzyme activity, these results demonstrated the possibility to enhance the production of gluconic acid and H_2O_2 directly from cellulose by DNA-guided enzyme assembly.

1. Introduction

Fuel cells are potential alternative to thermo-mechanical power generation processes by directly converting chemical fuels into electricity. Even though the technology has been around for decades, fuel cells are still not economically competitive because expensive and non-renewable noble metals are typically used as catalysts. In addition, metallic catalysts must be used at high temperatures and can be deactivated by trace amounts of impurities such as CO and sulfur in the fuels (Kim et al., 2006). On the other hand, biofuel cells (Minteer et al., 2007; Ramanavicius et al., 2008) that utilize enzymes can effectively catalyze redox reactions of abundant raw materials (e.g. glucose) to electrical energy under ambient conditions and neutral pH. In contrast to noble metals, enzymes are renewable and the cost of production can be very low, as enzymes can be economically produced easily by large-scale fermentation.

Past efforts on enzyme fuel cells have been focusing on the use of an individual enzyme to oxidize the substrate (Moehlenbrock et al., 2010). Unfortunately, the use of a single enzyme often limits the number of released electrons due to incomplete fuel oxidation. For instance, the conversion of methanol to formaldehyde by alcohol dehydrogenase releases only two of the six available electrons, while the complete oxidation of methanol to CO_2 using a sequential three-enzyme oxidation system generates six electrons for fuel-cell applications (Addo et al., 2010). In an effort to address this shortcoming, many researchers have incorporated multi-enzyme cascades for more complete oxidization of fuels that are both abundant and renewable (Liu et al., 2013; Siu et al., 2015; Sokic-Lazic et al., 2010).

Cellulose, one of the most abundant natural resources on earth, has been the focus of considerable interest as a renewable energy source (Liao et al., 2016). Although extensive research efforts have been made toward the development of glucose oxidase (GOX)-based fuel cells using glucose as the fuel (du Toit and Di Lorenzo, 2014; Korkut and Kilic, 2016; Willner et al., 1996), progress toward the use of cellulose as a substrate has been lagging. Recently, cellulose has been used as the fuel in a microbial fuel cell (MFC) for the direct electricity generation (Ren et al., 2007; Rezaei et al., 2007) using both cellulolytic and exoelectrogenic microorganisms. More importantly, it has been shown that addition of cellulases to increase the hydrolysis of cellulose can substantially improve the overall power output to a level achieved using the same amount of glucose (Rezaei et al., 2008). This result clearly indicates the importance of cellulose hydrolysis on the overall fuel cell performance.

Cellulosomes are multi-enzyme systems found in many anaerobic bacteria for efficient degradation of cellulose to glucose (Bayer et al., 2004). Endoglucanases, excoglucanases, and β-glucosidases are organized in close proximity on a scaffold via the high-affinity cohesin-dockerin interaction for synergistic digestion of cellulose (Bayer et al., 2008). Inspired by cellulosome systems, synthetic protein scaffolds based on cohesin-dockerin interaction have been developed for efficient

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enzymatic reaction (Sun and Chen, 2016; Sun et al., 2014; Tsai et al., 2009). However, truncation of protein scaffold becomes significant when scaffold sizes increase, which limits the complexity of synthetic enzymatic systems (Morais et al., 2012).

DNA is a promising scaffold for immobilization of enzymes based on its programmable hybridization rule and predictable hybridized structures (Pinheiro et al., 2011). More importantly, DNA scaffolds provide the unique benefit of site-specific enzyme organization, which could have a significant effect when the diffusion of intermediates is the determining factor for cascade enzymatic reactions (Han et al., 2012; Piperberg et al., 2009; Sun and Chen, 2016). Recently, a DNA-guided approach for enzyme fuel cell applications has been reported in which the mediator ferrocene and glucose oxidase (GOX) were coupled to a gold electrode based on DNA hybridization (Müller and Niemeyer, 2008; Piperberg et al., 2009). By simply switching the positioning of ferrocene on the DNA scaffold, a strong effect on the rate of glucose oxidation was observed. This example demonstrates the importance of using DNA to control the order of assembly for enzyme fuel cell applications.

Motivated by these examples, our goal is to demonstrate the possibility of using site-specific organization of cellulases and GOX onto a synthetic DNA template for the efficient conversion of cellulose to gluconic acid and H_2O_2 (Fig. 1). In our design, three cellulosomal components, CelA (endoglucanase), CelE (exoglucanase), and CBM (cellulose binding module), were used to first convert cellulose to the disaccharide, cellobiose, which was subsequently converted to glucose by the last cellulosomal component, BglA (β-glucosidase) and finally to gluconic acid and H_2O_2 by glucose oxidase (GOX). Although the synergistic effect of synthetic cellulosome on cellulose hydrolysis has been well documented (Tsai et al., 2013a; Tsai et al., 2013b), it was not clear whether a similar beneficial effect could be obtained by channeling glucose between BglA and GOX by DNA-guided assembly. As the current generated by GOX-based fuel cells depends on how fast H_2O_2 is generated (Piperberg et al., 2009), we focused our investigation on the effect of enzyme assembly on enhancing H_2O_2 generation.

