Bioresource Technology 146 (2013) 118–125

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of temperature on lignin-derived inhibition studied with three structurally different cellobiohydrolases

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HIGHLIGHTS

- Temperature increased inhibition arising from unproductive enzyme adsorption.
- Inhibition became evident at different temperatures for different enzymes.
- *Te*Cel7A-CBM1 was most lignin-tolerant among the studied enzymes.
- *Tr*Cel7A was least lignin-tolerant among the studied enzymes.

ARTICLE INFO

Article history:
Received 30 May 2013
Received in revised form 13 July 2013
Accepted 15 July 2013
Available online 20 July 2013

Keywords:
Enzymatic hydrolysis
Lignin
Adsorption
Cellulase
Enzyme stability

ABSTRACT

Non-productive enzyme adsorption onto lignin inhibits enzymatic hydrolysis of lignocellulosic biomass. Three cellobiohydrolases, *Trichoderma reesei* Cel7A (*Tr*Cel7A) and two engineered fusion enzymes, with distinctive modular structures and temperature stabilities were employed to study the effect of temperature on inhibition arising from non-productive cellulase adsorption. The fusion enzymes, *Te*Cel7A-CBM1 and *Te*Cel7A-CBM3, were composed of a thermostable *Talaromyces emersonii* Cel7A (*Te*Cel7A) catalytic domain fused to a carbohydrate-binding module (CBM) either from family 1 or from family 3. With all studied enzymes, increase in temperature was found to increase the inhibitory effect of supplemented lignin in the enzymatic hydrolysis of microcrystalline cellulose. However, for the different enzymes, lignin-derived inhibition emerged at different temperatures. Low binding onto lignin and thermostable structure were characteristic for the most lignin-tolerant enzyme, *Te*Cel7A-CBM1, whereas *Tr*Cel7A was most susceptible to lignin especially at elevated temperature (55 °C).

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1. Introduction

Environmental and societal concerns arising from the utilization of fossil resources have renewed interest in lignocellulose as a renewable feedstock for fuel and chemical production. Biochemical processing of lignocellulose targets at enzymatic hydrolysis of the structural polysaccharides, cellulose and hemicelluloses, to mono- and oligomeric sugars that can be converted to desired chemicals.

Some of the leading pretreatment technologies, such as steam pretreatments, preserve lignin in the material (Mosier et al., 2005). Residual lignin restricts enzymatic hydrolysis of lignocellulosic biomass and three distinct mechanisms are suggested to contribute to the lignin-derived inhibition. Firstly, lignin physically shields cellulosic fibrils from an enzymatic attack (Mooney et al., 1998). In addition, lignin adsorbs hydrolytic enzymes (Palonen et al., 2004; Sutcliffe and Saddler, 1986) and soluble lignin-derived compounds can act as enzyme inhibitors (Jing et al., 2009). Enzyme binding onto lignin is a major obstacle in lignocellulose processing; binding lowers hydrolysis yields and rates (Chernoglazov et al., 1988) and prevents enzyme recycling after a completed hydrolysis (Lu et al., 2002). Recently, Rahikainen et al. (2011) suggested that temperature-induced enzyme unfolding on lignin-rich surface causes irreversible binding and loss of activity. It was hypothesised that a rigid enzyme structure, inherently resistant to unfolding, could better withstand the presence of lignin than a less rigid protein structure (Rahikainen et al. 2011).

Trichoderma reesei Cel7A cellobiohydrolase (*Tr*Cel7A, former CBHI) is a well-characterised cellobiohydrolase with a bidomain structure. The enzyme comprises of a glycosyl hydrolase family 7 (GH7) catalytic domain (Divne et al., 1994) linked to a family-1 carbohydrate-binding module (CBM) (Kraulis et al., 1989) through a highly O-glycosylated linker peptide. *Talaromyces emersonii* Cel7A

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0960-8524/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.biortech.2013.07.069

cellobiohydrolase (*Te*Cel7A, former CBH IB) is a single-domain thermostable enzyme that does not possess a CBM (Tuohy et al., 2002). The catalytic domains of *Tr*Cel7A and *Te*Cel7A are 66% identical in their amino acid sequence (Voutilainen, 2011), but they have distinctly different thermal stabilities; *Te*Cel7A has almost 10 °C higher unfolding temperature (T_m) compared to *Tr*Cel7A (Voutilainen et al., 2008, 2010).

Different types of CBMs have been fused to the single-domain *Te*Cel7A through a linker peptide in order to improve the activity of the enzyme on cellulosic substrates (authors SNR and AK, manuscript in preparation). Two of the fusions were employed here to study the effect of enzyme structure and thermostability on lignin-derived inhibition: *Te*Cel7A-CBM1 with a family-1 CBM from *T. reesei* Cel7A (PDB accession number 1CBH) and *Te*Cel7A-CBM3 with a family-3 CBM from the cellulosomal scaffoldin subunit of *Clostridium thermocellum* (PDB accession number 1NBC). Both CBMs possess a planar (type A) substrate binding site topography with affinity to crystalline cellulose (Boraston et al., 2004), otherwise the CBM structures are very different. CBM3 is relatively large (155 amino acids) and it has a calcium ion as an integral part of the CBM structure (Tormo et al., 1996). On the contrary, CBM1 is small (36 amino acids) and the structure is stabilised by two disulphide bridges that are absent in the CBM3 structure (Kraulis et al., 1989).

