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Improved stability and reusability of endoglucanase from *Clostridium thermocellum* by a biosilica-based auto-encapsulation method



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ABSTRACT

The functional improvement of endoglucanase (EG), a key cellulose-hydrolyzing biocatalyst, is imperative for the practical use of cellulosic materials such as lignocellulose, stove and straws. Here, we employed a bio-inspired silica-encapsulation method to improve the stability and reusability of EG. We introduced a new silica-forming peptide (SFP) from *Ectocarpus siliculosus* at the C-terminus of EG to generate a recombinant fusion protein, EG-SFP, with auto-silicifying ability. We obtained an EG-SFP-encapsulated silica matrix (EG-SFP@Silica) via the EG-SFP-mediated auto-silicification process under ambient conditions. The immobilization efficiency was 90%. The introduction of SFP did not significantly affect the functionality of EG, and moreover, EG-SFP@Silica demonstrated higher thermostability by 5 °C than free EG-SFP or EG. In addition, EG-SFP@Silica retained 90% of its initial residual activity with up to 18 uses. These results provide a platform for the development of a practical enzymatic hydrolysis process for cellulosic materials.

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1. Introduction

The production of bio-chemical or bio-fuel based on the enzymatic degradation of cellulosic materials is an important area of interest in biomass technology development [1]. Enzymatic cellulose degradation is carried out by synergistic actions of three major cellulases: endoglucanase (EG, EC 3.2.1.4), exoglucanase (EX, EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21) [2]. The enzyme-based bioconversion of cellulosic materials suggests the potential for higher yields, higher selectivity, lower energy costs, and relatively-mild operating conditions. It is therefore considered a prominent method of generating alternative energy resources based on the inexpensive, plentiful, renewable and short term carbon cycle process [3,4]. However, despite its many advantages, the enzymatic process still has limitations, specifically regarding its application in industrial settings, since long-term and reliable cellulase hydrolysis activity is required for large scale cellulose degradation. Therefore, more stable and efficient enzymatic methods should be developed for sustainable and economical bioprocessing of cellulosic materials [5].

Enzyme immobilization is an attractive method for improving the performance of enzymatic hydrolysis. The immobilization of cellulase on various support matrices such as magnetic nanoparticles, silica, polyurethane, and chitosan results in enhanced stability and reusability of cellulase [6–9]. Moreover, immobilized enzymes can be separated from a solution, thus becoming available for continuous use as a bioreactor, reducing the enzyme sensitivity to external environments, and retaining their catalytic activity. However, the conventional methods for enzyme immobilization require harsh conditions (high salt or non-physiological steps) or several chemical treatments, which result in low immobilization efficiency and decreased enzymatic activity [10].

In nature, biogenic silica (biosilica) formation occurs via silica-forming biomolecules such as proteins (e.g., silicatein A found in sponge) or peptides (e.g., silaffin R5, a widely known silica-forming peptide found in diatom) [11]. Biogenic silicification (biosilicification) is a variation of the sol-gel silica polycondensation process, which occurs under ambient conditions, and is facilitated by silica-forming proteins or peptides (SFP) [12]. Biosilicification by SFP can be applied as a bio-inspired method for appropriate encapsulation of enzymes under mild conditions [13,14]. This bio-inspired immobilization technique can overcome the limitations of chemical-based immobilization methods such as adverse effects on enzymatic performance, and lead to high loading efficiency and improved enzymatic activity [15]. In fact, silica matrices are ideally suited for enzyme immobilization, due to their high stability and

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rigidity, functionality, biocompatibility, corrosion resistance, and optical properties [16].

In the present study, to improve the stability and reusability of cellulase, we applied a biosilica-based auto-encapsulation method for an efficient immobilization of cellulase [17,18]. Thermophilic β -1,4 Endoglucanase (EG) from *Clostridium thermocellum* was selected as model cellulase [19] and EctP2 from *Ectocarpus siliculosus*, a new peptide similar to silaffin R5, was selected as a new silica-forming peptide (SFP). A recombinant fusion protein, EG-SFP, was designed to mediate the auto-encapsulation process via biosilicification by SFP and produced a cellulase-encapsulated silica matrix (EG-SFP@Silica). We investigated the immobilization efficiency, structural properties, hydrolysis activity, thermal stability, and reusability of EG-SFP@Silica compared to the free enzyme. This biosilica-based auto-encapsulation method of cellulase immobilization will be useful for the development of an efficient enzymatic hydrolysis process for cellulosic materials.

2. Materials and methods

2.1. Strains, plasmid, and chemicals

Escherichia coli BL21 (DE3) (Agilent Technologies, Santa Clara, CA, USA) was used as the host for the protein overexpression system. EctP2 peptides (10 mg) and *EctP2* genes (4 μ g, in a pUC57 vector) were synthesized by Genscript (Piscataway, NJ, USA). The pET42b+ and pETDuet-1 expression vectors were obtained from Novagen (Madison, WI, USA). Carboxymethylcellulose (CMC) sodium salt, sodium phosphate (monobasic and dibasic), glutaraldehyde solution 50 wt.% in H₂O and tetramethyl orthosilicate (TMOS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein Assay Dye Reagent Concentration were from Bio-Rad (Hercules, CA, USA) and amine-functionalized silica-bead, Sicastar[®]NH₂ 200 nm was from Micromod Partikeltechnologie GmbH (Rostock, Germany). All other reagents were of analytical grade.

