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Casein-based scaffold for artificial cellulosome design

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ABSTRACT

Cellulosomal systems are known as highly efficient biocatalysts in the degradation of lignocellulosic biomass in nature, but they remain unsuitable for industrial applications. In seeking alternatives to natural cellulosomes, casein was chosen as a scaffold for cellulase clustering. Casein is recognized as an excellent substrate for microbial transglutaminase (MTG) because it contains naturally reactive glutamine and lysine residues. A substrate peptide containing an MTG-reactive lysine residue was inserted into the C-terminus of the endoglucanase Cel5A and Cel6A from *Thermobifida fusca* using genetic engineering. The engineered cellulases, EG(Cel5A) and EG(Cel6A), were conjugated onto casein in different ratios by an MTG-mediated site-specific protein crosslinking reaction. Overall, a more than two-fold increase was observed when EG(Cel5A) was conjugated onto *N*,*N*-dimethylcasein, but a small or no change was observed for EG(Cel6A).

1. Introduction

Multi-enzymatic systems called cellulosomes were identified as powerful biocatalysts for lignocellulosic conversion [1]. In general, the intrinsic disposition of different enzymes in a scaffold and their proximity to the target substrate could generate a unique synergism, which can effectively enhance cellulose degradation [2]. Although cellulosomes are efficient catalysts in nature, these natural multi-enzymatic systems are found in a restricted number of microorganisms, and the mass production and application of these systems at industrial scale remains a challenge. The rational engineering of cellulosomes, using the cohesin–dockerin interaction, enabled the creation of novel types of cellulosome systems, mixing enzymes from different organisms into one single scaffold; it is also possible to control the specific location of enzyme binding in the scaffold [3,4]. Furthermore, novel biomaterials combined with different (hemi)cellulases through bioconjugation techniques have been used to create multi-enzymatic systems (for a review see [5]). More recently, the use of HaloTag for DNA-conjugation was demonstrated to be efficient in the creation of artificial cellulosomes [6]. New discoveries in genomics and proteomics have unraveled the full protein content of cellulosomes and their organization, which has brought the development of artificial cellulosomes to new levels of understanding of these complex organizations (for a review see [7]).

Owing to its amphiphilic nature and capacity to self-organize into micelles in aqueous solution, the use of casein has been considered in several applications [8–11]. Researchers have demonstrated the use of casein as a cellulase stabilizer during the saccharification of lignocellulosic materials. The adsorption of casein onto lignocellulosic biomass could reduce irreversible cellulase adsorption [12]. Eckard and co-workers have also demonstrated the potential application of casein combined with polyethylene glycol for enzyme recycling to increase lignocellulosic ethanol yields [13].

Here, we explored a new strategy to develop artificial cellulosomes using casein as a proteinaceous scaffold. Casein has natural reactive glutamine residues for microbial transglutaminase (MTG)-mediated crosslinking of genetically modified cellulases containing lysine residues and could be of interest for the industry due to its ease of manipulation and low cost. Overall, β-casein contains 20 glutamine residues and 12 lysine residues, while DM-casein also contains 20 glutamine residues, but the majority of lysine residues were chemically modified (> 90% methylated). Three out of 20 Qs and 12 Ks in β-casein, as well as DM-casein, could be recognized by MTG as previously reported [14]. We prepared different combinations of enzyme–casein conjugates based on MTG-mediated protein crosslinking reactions with engineered cellulases, EG(Cel5A) and EG(Cel6A) from *Thermobifida fusca*, each containing an MTG-reactive lysine residue. Crystalline cellulose (Avicel) was used as a substrate to analyze the potential of casein scaffolds to enhance enzymatic saccharification and the synergistic interaction between the different cellulases in casein assemblies.

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2. Material and methods

2.1. Biomass material and enzymes

Avicel PH-101, β-casein or *N,N*-dimethylcasein (DM-casein) from bovine milk (> 90% methylated), were obtained from Sigma–Aldrich (St. Louis, MO, USA), and used without further purification. Microbial transglutaminase (MTG) was supplied by Ajinomoto Co., Ltd. (Tokyo, Japan). Beta-glucosidase from *T. fusca*, Tfu0937, was expressed and purified as previously described by Mori et al. [15]. Endoglucanases fused with a substrate peptide containing a reactive lysine residue were expressed and purified as described in the supplementary material. Control tests were done in order to assure that both endoglucanases, EG(Cel5A) and EG(Cel6A), did not possess reactive glutamine residues (Fig. S1).

2.2. Site-specific conjugation of recombinant endoglucanases, Tfu0901-CK or Tfu1074-CK to β-casein or N,N-dimethylated casein by MTG

