Journal of Molecular Catalysis B: Enzymatic 126 (2016) 1–9

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

EndoG: A novel multifunctional halotolerant glucanase and xylanase isolated from cow rumen

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ARTICLE INFO

Article history:
Received 24 August 2015
Received in revised form
29 December 2015
Accepted 11 January 2016
Available online 14 January 2016

Keywords:
Bioethanol
Lignocellulose saccharification
Halotolerance
Glucanase
Xylanase

ABSTRACT

Lignocellulosic materials represent a sustainable source of chemicals and fuels because of their low cost and ample supply. The current limitations on bioconversion of lignocellulosic biomass include poor enzyme stability and inhibition by secondary or final products. Here, we report the biochemical characterization of a novel, rumen metagenome-derived glucanase and xylanase, named EndoG. From the deduced amino acid sequence it was assigned to glycoside hydrolase family 5. EndoG showed similarity to non-characterized proteins, and its parental organism is likely related to the genus *Prevotella*. The 1146pb ORF encoding EndoG was over-expressed in *Escherichia coli* and the protein purified. The recombinant EndoG displayed a wide range of pH activity with a maximum at pH 5.0 and at least 65% activity at pH between 4.5 and 7.5. The enzyme was highly stable at 55 °C for 1 h, and retained 81% activity at 4 M NaCl. EndoG was also active in the presence of diverse divalent cations, detergents, EDTA, acetate, furfural, imidazolium ionic liquids, and ethanol. Glucose or cellobiose had no effect in EndoG performance. EndoG behaved as a multifunctional endo- and exo-glucanase, as well as xylanase, displaying activity on 4-methylumbelliferyl-β-D-cellobioside, *p*-nitrophenyl-β-D-cellobioside, carboxymethylcellulose (CMC), phosphoric acid swollen cellulose, avicel, xylan, lichenan and sugar cane bagasse. To evaluate its biotechnological potential, ethanologenic *E. coli* MS04 cells expressing either EndoG or a *Thermobifida fusca* β-glucosidase were co-cultured in minimal media with avicel or CMC as the sole carbon source attaining 2.0 g/L and 3.3 g/L of ethanol, respectively.

1. Introduction

Lignocellulosic materials are a sustainable feedstock for chemicals and fuels because of their relative low cost and plentiful supply. The main impediment to more widespread utilization of this important resource is the absence of low-cost technology for overcoming the recalcitrance of cellulosic biomass [1]. Lignocellulose conversion commonly involves (i) a chemical or physical pretreatment of biomass, (ii) chemical or enzymatic saccharification of pretreated biomass, and (iii) fermentation or conversion of the resulting sugars into the end products by specialized microorganisms [2].

Cellulose can be degraded into glucose by chemical or enzymatic transformations [3]. To date, enzymatic hydrolysis is the most convenient and eco-friendly method for cellulose hydrolysis resulting in appreciable sugar yields. The enzymatic hydrolysis may take place in a separate step followed by fermentation, called separate hydrolysis and fermentation (SHF) process, or it may take place together with the fermentation in a simultaneous saccharification and fermentation of sugars (SSF) process. The ultimate objective would be a one-step consolidated bioprocess (CBP) of lignocellulose, in which all of these steps occur in a single reactor, where a single microorganism or microbial consortium converts pretreated biomass into products without exogenously added enzymes [4].

Based on their catalytic action modes, there are three major types of cellulase activities: β-1,4-endoglucanase (EC 3.2.1.4), exoglucanase, including cellobiohydrolase (EC 3.2.1.91) and cellodextrinase (EC 3.2.1.74), and β-glucosidase (EC 3.2.1.21). Endoglucanases randomly cut internal sites on cellulose surfaces,

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http://dx.doi.org/10.1016/j.molcatb.2016.01.004
1381-1177/

generating new chain ends. Cellobiohydrolases and cellodextrinases act in a processive manner on the reducing or non-reducing ends of cellulose chains releasing cellobiose as their major product. β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose [5]. Cellulases are classified into different glycoside hydrolase (GH) families based on amino acid sequence [5]. The most recent nomenclature describes 133 families of GH which are organized into 14 clans as recorded in the CAZy server [6]. In addition to cellulases, endo-1,4-β-xylanases (EC 3.2.1.8), xylan 1,4-β-xylosidases (EC 3.2.1.37) and other accessory enzymes are also necessary to degrade the hemicellulose matrix.

The current limitations on the enzymatic conversion of lignocellulosic biomass include suboptimal stability and sensitivity of some cellulases to inhibitory agents and reaction byproducts [7,8]. Improving the yield and rate of cellulase-mediated hydrolysis is currently one active research field. The demand is high for new cellulases with better properties such as higher catalytic efficiency on insoluble cellulosic substrates, better temperature range, increased stability, and higher tolerance to end-product inhibition [9–11].

Continuous prospecting and bioengineering efforts should provide novel enzymes with higher specific activity and with lower susceptibility to inhibitors [12,13]. Metagenomic methodologies allow for the recovery of phylogenetic information and also of functional data directly from the DNA isolated from environmental samples [14]. For example, sequence-based metagenomic applied to the bovine rumen has revealed a cellulase-rich environment and a promising source of novel industrial cellulases [15–17]. In addition, function-based metagenomic is a cultivation independent technique that allows the isolation and recovery of enzymatic or metabolic functions of interest from the microbial community living in an ecological niche. It involves direct isolation of DNA from the environment, cloning and expression of the metagenome in a heterologous host [18].

