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Lignin boosts the cellulase performance of a GH-61 enzyme from *Sporotrichum thermophile*

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ABSTRACT

An enzyme belonging to the glycoside hydrolase family 61 from the thermophilic fungus *Sporotrichum thermophile*, was functionally expressed in the methylotrophic yeast *Pichia pastoris* under the transcriptional control of the alcohol oxidase (*AOX1*) promoter. The enzyme hydrolyzed barley β-glucan, carboxymethyl cellulose, lichenan, wheat arabinoxylan and birchwood xylan showing optimal activity at pH 8 and 65 °C. A 2:1 mixture of Celluclast 1.5 L and *St*Cel61a was capable of increasing the degree of spruce conversion by 42%. The use of substrates with varying lignin content permitted the detection of a dependence of the enhancing capacity of *St*Cel61a on the radical scavenging capacity of the different lignocellulosics. In the presence of a reductant, *St*Cel61a boosted the efficiency of a mixture of purified cellulases (EGII, CBHI, β-GLUC) by 20%. The synergistic activity exhibited by *St*Cel61a and its dependence on reducing substances provide guidelines for process design towards the production of economically viable bioethanol.

1. Introduction

Lignocellulosic biomass, an abundant and renewable carbon source, has the potential to replace starch as a feedstock for the production of fuel ethanol. The most common forms of carbon found in lignocellulosic biomass are cellulose, a biopolymer of glucose, and hemicellulose, a heterogeneous polymer of hexose and pentose sugars including glucose, mannose, galactose, xylose and arabinose, whose exact composition depends on its source. Cellulose, which is the most abundant carbohydrate available in plant biomass, can be utilized in many ways, one example being its conversion to second generation (lignocellulosic) bioethanol.

Complete cellulose hydrolysis occurs enzymatically through the synergistic action of at least three types of enzymes, which are the endoglucanases (E.C. 3.2.1.4), the cellobiohydrolases or exoglucanases (E.C. 3.2.1.91) and the β-glucosidases (E.C. 3.2.1.21) (Henrissat et al., 1985). These enzymes are classified into different glycoside hydrolase (GHs) families in the continually updated Carbohydrate-Active enZymes (CAZy) database, which is a knowledge-based resource specialized in the enzymes that build and breakdown complex carbohydrates and glycoconjugates (http://www.cazy.org/).

Currently, there are more than 200 CAZy entries for GH family 61, all from eukaryotic sources, but major activities or functions of this group are still unclear. Proteins from *Aspergillus nidulans* (Bauer et al., 2006), *Aspergillus kawachii* (Koseki et al., 2008), *Hypocrea jecorina* Cel61A (Karlsson et al., 2001; Liu et al., 2006) and Cel61B (Karkehabadi et al., 2008), GH61B and GH61E from *Thielavia terrestris* (Harris et al., 2010) and PlEGL1 from *Pyrenochaeta lycopersici* (Valente et al., 2011) display weak endoglucanase activity; however, the reported activities are considerably lower than those of other endoglucanases such as Cel7B from *H. jecorina* (Karlsson et al., 2001) and AN1285.2 from *A. nidulans* (Bauer et al., 2006), suggesting that it is highly unlikely that these GH-61 proteins function as major cellulases in nature. In addition, CEL1 of *Agaricus bisporus* has been reported to show no detectable glucanase activity (Armesilla et al., 1994), while no information regarding enzymatic activity is reported for the rest of the GH-61 proteins published to date.