The impact of temperature on lignin-binding and lignin-derived inhibition was studied using isolated lignins from steam pretreated spruce and wheat straw and the three GH7 cellobiohydrolases, *Tr*Cel7A, *Te*Cel7A-CBM1 and *Te*Cel7A-CBM3, with different modular structures and temperature stabilities.

2. Methods

2.1. Lignocellulosic materials

Hydrothermally pretreated wheat straw and steam pretreated spruce were provided by Inbicon A/S (Kalundborg, Denmark) and Lund University (Sweden), respectively. Wheat straw was cut, soaked in water containing 3 g/l of acetic acid and heated up by steam to 190 °C for 12 min (Petersen et al., 2009). Spruce chips were impregnated with 3 wt.% SO_2 and heated up by steam to 215 °C for 5 min (Stenberg et al., 1998). The pretreated substrates were washed with distilled water and stored frozen at −20 °C prior to use.

The lignin-rich enzymatic hydrolysis residues (designated as EnzHR lignins) were isolated from the hydrothermally pretreated wheat straw and steam pretreated spruce using enzymatic hydrolysis of polysaccharides and subsequent protease treatment to remove solid-bound cellulase contaminants (Rahikainen et al., 2011). The freeze-dried EnzHR lignin preparations were stored at room temperature prior to use. Microcrystalline cellulose PH-105 (Avicel) was purchased from Serva GmbH (Heidelberg, Germany).

2.2. Characterisation of the lignocellulosic materials

The pretreated lignocelluloses and the isolated EnzHR lignins were characterised for carbohydrate content, nitrogen content and specific surface area (Table 1). Carbohydrates were analysed as monosaccharides after a two-step sulphuric acid hydrolysis (Laboratory Analytical Procedure, LAP-003, National Renewable Energy Laboratory) using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Dionex ICS-3000 liquid chromatograph (Dionex Corp., Sunnyvale, CA, USA) was used for the analysis according to a previously published protocol (Tenkanen and Siika-aho, 2000) with minor modifications. Column equilibration was carried out with 15 mM NaOH and monosaccharide separation was performed by isocratic elution with water. Nitrogen contents were measured at Analytische Laboratorien Prof. Dr. H. Malissa und G. Reuter GmbH (Lindlar, Germany) with a standard method ASTM D-5291. The method is based on sample combustion and analysis of the combustion gases. Specific surface areas were determined with the Brunauer–Emmett–Teller (BET) method using a TriStar 3000 instrument (Micrometrics Instrument Corp., Norcross, GA, USA).

2.3. Enzymes and their purification

The fusion enzymes were cloned and expressed in-house in *Saccharomyces cerevisiae* (cloning and expression to be published by authors SNR and AK). In the fusions, *Te*Cel7A catalytic domain was fused to a CBM from *T. reesei* Cel7A (*Te*Cel7A-CBM1) or to a CBM from the cellulosomal scaffoldin subunit of *C. thermocellum* (*Te*Cel7A-CBM3) via a 27 amino acid linker peptide from *Tr*Cel7A. Enzyme purification was carried out with a two-step procedure employing first anion exchange chromatography (Voutilainen et al., 2010) followed by a hydrophobic interaction chromatography (HIC) step for *Te*Cel7A-CBM1 and a size exclusion chromatography (SEC) step for *Te*Cel7A-CBM3. HIC was performed with a HiTrap Phenyl FF (high substituted) column (GE Healthcare, UK) using 10 mM sodium acetate buffer (pH 5) and a decreasing 500–0 mM ammonium sulphate gradient. SEC was carried out using a HiLoad 16/60 Superdex 200 column (Amersham Biosciences, Uppsala, Sweden) and isocratic elution with 50 mM sodium acetate buffer (pH 5) containing 150 mM NaCl. *Tr*Cel7A was purified from the culture supernatant of *T. reesei egl1*$^-$, *egl2*$^-$ deletion strain as described in Rahikainen et al. (2013). Thermostable *Thermoascus aurantiacus Ta*Cel5A endoglucanase II, *Ta*Xyn10A xylanase and *Ta*-Cel3A β-glucosidase were kindly provided by Roal Oy (Rajamäki, Finland). Each of the *T. aurantiacus* enzymes was expressed in a *T. reesei cbh1*$^-$, *cbh2*$^-$, *egl1*$^-$, *egl2*$^-$ deletion strain. The better thermal stability of *T. aurantiacus* enzymes compared to most *T. reesei* enzymes enabled purification by a simple heat treatment at 60 °C (2 h, pH 6). In the heat treatment, most *T. reesei* enzymes were precipitated and could be separated by centrifugation, whereas the thermostable target enzyme remained in solution.

Table 1
Characterisation of the pretreated lignocellulosic materials and the isolated EnzHR lignins. nd = not determined.

	Glucan (wt.%)	Xylan (wt.%)	Mannan (wt.%)	Galactan (wt.%)	Total polysaccharides (wt.%)	Klason lignin (wt.%)	Nitrogen (wt.%)	BET surface area (m^2/g)
Steam pretreated spruce	57	0.3	0.1	<0.1	52	32.5[a]	<0.05	12.0
Hydrothermally pretreated wheat straw	65	4	0.2	0.1	63	26.4[b]	0.33	2.9
Spruce EnzHR lignin	7.6	<0.1	0.3	<0.1	7	nd	0.23	3.7
Wheat straw EnzHR lignin	3.3	0.4	<0.1	0.9	5	nd	1.47	4.2
Avicel (PH-105)	nd	nd	nd	nd	nd	nd	nd	1.5

[a] Data from Várnai et al. (2010).
[b] Data from the University of Copenhagen.

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