Firstly aqueous casein dispersion (1 mg/mL) was prepared by dissolving casein in 50 mM phosphate buffer (pH 7.0). The molecular weights used for calculating the ratios of casein and endoglucanase were: 26 kDa for β-casein, 28 kDa for DM-casein, 51 kDa for EG(Cel5A) and 49 kDa for EG(Cel6A). EG(Cel5A) was mixed with β-casein or DM-casein in two different molar ratios, EG(Cel5A):DM-casein, 1:2 or 1:6; which corresponded to 1.0 or 3.3 (mg:mg) of DM-casein:EG(Cel5A), in the presence of MTG (1.0 U/mL) in 50 mM phosphate buffer (pH 7.0). One unit of MTG catalyzes the formation of 1 μmole of hydroxamate per minute from Z-Gln-Gly and hydroxylamine at pH 6.0 at 37 °C [16]. In each reaction, the total enzyme concentration was held constant at 2 μM. The MTG reaction was incubated for 1 h at 40 °C. After the incubation, *N*-ethylmaleimide was added to 1 mM to terminate the MTG reaction. To identify the conjugates, a total of 15 μg protein samples were mixed with the standard sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (12 vol% 2-mercaptoethanol, 4 wt% SDS, 20 vol% glycerol in 100 mM Tris-HCl, pH 6.8) and separated on a 3–8% gradient gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250. EG(Cel5A) and DM-casein were mixed in other ratios: DM-casein:EG(Cel5A) (mg:mg) 0.1, 0.2, 1.0, 1.6, 3.3 and 6.6. EG(Cel6A) was mixed with DM-casein in the selected molar ratio of 1:6, (EG[Cel6A]:DM-casein), or 3.3 (mg:mg) of DM-casein:EG(Cel6A). Both enzymes EG(Cel5A) and EG(Cel6A) were combined with DM-casein, in the same ratio of 3.3, DM-casein:(EG[Cel5A] + EG[Cel6A]). A ratio of 1:1 was used when EG(Cel5A) and EG(Cel6A) were mixed. The degree of conjugation was calculated based on the band intensity using ImageJ software [17].

2.3. Size-exclusion chromatography analysis

The size exclusion chromatography analysis was performed with a Bio-Logic, Duo-Flow chromatography system containing a QuadTec UV–vis detector (Bio-Rad, USA). The Superdex 200 Increase 10/300 GL column (GE Healthcare Life Sciences, UK), providing a wide fractionation range from 10 to 600 kDa, was used (Fig. S2). The buffer solution, 100 mM Tris- HCl (pH 8.0), was used as mobile phase with a flow rate of 0.75 mL/min. Samples of 50 μL were injected for UV detection. The molecular weight distributions of samples were estimated using a Gel Filtration LMW or HMW Kit (GE Healthcare Life Sciences).

2.4. Enzymatic hydrolysis of crystalline cellulose

The hydrolysis experiments were carried out with 0.5 wt% of Avicel in 1 mL of 50 mM sodium phosphate buffer (pH 7.0). The enzyme concentration was held constant at 100 nM for both total cellulase and total beta-glucosidase in each reaction tube. A ratio of 1:1 was used when two cellulases were mixed. All reactions were carried out at 50 °C with stirring (1000 rpm) for 48 h. A dinitrosalicylic acid (DNS) assay was performed to measure the reducing ends of cellulose after the enzymatic reaction [18]. At the end of the enzyme reaction, 100 μL of reagent containing 1.3 M 3,5-dinitrosalicylic acid, 1 M potassium sodium tartrate, and 0.4 N NaOH was added to 100 μL of the reaction mixture containing substrate and incubated at 99 °C for 5 min to label the reducing ends of the hydrolyzed cellulose. DNS assay enables the detection of reducing end produced on solid substrates, and it is appropriated to analyze the activities of the conjugated endoglucanases with Avicel. Nevertheless, with the DNS assay, the precise measurement of the amount of reducing ends in low concentration range could be difficult due to the large experimental errors. Thus, the addition of beta-glucosidase was done and the oligosaccharides produced in the reaction solution were converted to glucose, therefore detection accuracy was improved. The increase in the reducing ends concentration sufficiently reflected the catalytic activity of conjugated endoglucanases, given that the enzymatic reaction at solid-liquid interface is the rate-limiting step. Statistical analyses were done using GraphPad Prism6 (GraphPad Software, Inc., La Jolla, CA). Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons.

2.5. Calculation method of degree of synergy

The calculation of degree of synergy used the equation [19]:

$$\text{Degree of synergy} = Y_{1+2}/(\alpha Y_1 + \beta Y_2),$$

where α and β correspond to the molar ratios of the enzymes. For the binary mixture, α and β are 0.5. Y_{1+2} represents the yield of reducing sugar achieved by the two enzymes working simultaneously. Y_1 and Y_2 indicate the yields of reducing sugar achieved by each enzyme working individually.

3. Results and discussion

3.1. Casein as a potential scaffold for artificial cellulosome design

The use of naturally occurring biopolymers could be a potential strategy to produce artificial cellulosomes. Proteinaceous biopolymers have many advantages over synthetic polymers, such as biocompatibility, biodegradability and non-toxicity, and they are naturally produced or easy to produce at relatively low cost. In addition, the use of casein as a scaffold has the advantages of offering a simple and direct conjugation process for enzymes and being inexpensive, especially compared with other materials, such as synthetic polymers, streptavidin or DNA [15,20–22].

To test the potential application of casein as a scaffold for the development of artificial cellulosomes, a substrate peptide containing a lysine MTG-reactive residue was inserted into the C-terminus of the endoglucanase EG(Cel5A). Glutamine and lysine residues are extremely reactive in the casein structure, creating an excellent substrate for MTG. Two different types of casein, β-casein or DM-casein, were tested. The β-casein molecule contains reactive glutamine residues that can crosslink with the lysine residue of the modified enzyme using the MTG reaction. It also contains intrinsic reactive lysine residues, and self-crosslinking can occur in the β-casein structure by MTG addition, resulting in a casein biopolymer [23]. Conversely, DM-casein contains only reactive glutamine residues that are free to crosslink with the modified enzyme containing lysine residues, allowing control of the number of enzymes per molecule of casein. Nonetheless, self-assembly of casein molecules could occur in solution, and DM-casein–EG conjugates could be found as part of casein assembly (Fig. 1). It has already been demonstrated that casein has a strong tendency to self-assemble into casein micelles due to its amphiphilic properties, especially in a specific range of pH, including pH 7, which was used in this study [24].