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Insights into enhanced thermostability of a cellulosomal enzyme

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ABSTRACT

Improved stability of cellulosomal enzymes is of great significance in order to provide efficient degradation of cellulosic derivatives for production of biofuels. In previous reports, we created a quadruple mutant of the endoglucanase Cel8A from *Clostridium thermocellum* resulting from a combination of both random error-prone PCR and a bioinformatics-based consensus mutagenesis approach. The quadruple mutant exhibited an increased half-life of activity by 14-fold at 85 °C with no apparent loss of catalytic activity compared to the wild-type form. Connection of the wild-type enzyme to its respective cohesin partner conferred increased thermostability, but no increase was observed for the cohesin-complexed mutant enzyme. The mutant and the wild-type enzymes were integrated into divalent chimeric scaffoldins with a family 48 exoglucanase partner, and the cellulose-degradation activities of resultant designer cellulosomes were examined. Despite the heightened thermostability of the mutant as a free enzyme, its substitution for the wild-type endoglucanase within the cellulosome context failed to exhibit an improvement in overall degradation of cellulose.

1. Introduction

Degradation of cellulosic biomass into soluble sugars is a pivotal step for conversion into desired chemicals and biofuels.[1,2] Despite the extensive research that has been carried out during the past decades, conversion of cellulosic waste and/or dedicated crops still presents significant technological challenges in order to be cost effective for replacement of traditional fossil fuels.[3] One of the major obstacles is the enzyme-mediated hydrolysis of cellulose, due to its tightly packed microcrystalline organization.[4] Three major classes of enzymes are known to hydrolyze cellulose derivatives: endoglucanases that randomly cleave cellulose chains internally, exoglucanases that cleave only the termini of exposed cellulose chains producing cellobiose, and β-glucosidases that finally cleave cellobiose into glucose.[5] Nevertheless, these available enzymes in nature require very long periods of incubation and excessive enzymatic loadings to suit industrial needs, and great efforts are currently being spent in order to optimize their efficiency.[6]

One of the most efficient types of cellulase systems in nature involves their integration into an extracellular multi-enzymatic complex named the cellulosome. Cellulosomes consist of a non-catalytic 'scaffoldin' subunit that serves to integrate the various enzymes into the complex by virtue of two complementary recognition modules termed 'dockerin' and 'cohesin'.[7,8] The non-catalytic scaffoldin subunit comprises a string of repeating cohesin modules, plus a single carbohydrate-binding module (CBM). Cellulosomal enzymes contain a catalytic module and an appended dockerin module, which binds tightly to the cohesins of the scaffoldin. The incorporation of these enzymes into the scaffoldin subunit results in significant activity enhancement, suggesting that the process of the cohesin–dockerin liaison confers optimal conformational arrangement of the different catalytic units.[9]

During the course of our studies on the cellulosome systems of anaerobic thermophilic bacteria, we have advocated the development of designer cellulosomes as a possible approach for obtaining high-titer cellulase cocktails. Designer cellulosomes are composed of a chimeric structural integrating cohesin-containing component (the scaffoldin) and selected enzymes, which bear matching dockerin modules. In this approach, recombinant chimeric scaffoldins are used as artificial nanodevices allowing the control of the enzyme composition and architecture of the resultant designer cellulosome. Thus, we can produce the various components in high quantities in heterologous microbial hosts and allow them to self-assemble by virtue of divergent cohesin–dockerin interactions.[10–13] The rationale for employing such artificial enzyme complexes is that the native cellulosomes are particularly efficient degraders of crystalline cellulosic substrates, but their production in native anaerobic systems is inadequate for industrial purposes.

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The use of thermophilic enzymes for enhanced degradation of cellulosic substrates has long been proposed as an approach for achieving a cost-effective catalytic process.[14] This strategy has spawned a broad search for thermophilic cellulolytic microbes and their enzymes. Alternatively, methodologies for obtaining improved enzymes have focused on increasing the thermostability of native cellulases.[15–17] In this context, we have explored the possibility of improving enzyme performance by increasing the thermostability of endoglucanase Cel8A, a prominent cellulosomal enzyme from *Clostridium thermocellum*. Since this bacterium thrives at 60 °C, it is naturally thermophilic, and its enzymes are considered thermostable in their native state. We were thus interested in determining whether we could increase the performance of the Cel8A endoglucanase further by increasing its thermostability. For this purpose, we produced a series of Cel8A mutants that exhibit enhanced thermostability properties, by using either random error-prone PCR and/or a semi-rational bioinformatics-based consensus mutagenesis approach, taking advantage of the large number of known homologous protein sequences.[15] The most efficient Cel8A variant comprised a quadruple mutation (termed the QM mutant) and displayed an increased half-life (at 85 °C) 14-fold that of the wild-type enzyme.[16]