2. Materials and methods

2.1. Protein expression and conjugation with DNA linkers

The four cellulosomal components (CelA-ELP-Halo, CBM-ELP-Halo, CelE-ELP-Halo, BglA-ELP-Halo) were constructed as reported. (Sun and Chen, 2016) All four proteins were expressed in *E. coli* BLR in TB medium supplemented with 50 μg/ml kanamycin at 37 °C. The cultures were transferred to 25 °C shaker for overnight leaky expression when the OD_{600} reached 1. Cells were harvested and resuspended in PBS for sonication. The cell debris was removed by centrifugation for 10 min at 4 °C. Two cycles of ELP purification were conducted to purify the proteins from cell lysis. 1 M Na_2SO_4 was added to protein samples to induce inverse-phase transition of ELP. The proteins samples were incubated at 37 °C for 10 min and centrifuged for 10 min in 37 °C. The supernatants were removed and the pellets containing ELP proteins were resuspended in PBS and incubated on ice for resolubilization. Centrifugation at 4 °C was used to remove insoluble proteins in the pellet and the supernatant containing ELP proteins were collected for DNA conjugation.

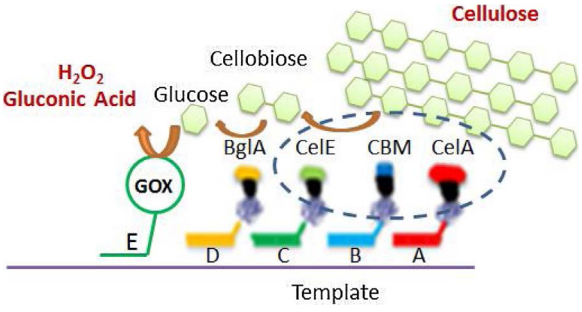

Fig. 1. Schematic of a DNA-guided five-enzyme cascade for direct conversion of cellulose to gluconic acid and H_2O_2.

The DNA linkers were ordered with a 5′ amine group. They were first modified with a chlorohexane (CH) ligand (Promega P675A) for HaloTag attachment by mixing the CH ligand and DNA linkers at a molar ratio of 30:1. The mixture was incubated at room temperature for 4 h. To purify CH-conjugated DNA and remove excessive CH ligands, a 3000 DA ultrafiltration column (Vivaspin 500, Sartorius Stedim Biotech) was used for purification. The purified DNA linkers were then mixed with ELP purified cellulase components for conjugation via HaloTag at a molar ratio of 3:1 overnight at 4 °C. The excessive DNA linkers were removed by ELP purification. 10% SDS-PAGE was used to check the conjugation efficiency.

Glucose oxidase (GOX) from *Aspergillus niger* was purchase from Sigma-Aldrich (G2133). The DNA linker for GOX was modified with a 5′ thiol group. Sulfo-EMCS (Sigma-Aldrich 803235) was used as the cross linker for conjugation. Thiolated DNA linkers at 25 μM were incubated with 25 mM DTT for 2 h at room temperature to reduce possible disulfide linkage between DNA linkers. A 3000 DA ultrafiltration column was used to purify DNA linkers. GOX at 50 μM was mixed with 5 mM sulfo-EMCS in PBS buffer (pH 7.4) at room temperature for 6 h.

A 50 kDa centrifugal column (EMD Millipore, Amicon Ultra-UFC505096) was used to remove the excessive sulfo-EMCS. The treated DNA linkers and purified GOx were mixed at 1:1 molar ratio for 2 h at room temperature. The conjugation efficiency of DNA linker to GOx was evaluated by 8% native gel running at 100 V for 1 h.

2.2. Proteins assembly onto the DNA template

The sequences of DNA template and linkers are list in Table 1 with the corresponding regions in same color coding.

To assemble DNA-conjugated proteins onto the DNA template, the proteins and DNA template were mixed at 1:1 molar ratio for 1 h at room temperature. Binding was detected by electromobility shift assays by running the samples on a 0.8% agarose gel at 90 V for 30 min.

2.3. Measurements of enzyme activities

To evaluate the H_2O_2 production rate from BglA and GOX, 50 nM enzymes were incubated with 20 mM cellobiose (equivalent to 40 mM glucose) and the H_2O_2 produced was detected kinetically at OD570 nm using the glucose oxidase activity assay kit (Sigma MAK097).

To evaluate the glucose production rate from the four cellulosomal components, 1 μM enzymes were incubated with 8 g/l phosphoric acid swollen cellulose (PASC, equivalent to 40 mM glucose). PASC was prepared from Avicel PH101 (Sigma) as previously described (Tsai et al., 2009). The glucose concentration was measured using a glucose (HK) assay kit (Sigma GAHK20) at various time points. The pH of the reaction buffer was adjusted using 100 mM citric acid-sodium citrate buffer.

To measure H_2O_2 production rate from the 5-enzyme system, 1 μM enzymes were incubated with 8 g/l PASC. The H_2O_2 concentration samples were collected periodically measured using the glucose oxidase activity assay kit.

3. Results and discussion

3.1. Protein conjugation with DNA linkers

We have previously demonstrated that CelA (endoglucanase from *Clostridium Thermocellum*), CBM (the carbohydrate binding module from *Clostridium Thermocellum*), CelE (exoglucanase from *Clostridium* Cellulolyticum), and BglA (β-glucosidase from *Clostridium*