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Enhancement of acetyl xylan esterase activity on cellulose acetate through fusion to a family 3 cellulose binding module

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ABSTRACT

The current study investigates the potential to increase the activity of a family 1 carbohydrate esterase on cellulose acetate through fusion to a family 3 carbohydrate binding module (CBM). Specifically, *Ct*CBM3 from *Clostridium thermocellum* was fused to the carboxyl terminus of the acetyl xylan esterase (AnAXE) from *Aspergillus nidulans*, and active forms of both AnAXE and AnAXE–*Ct*CBM3 were produced in *Pichia pastoris*. *Ct*CBM3 fusion had negligible impact on the thermostability or regioselectivity of AnAXE; activities towards acetylated corncob xylan, 4-methylumbelliferyl acetate, *p*-nitrophenyl acetate, and cellobiose octaacetate were also unchanged. By contrast, the activity of AnAXE–*Ct*CBM3 on cellulose acetate increased by two to four times over 24 h, with greater differences observed at earlier time points. Binding studies using microcrystalline cellulose (Avicel) and a commercial source of cellulose acetate confirmed functional production of the *Ct*CBM3 domain; affinity gel electrophoresis using acetylated xylan also verified the selectivity of *Ct*CBM3 binding to cellulose. Notably, gains in enzyme activity on cellulose acetate appeared to exceed gains in substrate binding, suggesting that fusion to *Ct*CBM3 increases functional associations between the enzyme and insoluble, high molecular weight cellulosic substrates.

1. Introduction

Cellulose is an abundant and renewable polysaccharide produced by plants as well as tunicates and certain bacteria. As a homopolymer of glucose, cellulose constitutes one of the simplest polysaccharides although the architecture of cellulose fibrils can vary depending on source and processing history [1]. Cellulose is also regarded as one of the most commercially important polysaccharides, being used in a wide range of applications including textiles, paper, and packaging materials. Moreover, the potential to generate cellulose derivatives, including cellulose acetate and other cellulose esters, allow many more applications ranging from textiles, to renewable plastics, pharmaceuticals and food products.

As reviewed by Klemm et al. [2] and more recently by Edgar and co-authors [3], the choice of substituent as well as the ability to control the regioselectivity of derivatization reactions is important to fine-tune the properties of cellulose derivatives. While chemical routes to site-specific derivatizations are feasible, the similar reactivity of hydroxyl groups within anhydroglucose units (AGUs) means that several reaction steps are required which can compromise the degree of polymerization or crystallinity of the cellulose substrate [2]. Alternatively, a combination of chemocatalytic and enzymatic reactions can be applied, which benefits from the versatility and specificity of the respective processes.

Both indirect and direct chemo-enzymatic approaches have been developed to modify cellulose surfaces. Indirect approaches include first modifying hemicelluloses, such as xyloglucan or galactoglucomannan, which then adhere to cellulose surfaces through hydrogen bonding and hydrophobic interactions [4–6]. Enzymes have also been used to directly modify the surface chemistry of cellulose microfibrils. For example, lipases can promote stereo and regioselective acylation and deacylation of carbohydrates, including cellulose [7–13].

Rather than regioselective addition of new functionalities, enzymes can be employed as a second step to selectively remove functional groups that are chemically introduced at C2, C3 and C6 positions of AGUs. In particular, carbohydrate esterases have been evaluated for this purpose. Currently, the carbohydrate active enzyme (CAZy) classification system (www.cazy.org) [14] assigns

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carbohydrate esterases (CEs) into 16 CE families. Among these, acetyl esterases and acetyl xylan esterases (AXEs) targeting polymeric or oligomeric substrates have been classified into 8 CE families, namely CE1–7, CE12 and CE16, where AXEs are found in CE1–7, and CE12, and family CE16 comprises acetyl esterases. The diversity of AXEs in particular reflects the diversity of acetyl groups present in native forms of non-cellulosic plant polysaccharides [15]. And as described in several recent studies and comprehensive reviews [16–19], AXEs from different CE families can differ in terms of their regioselectivity towards acetylated xylan. Briefly, characterizations to date indicate that esterases from families CE1, CE5 and CE6 can deacetylate mono- or di-acetylated methyl β-xylopyranoside [17], whereas family CE4 esterase target *O*-2- and *O*-3-monoacetylated xylopyranosyl residues [20] and family CE16 esterases preferentially deacetylate *O*-3 and *O*-4 positions at the non-reducing end of acetylated xylooligosaccharides [16,18].

The regioselectivity of CE1, CE4 and CE5 esterases has also been reported using cellulose acetate, and used to produce 6-*O*-acetylated cellulose, 2,6-*O*-acetylated cellulose, and 3,6-*O*-acetylated cellulose, respectively [21]. Accordingly, carbohydrate active esterases from multiple CE families could be applied to control cellulose solubility as well as de-protect specific hydroxyl positions for site-specific chemical modification. Notably however, the extent of deacetylation can depend on the degree of substitution (DS) of the cellulose substrate. For example, Altaner et al. [21,22] report an esterase isolated from a commercial cellulase preparation (Cellulase AP3, Novozymes) that is inactive on cellulose acetate with DS 2.3, but removes 5%, 30% and 60% of acetyl groups from C2 and C3 positions of more soluble forms of cellulose acetate with DS 1.8, 1.4 and 0.9, respectively.