In the present work, we have employed the designer cellulosome strategy as a conceptual platform in order to examine the comparative compatibility of the mutant enzyme to act synergistically within an enzyme complex.[18] Previous studies have demonstrated that integrating dockerin-bearing enzymes into these artificial cellulosomal structures has great potential for enhanced enzymatic activity, due to the overall synergistic combination of proximity and targeting effects.[19,20] The previously characterized thermostable endoglucanase mutant (QM or herein designated 8A*) was thus integrated into various chimeric scaffoldins in order to determine whether it would exhibit enhanced synergy, compared to the wild-type Cel8A enzyme, with a Cel48 exoglucanase partner. The results indicated that, under the defined experimental conditions used herein, the enhanced thermostability of the mutant failed to provide additional improvement in the observed synergistic activity within the context of the designer cellulosome.

2. Materials and methods

2.1. Cloning

Wild-type enzymes, chimeras, and recombinant scaffoldins were cloned as described previously.[21,22] All enzyme constructs were designed to contain a His tag for the subsequent purification. PCRs were performed using ABgene Reddymix ×2 (Advanced Biotechnologies Ltd, United Kingdom), and DNA samples were purified using a HiYield gel/PCR fragment extraction kit (Real Biotech Corporation [RBC], Taiwan).

Plasmid pET28aCel8A containing the cel8A gene (NCBI accession no. AAA83521) from *C. thermocellum* ATCC 27405 was used to construct a library of various mutants as previously described in.[15] Especially, the mutant 8A* (QM) we used in this study is a combination of random error-prone PCR and bioinformatics-based approach.[16]

Scaf T, Scaf BT, and Scaf TF were assembled from a cohesin module and a CBM, which were cloned from the appropriate genomic DNAs as previously described.[23,24]

2.2. Protein expression and purification

Wild-type enzymes and chimeras were prepared as described previously.[22,23,25] Recombinant scaffoldins were purified on phosphoric acid swollen cellulose (PASC) by the previously described method.[21] The purity of each recombinant protein was tested by SDS–PAGE on 12% acrylamide gels. The concentration of the purified protein was estimated by absorbance at 280 nm, based on the known amino acid composition of the desired protein using the Protparam tool (http://www.expasy.org/tools/protparam.html). The proteins were then stored in 50% (v/v) glycerol at −20 °C.

2.3. Non-denaturing PAGE

To check the extent of interaction and determine exact equimolar ratios between the cohesin-bearing scaffoldin and dockerin-bearing enzymes, a differential mobility assay on non-denaturing gels was used. Protein samples (4–8 μg each) were added to Tris-buffered saline (TBS) (pH 7.4), supplemented with 10 mM $CaCl_2$ and 0.05% Tween 20, to a total volume of 30 μL. The tubes were incubated for 2 h at 37 °C. Sample buffer (7.5 μL in the absence of SDS) was added to 15 μL of the reaction mixture, and the samples were loaded onto non-denaturing gels (4.3% stacking gels/9% separating gels).

2.4. Affinity pull-down assays

Different complexes (each including a CBM-containing protein) were formed at desired ratios and then subjected to interaction with Avicel cellulose substrate before being separated by centrifugation. Unbound fractions were collected in the supernatant fluids. Proteins that bound to the cellulosic substrate (in the pellet) were resuspended by vortexing in SDS-containing buffer. Both unbound and bound fractions were subjected to SDS–PAGE (10% acrylamide gels).

2.5. Enzymatic activity

Degradation of the crystalline cellulosic substrate (Avicel) was assayed using enzyme concentrations of 0.5 μM in a final volume of 200 μL. The suspensions were incubated at 50 or 60 °C for 72–96 h, in the presence of 80 μL of 10% (w/v) Avicel. Samples were shaken for the duration of the incubation. Reactions were terminated by immersing the tubes in ice water, and the samples were centrifuged at maximum speed to remove the substrate. Dinitrosalicylic acid (DNS) solution (150 μL) was added to 100 μL supernatant fluids,[26] and the reaction mixture was boiled for 10 min. Optical density was measured at 540 nm, and activity was determined from a glucose standard curve. The blank was determined as the optical density obtained by an enzyme-free control. All assays were performed in triplicate.

2.6. Thermal stability of enzymes

Enzyme thermostability was monitored in 50 mM sodium acetate buffer (pH 6.0). Enzymes were incubated at 85 °C for the desired time periods, and residual endoglucanase activity was determined. The rate of inactivation was determined by removing samples for assay of enzymatic activity. The inactivation rate constant was calculated using the equation $\log(\%\ \text{remaining activity}) = 2.303 \times K_i \times t$, where K_i is the inactivation constant and t is time.[27] The half-lives of enzyme activity were deduced from the resultant plots.

3. Results

3.1. Construction and expression of recombinant proteins

The recombinant proteins designed for use in this study are shown schematically in Figure 1. The wild-type form of