博士論文（要約）

Design of an artificial mini-cellulosome using a heterotrimer protein as a scaffold

(ヘテロ三量体タンパク質を足場とする
人工セルロソームに関する研究)

Schmitt Katharina Karin Ingrid

シュミット カタリーナ カリン イングリッド

The high demand for regenerative biofriendly energy sources has led to intensive studies on the processing of lignocellulosic biomass for commercially scale application [1]. Found in all plants, lignocellulose is responsible for the stable structure of the cell wall, consisting of a cellulose core structure, wrapped tightly in a meshwork of other plant components, predominantly, covalently interlinked hemicellulose and lignin polymers [2,3].

Cellulose, the most abundant polymer in nature, is readily available from unused non-food resources, like municipal, agriculture and forestry residues or algae and switchgrass [5,6]. It is a promising carbohydrate source for biofuel production such as ethanol, fatty acids or long-chain alkyls [7] and other feedstocks such as gluconic acid [8] or sorbitol [9]. However, the degradation of natural cellulosic material is challenging. To obtain pure cellulose the densely packed surrounding plant polymers have to be removed. These exposed crystalline microfibril structures, formed by intra- and intermolecular hydrogen bonds between interlaced cellulose strands, pose another obstacle. Nevertheless, cellulose is effectively digested by specified microorganisms utilizing a concert of glycoside hydrolases together with non-hydrolytic deconstructive enzymes such as monooxygenases [10–12].

The cellulosome, which was first characterized in *Ruminiclostridium thermocellum*, formerly annotated as *Clostridium thermocellum*, is a highly efficient cellulolytic multi-enzyme complex presented on the cell wall of the bacterium [13,14]. An environment-adapted selection of cooperating lignocellulose degrading enzymes are expressed and loaded on the central scaffold protein via interaction between dockerin domains of the enzymes and cohesins of the scaffoldin [15]. In addition to the catalytic modules, the cellulosome contains a basic carbohydrate binding module (CBM), which promotes specific binding to the substrate. The high cellulose digestion efficiency is ensured by the incorporation of distinctively active enzymes onto the scaffold, where they are favorably close to each other and also to the target substrate [16].

Researchers, developing approaches to efficiently degrade cellulosic biomass, are mimicking the cellulosome system by forming artificial multi-enzyme complexes. Catalytic active or binding proteins are assembled on small protein chains, nanospheres or DNA scaffolds by strong binding [4,17,18]. Hereby, close proximity of enzymes on a common backbone has been proven to be beneficial for cooperation and therefore improved cellulose digestion. Furthermore, it was shown that the integration of CBM domains into the cellulolytic complex plays an essential role in substrate specific targeting, resulting in enhanced degradation rates. In all cases cellulosic material was successfully deconstructed, though the approaches exhibit some drawbacks. Random protein integration on bulky nanospeheres could cause steric hindrance or unfavorable substrate binding and reaction conditions to stably build a DNA scaffold could destabilize enzyme structures, resulting in low

activities.

In this study, I constructed an artificial mini-cellulosome using a heterotrimeric protein scaffold, the proliferating cell nuclear antigen (PCNA) from *Sulfolobus solfataricus*. The PCNA, which acts naturally as a DNA sliding clamp incorporating DNA-processing enzymes [19], is composed of three subunits, PCNA1, PCNA2 and PCNA3, which self-assemble in a distinctive manner. PCNA1 and PCNA2 form an initial highly stable heterodimer (K_d = 12×10^{-6} μM^{-1}), followed by PCNA3 integration. The PCNA3 association into the heterotrimer is weak due to a lower binding affinity towards the heterodimer PCNA1 and PCNA2 ($K_d = 27 \times 10^{-2}$ μM^{-1}) [19]. Fusion to the PCNA subunits enables endoglucanase, exoglucanase and CBM to form a protein complex, in which cooperating functional components are displayed on a PCNA scaffold.