2.2. Construction of expression vectors

The cDNA sequence of EG was obtained from *C. thermocellum* ATCC27405 (Genbank accession number: txid203119) and its codon-optimized sequence was synthesized by GeneScript, and was used to efficiently expression *E. coli*. The gene fragment of EG containing NdeI/XhoI was obtained and cloned into NdeI/XhoI-digested pE42b+ vector (pET-EG) as shown in Fig. 1A. The silica forming peptide (SFP) used was the 13 mer polypeptide, SSKKSGERHHRSA, from *E. siliculosus* (EctP2). The SFP peptide gene sequence was cloned into a pETDuet-1 vector (resulting plasmid: pETD-SFP). The EG gene fragment containing BamHI/PstI was obtained by polymerase chain reaction (PCR) and cloned into the BamHI/PstI-digested pETD-SFP vector to produce pETD-EG-SFP. All cloned sequences were confirmed by a sequencing service (Cosmo Genetech, Seoul, Korea).

2.3. Enzyme expression and purification

The *E. coli* BL21 (DE3) harboring pETD-EG-SFP was inoculated into 5 ml of Luria-Bertani (LB) broth containing ampicillin (50 mg/ml), and cultured at 37 °C for 12 h in shaking incubator (Vision science, Daejeon, Korea). The pre-cultured solution was transferred to a large culture up to 0.6 of optical density (OD) followed by the addition of 0.1 mM of isopropyl- β -D-thiogalactoside (IPTG), and then incubated at 20 °C overnight. After culturing, the cells were harvested by centrifugation at 10,000 \times g for 20 min at 4 °C and the supernatant was removed. The precipitated cells were frozen at -20 °C to deteriorate the cell wall and allow easy

protein extraction from the cell. The cell lysis buffer consisted of a protease inhibitor (100 \times , 10 μ l), phenylmethylsulfonyl fluoride (PMSF) (100 mM, 100 μ l), lysozyme (10 mg/ml, 100 μ l), and DNase (25 U, 10 μ l) in Tris-HCl (pH 8.0). The precipitated cells and lysis buffer were mixed and incubated at room temperature for 15 min. Bacterial suspensions were sonicated on ice using a sonicated with 20% amplitude, at 1 s intervals for 5 min. The sonicated bacterial solutions were then centrifuged at 14,000 rpm for 20 min at 4 °C and supernatants were collected. SFP-fused endoglucanase (EG-SFP) was His-tagged at its N-terminus. Therefore, the enzyme was purified by HisPur[™] Ni-resin according to the manufacturer's protocol (Thermo Fisher Scientific Inc, MA, USA). A brief description of cellulase purification is as follow; the 5 ml of slurry (Ni-resin) was equilibrated by 50 mM phosphate containing 150 mM sodium chloride (pH 8.0). The supernatant including proteins was added to the resin for binding between proteins and Ni-resin and non-his-tagged proteins washed out by 20 mM imidazole dissolved in equilibrium buffer (50 mM phosphate containing 150 mM sodium chloride, pH 8.0). The enzyme (EG) including his-tag was eluted by 250 mM imidazole dissolved in equilibrium buffer. Amicon[®] Ultra Centrifugal Filters (Merck Millipore, Darmstadt, Germany) were used to remove the remaining imidazole and change buffers. Protein expression was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein was quantified using a Bradford assay (Bio-Rad, USA). The 10 μ l of purified enzyme (EG) was added to 200 μ l of Bio-Rad Protein Assay Dye Reagent Concentration and thoroughly mixed by pipette. The protein quantification was measured using spectrophotometer of Tecan system (San Jose, CA, USA) at 595 nm.

2.4. Auto-encapsulation of SFP-fused endoglucanase

EG-SFP was immobilized inside silica-particles formed via an auto-encapsulation reaction with TMOS. The auto-encapsulation reagents were 1 M TMOS in 1 mM HCl, 0.1 M phosphate buffer (pH8.0), and 10 μ M EG-SFP to produce EG-SFP-encapsulated silica matrix (EG-SFP@Silica). The volume ratio of buffer: TMOS: EG-SFP was 8:1:1. After a 12 h reaction, the produced EG-SFP@Silica was centrifuged for 5 min at 10,000 rpm to be separated. The supernatant was removed and 1 ml of 50 mM phosphate buffer (pH 8.0) was added to the precipitates. The re-suspended mixture was then centrifuged and washed. This procedure was repeated twice. The immobilization efficiency (%) was calculated as following formul; immobilization efficiency (%) = 100 \times (the amount of the immobilized cellulase)/(the initial amount of cellulase measured before immobilization). The amount of the cellulase was estimated by a Bradford assay (Bio-Rad, USA)

2.5. Chemical immobilization of endoglucanase on silica particle

Sicastar[®]NH₂, the silica particle (200 nm) with amine group (NH₂) on the surface, was mixed with 0.2% of glutaraldehyde (GA) solution in final concentration and allowed at room temperature with continuous mixing. After 30 min, the GA-treated silica (GA-Silica) was separated by centrifugation for 5 min at 10,000 rpm. The precipitated GA-Silica was washed twice with deionized water by repeating re-suspension and centrifugation. The 100 μ l of EG was added to GA-Silica and the reaction volume was made up to 1 ml with 50 mM phosphate buffer (pH 8.0). The reaction was performed for 6 h at room temperature with continuous mixing. The precipitated EG@GA-Silica was washed twice with 50 mM phosphate buffer (pH 8.0) by repeating re-suspension and centrifugation [19].

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