It has been reported that some members of this family could significantly enhance the effectiveness of common cellulases in

Abbreviations: GH, glycoside hydrolase; CAZy, Carbohydrate-Active enzymes; β-GLUC, β-glucosidase; CBH, cellobiohydrolase; EG, endo-β-glucanase; PWS, pretreated wheat straw; PS, pretreated spruce; PP, pretreated pulp; CMC, carboxy methylcellulose; HEC, hydroxyethylcellulose; HPMC, hydroxypropyl methylcellulose; ILTC, ionic liquid treated cellulose; TCEP, tris (2-carboxyethyl)phosphine; LB, Luria–Bertani; PCR, polymerase chain reaction; IMAC, immobilized metal–ion affinity chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; pI, isoelectric point; DNS, dinitrosalicylic acid; ORF, open reading frame; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

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the breakdown of complex lignocellulosic substrates (Harris et al., 2010). These enzymes by themselves showed no significant detectable hydrolytic activity on the same substrates indicating that the enhancement was unlikely to be the result of intrinsic cellulolytic activity exhibited by GH-61 family members.

The first three-dimensional structure of a GH-61 member (Cel61B from *H. jecorina*) revealed the absence of ligand-binding clefts or tunnels lined with aromatic side chains, which are typical of carbohydrate-active enzymes (Karkehabadi et al., 2008). A structure-aided sequence alignment of all GH family 61 proteins identified a highly conserved group of residues on the surface of Cel61B, a site occupying a hexacoordinately bound intramolecular nickel atom. Careful analysis did not reveal any identifiable GH active center, while structural comparison search found similarities to the fold of CBP21, a chitin-binding protein from *Serratia marcescens* that belongs to CBM family 33 (Vaaje-Kolstad et al., 2005). A second structure of a GH-61 protein from *T. terrestris* was solved, which was also devoid of conserved, closely juxtaposed acidic side chains that could serve as general proton donor and nucleophile/base in a canonical hydrolytic reaction like in case of GHs (Harris et al., 2010).

CBP21 from *S. marcescens* is able to enhance crystalline chitin turnover via a novel mechanism that involves a hydrolytic and an oxidative step (Vaaje-Kolstad et al., 2010). CBP21 liberates oxidized fragments when incubated with crystalline chitin and this activity increases dramatically in the presence of reductants such as ascorbic acid. The same group also reported the ability of another CBM33 containing protein, CelS2 from *Streptomyces coelicolor* to release oxidized cellooligosaccharides when applied on crystalline cellulose and to enhance the efficiency of cellulases in the degradation of this recalcitrant substrate (Forsberg et al., 2011). According to the authors, a similar mechanism could be proposed for GH-61 proteins that share common structural features with CBP21.

Interestingly, another study revealed that the combination of a *Thermoascus aurantiacus* GH-61 with a *Humicola insolens* cellobiose dehydrogenase resulted in cellulose cleavage, yielding a mixture that included reducing end-oxidized and non-reducing end-modified cellooligosaccharides. In addition, the authors showed a synergistic effect of both *T. terrestis* GH-61 and CDH on microcrystalline cellulose hydrolysis by *T. terrestris* canonical cellulases (Langston et al., 2011). It was hypothesized that an oxidoreductive cellulolytic system co-exists with the well-studied fungal cellulases resulting in efficient lignocellulose conversion. It is therefore obvious that further studies on the function of GH-61 proteins could be of major importance for overcoming the economic barriers to industrial biomass utilization.

The filamentous fungus *Sporotrichum thermophile* is a highly proficient decomposer of cellulose, with a specific growth rate on insoluble cellulose similar to its specific growth rate on glucose (Bhat and Maheshwari, 1987; Topakas et al., 2003). In spite of distinctly lower *S. thermophile* cellulase activity compared to that of a hyperproducing cellulase mutant of *H. jecorina*, the thermophilic fungus grew more rapidly and degraded cellulose faster than the *H. jecorina* mutant (Bhat and Maheshwari, 1987). This observation raised the question as to whether the levels of cellulases produced are of prime importance in determining the rate or extent of cellulolysis. It seems that unknown catalytic activities and mechanisms act synergistically together with the known endoglucanase, exoglucanase and β-glucosidase activities for the efficient degradation of the cellulose fraction of the plant cell wall. Recently, the full genome of *S. thermophile* has been sequenced (http://genome.jgi-psf.org/, v2.0, DOE Joint Genome Institute), which allows the development of a complementary powerful tool for enzyme discovery. Automated annotation showed a plethora of genes encoding carbohydrate-active enzymes, indicating the potential of *S. thermophile* to degrade plant cell walls. Among the GHs a number of putative family 61 hydrolases were found.