In this work, we cloned and expressed in *Escherichia coli* a metagenomic-derived bovine rumen cellulolytic enzyme with both glucanase and xylanase capabilities. The recombinant enzyme was fully characterized. This novel cellulase was thermostable, tolerant to high concentrations of inorganic salts, imidazolium ionic liquids (ILs), inhibitors (such as furfural or acetate) and final products (glucose and cellobiose). This enzyme has potential applications in industrial biocatalysis as demonstrated by the conversion of avicel and CMC into ethanol.

2. Materials and methods

2.1. Cloning, expression of EndoG and protein purification

endoG was initially identified in one clone of a bovine rumen metagenomic library [19]. The clone containing *endoG* was fully sequenced and the predicted gene was amplified by polymerase chain reaction (PCR) with sense (5′-AGGCA**CCATGG**CTATGAAAAAGATTCTTTATTTCTTTGC-3′) and antisense (5′-ATATT**AAGCTT**TTTATCAAGCCCTTTGGCACCC-3′) primers. NcoI and HindIII sites were introduced in the sense and antisense primers, respectively (shown in bold). PCR was performed using Pfu polymerase (Thermo-Scientific, USA). To increase cloning efficiency the PCR fragments were first ligated into pBluescript SK+, then excised with NcoI and HindIII and ligated into pET28a (Novagen, USA) creating a C-terminal 6xHis Tag fusion. The native putative signal peptide on the N-terminal end was unaffected. Recombinant EndoG-6xHis, rEndoG, was expressed in *E. coli* BL21 (DE3) pLysS (Novagen, USA) in 2X YT medium (16 g/L tryptone, 10 g/L yeast extract and 5 g/L NaCl, pH 7.0). 50 mL were inoculated with 50 μL overnight culture, incubated at 37 °C until OD reached to 0.6 and induced during 4 h at 30 °C in the presence of 0.1 mM isopropyl thio-β-D-galactoside (IPTG). Cells were harvested, suspended in binding buffer (5 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8) and lysed by sonication. rEndoG was purified by affinity using a Ni-NTA resin and the hybrid protocol recommended by the manufacturer (Life Technologies, USA). Purity and integrity were checked by SDS-PAGE. Protein concentration was determined by the Bicinchoninic Acid Protein Assay Kit (Sigma–Aldrich, USA) using bovine serum albumin as standard.

2.2. Sequence and phylogenetic analyses

Signal peptide prediction was done using SignalP 4.1 server [20].

Closest homologs to EndoG were identified using NCBI BlastP [21]. Multiple sequences alignments were computed with MEGA 6 [22] with the Muscle method. Phylogenetic trees were constructed using the maximum likelihood method implemented in MEGA 6.

2.3. Enzymatic assay

Substrates 4-methylumbelliferyl-β-D-cellobioside (4-MUC) and p-nitrophenyl-β-D-cellobioside (pNPC) (Sigma–Aldrich, USA) were used for enzymatic characterization. Exoglucanase activity was determined as previously described [23] using 0.5 μg of rEndoG, and 2 mM of 4-MUC or pNPC as substrate. Reactions were carried out at 45 °C for 15 min. The release of 4-methylumbelliferone was followed by fluorescence (excitation 365 nm, emission 445 nm). The pNPC quantification was made spectrophotometrically at 410 nm ($\varepsilon = 1.6\ mM^{-1}\ cm^{-1}$).

2.4. pH and temperature effects on rEndoG activity and enzyme thermostability

The effect of pH on the activity of rEndoG was evaluated by carrying out enzymatic reactions in a mix of 0.05 M acetic acid, 0.05 M MES and 0.1 M Tris to ensure constant ionic strength [24]. pH was varied between 3.5 and 11.0 as needed. The effect of temperature on rEndoG activity was determined between 20 and 80 °C in 50 mM sodium phosphate pH 5.5 for 15 min. For the thermostability assay, rEndoG was pre-incubated at different temperatures for 10–60 min in the absence of substrates. The residual activity was determined at pH 5.5 and 45 °C for 15 min.

2.5. Effect of metals and other inhibitors

The effect of metal ions on the activity of rEndoG were determined at pH 5.5 and 45 °C with the following metal salts at final concentrations of 5 mM: $MgCl_2$, $CoCl_2$, $CaCl_2$, $NiCl_2$, KCl, $CuSO_4$, $MnCl_2$, $AgNO_3$, $ZnCl_2$, $HgCl_2$, $FeCl_2$, and $Cd(OAc)_2$. Analogously, acetone, methanol, propanol, butanol, toluene, Tween 20, glycerol, SDS and Triton X100 were evaluated at a final concentration of 5% (v/v), EDTA at 50 mM, and phenylmethanesulfonyl fluoride (PMSF) at 5 mM.

To determine the effect of glucose (up to 1 M), cellobiose (up to 10 mM), acetate (up to 2 M), ethanol (up to 15%), furfural (up to 150 mM) or NaCl (up to 4 M) on rEndoG activity the enzymatic reactions were carried out at pH 5.0 and 45 °C using 2 mM 4-MUC as substrate.

The effect of 0–15% (w/v) imidazolium ILs 1-*n*-buty-3-methylimidazolium chloride ([C_4mim]Cl), 1-*n*-butyl-3-methylimidazolium bromide ([C_4mim]Br), 1-ethyl-3-methylimidazolium bromide ([C_2mim]Br), and 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) on the activity of rEndoG was evaluated at 45 °C and pH 5.0 using pNPC as substrate.

When 4-MUC was used as a substrate, activity was reported as relative to the control reaction without any additive or treatment.