To facilitate the hydrolysis of insoluble substrates, certain enzymes comprise substrate binding modules that increase productive interactions between the water-soluble enzyme and water-insoluble substrate [23]. Carbohydrate binding modules (CBMs) are a particularly well characterized example of substrate binding modules that can increase enzyme action on polysaccharides including cellulose [24], especially when present at low concentrations [25]. The CAZy system currently classifies CBMs into 71 families; CBMs are also grouped into three types: (1) Type A which possess a flat binding site that can bind crystalline cellulose, (2) Type B which adopt a groove architecture better suited to binding amorphous polysaccharides, and (3) Type C which bind short oligosaccharides [26].

Several studies have demonstrated the potential of CBMs to improve cellulase performance on various forms of cellulose [27–30]. Moreover, the hydrophobic cleft of type A CBMs has been harnessed to improve enzyme action on synthetic polymers. For example, Ribitsch et al. [31] demonstrate nearly 4 times improvement in PET (polyethylene terephthalate) hydrolysis by a CE5 cutinase from Thermomyces cellullosylitica upon C-terminal fusion of a type A CBM. In fact, activity enhancements were greater upon CBM fusion than fusion of a polyhydroxyalkanoate binding module. Matama et al. [32] also demonstrate the potential to increase cutinase mediated deacetylation of cellulose diacetate and cellulose triacetate fabrics through C-terminal CBM fusion.

To advance the applied potential of carbohydrate esterases to facilitate regio-selective modification of cellulose materials, the current study investigates the ability of CBM3 fusion to enhance the activity of a CE1 acetylxylan esterase from *Aspergillus nidulans* (AnAXE, AN6093.2) on commercially available cellulose acetate with DS ~2.7. A family CE1 acetyl esterase was chosen given the ability of enzymes from this family to deacetylate *O*-2 and *O*-3 positions of high molecular weight plant polysaccharides. Further, the type A CBM3 from the *Clostridium thermocellum* cellulose scaffolding protein (CipA) [33] was chosen since CBM3 is known to bind crystalline cellulose in a reversible manner [27], and was recently shown to improve cellobiohydrolase activity on cellulosic substrates over CBM1 and CBM2 selections [30]. To our knowledge, this is the first characterization of an AXE-CBM3 enzyme and study to show that the addition of *Ct*CBM3 to AnAXE improves esterase binding and activity towards cellulosic substrates.

2. Materials and methods

2.1. Materials and strains

Corncob xylan was obtained from Intatrade (Germany), whereas *p*-nitrophenyl acetate (*p*NP-acetate), *p*-nitrophenol (*p*NP), 4-methylumbelliferyl acetate (4-MUA), 4-methylumbelliferone (4MU), cellobiose octaacetate, cellulose acetate (CA; 40 weight% acetyl substitution; degree of substitution ~2.7), and crystalline cellulose (Avicel) were purchased from Sigma–Aldrich. *Pichia pastoris* SMD1168H was purchased from Invitrogen (USA), and *Escherichia coli* XL-1 was purchased from Agilent Technologies (USA). *P. pastoris* X-33 expressing an acetyl xylan esterase (AXE) from *A. nidulans* (AN6093.2; UniProt ID Q5B037) with a C-terminal His_6-tag from a methanol inducible promoter was obtained from the Fungal Genetics Stock Center (http://www.fgsc.net/).

2.2. AnAXE gene constructs

The amino acid sequence of AnAXE from *A. nidulans* and the family 3CBM from the *C. thermocellum* scaffoldin CipA were used to design the AnAXE–*Ct*CBM3 fusion construct containing a C-terminal His_6-tag (Supplemental Fig. 1). The genetic construct was synthesized and cloned into the pJ912 expression plasmid (DNA 2.0, USA), which promotes methanol induced expression and secretion of the target protein. Plasmids were transformed into *E. coli* XL-1 for storage and regeneration, and linearized with *Smi*I (Fermentas) before being transformed into *P. pastoris* SMD1168H by electroporation. *P. pastoris* transformants were selected on buffered methanol-complex agar plates (BMMY agar: 1% yeast extract, 2% peptone, 2% agar, 100 mM potassium phosphate, pH 6.0; 1.34% yeast nitrogen base without amino acids (YNB); 4×10^{-5}% biotin; 0.5% methanol), and then screened for protein expression by immuno-colony blot as previously described [34]. Integration of the plasmid into the *P. pastoris* genome was verified by colony PCR.

2.3. Recombinant protein expression

P. pastoris transformants were grown at 30 °C with shaking at 250 rpm according to the manufacturer's instructions (Invitrogen). When the cell density reached an OD_{600} ~2, cells were harvested by centrifugation at 9000 × *g* for 5 min at room temperature and suspended in buffered minimal medium containing 0.5% methanol to OD_{600} ~2. *P. pastoris* transformants that expressed wild-type AnAXE were grown at 30 °C and 250 rpm for 3 days and 0.5% methanol was added every 24 h to induce recombinant protein expression. To reduce apparent proteolysis within the linker region, *P. pastoris* cultures expressing the AnAXE–*Ct*CBM3 fusion were cultivated at 20 °C and induced by daily addition of 0.5% methanol for 5 days.

2.4. Enzyme purification

Secreted protein in *P. pastoris* culture filtrates were precipitated using 70% ammonium sulfate. Precipitated protein containing AnAXE was suspended in binding buffer (50 mM sodium phosphate (pH 7.2), 500 mM NaCl, and 20 mM imidazole) before being applied to a pre-packed HIS Trap HP 1 mL column (GE Healthcare, Sweden). AnAXE was then selectively eluted using the binding