Entirely biochemical degradation of cellulosic biomass to monomeric glucose requires at least three glucosidase hydrolases: i) endoglucanases act on amorphous regions of cellulosic material to break glycosidic bonds and produce a variety of oligosaccharides, ii) cellobiohydrolases (exoglucanases) digest polysaccharides strands from the reducing or the non-reducing ends to generate cellobiose, and iii) β-glucosidase yields fermentable monomeric glucose by hydrolyzing cellobiose [20,21]. I chose cooperating cellulose-active modules from the thermophilic organisms *R. thermocellum*, which degrades symbiotically cellulosic organic material in mammalian rumen [22], and *Thermobifida fusca*, which is a soil inhabiting actinobactrium feeding on decaying organic material [23]

Before combining two enzymes and a binding module on a heterotrimeric PCNA mini-scaffold, I tested the binding behavior of a heterotrimeric (one) enzyme-CBM3 complex. An unprecedented reversible association and dissociation of the CBM with the enzyme complex was discovered by fusing CBM to the PCNA3 subunit, which has advantageous enzymatic potential comparing with that of conventional cellulosome with a tightly linked CBM and enzyme. It allowed increased mobility of the enzyme complex on the surface, improving simultaneously enzyme–substrate interaction and supporting the continuous enzyme progression.

The interaction between PCNA3 linked CBM3 and the enzyme-PCNA1∞PCNA2 heterodimer is less stable than the binding strength between PCNA1 and PCNA2. Therefore, after the complexed heterotrimer binds to the cellulose surface via the CBM domain and while the enzymatic reaction proceeds, the enzyme PCNA1∞PCNA2 heterodimer can disassemble from PCNA3-CBM3 to maintain the enzymatic mechanism driven progressivity. Because the disassembled heterodimer tends to associate with PCNA3, it “hops” to the next PCNA3-CBM3 molecule-bound site on the substrate and reforms a PCNA1∞PCNA2∞PCNA3 heterotrimer.

Compared with other mini-cellulosome designs, the complexation of enzyme and CBM linked by weak (PCNA1∞PCNA2)∞PCNA3 interactions would allow a dynamic association and dissociation of enzyme to the

substrate, which promotes both substrate proximity and free enzyme processivity. Increasing the amount of the non-catalytic CBM led to a further significant increase in degradation rates because more available substrate specific binding sites were offered on the cellulose surface by CBM

In natural cellulolytic systems, a synergistic enhancement of saccharification is accomplished by the cooperation of enzymes with complementing functions. Generally, a notable increase in hydrolysis rates is seen on crystalline cellulose, when cellobiohydrolases open up the highly packed fibers to make the substrate more accessible for endoglucanases, which show high turnover rates on amorphous regions. Endoglucanases produce new polysaccharide ends, which can be targeted by exoglucanases. Thus, I combined an endoglucanase domain with an exoglucanase domain on a PCNA1∞PCNA2 heterodimer and assembled the complete heterotrimer with PCNA3-CBM.

The assembling of two enzymes on a common heterodimer should increase the cellulolytic activity synergistically. The proximity to the substrate can enhance degradation rates further. The reversible integration of CBM was beneficial for enzymatic degradation because enzymes can move more freely according to their intrinsic mechanism. Random diffusion is prevented by a sufficient high affinity to the cellulose bound CBM, providing a beneficial substrate and enzyme–enzyme proximity. Natural enzyme compositions display a catalytic domain that is covalently linked to a CBM domain. This CBM module is required to bind specifically to the substrate and allow the active domain to align correctly on the substrate surface. In the case of processive cellulases, the catalytic domain binds a single polysaccharide strand and processively releases cellobiose. This process requires no further supportive substrate attachment. In fact, during processive degradation, CBM may hinder the progression of the enzyme because both modules are connected firmly, and CBM retains a stable attachment to the surface. Moreover, after the hydrolysis reaction terminates, the dissociation of the enzyme from the substrate surface might be hindered because of CBM-mediated cellulose binding. This action leads to a reduced enzyme processivity. In my approach, I bypassed the inseparable function of the CBM domain by integrating the enzyme and CBM domain on a PCNA scaffold, where CBM3 can reversible associate and dissociate, depending on the dynamic behavior of the attached enzyme. Therefore, the CBM can adjust to the current state of enzyme needs of promoting substrate attachment or offering improved mobility.

The combination of two enzymes and one reversibly integrated CBM3 led to an overall enhanced synergistic degradation activity, though the cellobiose ratio was slightly decreased. This result was presumably due to a reduced mechanistic processive activity caused by an altered enzyme–substrate interaction in a heterotrimeric complex.

Eventually, a cellulolytic multi-enzyme complex was constructed, which exhibited synergistic improvement of

solid, microcrystalline cellulose. The striking advantage of this degradative complex is the reversible association and dissociation of the CBM domain. Compared to a stable linkage of enzyme and substrate binding unit, the weaker interaction of these two modules in my approach lead to facilitated mobility of the enzyme unit while substrate proximity was still ensured, which improved degradation rates significantly.

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