In the present paper, the successful cloning of the complete genomic DNA sequence of a *S. thermophile* GH-61 gene and its expression in the methylotrophic yeast *Pichia pastoris* is reported. The recombinant protein was characterized with respect to its action on a variety of carbohydrate polymers and its ability to enhance saccharification of lignocellulosic substrates containing varying amounts of lignin was investigated. It has been demonstrated that the presence of lignin presents a significant source of antioxidants, which probably increase the activity of GH-61 by trapping liberated oxidized fragments. Also, the addition of reductants such as tris(2-carboxyethyl)phosphine (TCEP) or lignocellulosics methanol extract resulted in a significant boost to GH-61 activity.

2. Methods

2.1. Enzymes and chemicals

VentR® DNA polymerase was purchased from New England Biolabs (Beverly, MA). Perfectprep Gel Cleanup and Nucleospin Plasmid Kits were purchased by Eppendorf (Germany) and Macherey Nagel (Germany), respectively. Zero Blunt® PCR Cloning Kit, pPICZα vectors and EasySelect™ *Pichia* Expression Kit were purchased from Invitrogen (USA) while restriction enzymes were purchased from TAKARA (Japan). Celluclast derived from *T. reesei* was purchased from Novozymes A/S (Bagsværd, Denmark), while β-glucosidase from *Aspergillus niger* (β-GLUC), cellobiohydrolase (CBHI) and cellulase (endo-β-glucanase) (EGII) from *Trichoderma longibrachiatum* were purchased from Megazyme, Ireland.

The following substrates were used in the characterization experiments: carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) were from Fluka (Buchs, Switzerland), barley β-glucan, lichenan and wheat arabinoxylan were from Megazyme (Bray, Ireland), arabic gum from Acacia Tree, hydroxypropyl methylcellulose (HPMC), laminarin, TCEP hydrochloride solution and cellulose treated with ionic liquids (ILTC) were from Sigma, birchwood xylan was from Roth and Avicel cellulose was from Merck (Darmstadt, Germany). Hydrothermal pretreatment of wheat straw (PWS) was carried out at Inbicon, Skaerbaek, Denmark. Residence time in the hydrothermal reactor averaged 12 min with the reactor temperature maintained at 190 °C by injection of steam (Thomsen et al., 2006). The solid fiber fraction was analyzed according to the procedure of Xiros et al. (2009) and was found to contain 50.2% glucan, 3.8% hemicellulose, 25.5% acid insoluble lignin and 2.8% starch, based on dry material (w/w). Spruce steam-pretreated under weak acidic conditions (PS) at 190 °C for 5 min was obtained from SEKAB E-Technology, Örnsköldsvik, Sverige. Two pre-hydrolyzed soda pulps (PP1 and PP2) made from softwood (60% spruce and 40% pine chips) and delignified to different degrees were obtained from Innventia AB, Stockholm, Sweden; PP2 was subjected to oxygen delignification to further reduce the lignin content in the pulp. Steam pretreated spruce (PS) contained 51.4% glucan, 7.0% hemicellulose, 39.7% acid insoluble lignin and 1.6% acid soluble lignin. The high kappa pulp sample (PP1) contained 79.3% glucan, 13.4% hemicellulose and 6.9% acid-insoluble lignin and 0.4% acid soluble lignin. The composition of the low kappa pulp sample (PP2) was 96.3% glucan, 2.5% hemicellulose, 1.0% acid-insoluble lignin and 0.2% acid soluble lignin. All other chemicals were of analytical grade.

2.2. Strains, vectors and media

For cloning of the GH-61 gene from *S. thermophile* ATCC 42464 *Escherichia coli* One Shot® Top10 (Invitrogen, USA) and Zero Blunt®