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Stability of endoglucanases from mesophilic fungus and thermophilic bacterium in acidified polyols

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ABSTRACT

Recent developments in chemical pretreatments of lignocellulosic biomass using polyols as co-solvents (*e.g.*, glycerol and ethylene glycol) at temperatures less than 100 °C may allow the effective use of thermostable and non-thermostable cellulases *in situ* during the saccharification process. The potential of biomass saccharifying enzymes, endoglucanases (EG) from a thermophilic bacterium (*Thermotoga maritima*) and a mesophilic fungus (*Trichoderma longibrachiatum*), to retain their activity in aqueous buffer, acidified glycerol, and acidified ethylene glycol used as co-solvents at pretreatment temperatures at or below 100 °C were examined. The results show that despite its origin, *T. longibrachiatum* EG (*Tl*-EG) retained 75% of its activity after exposure to 100 °C for 5 min in aqueous buffer while *T. maritima* EG (*Tm*-EG) retained only 5% activity. However, at 90 °C both enzymes retained over 87% of their activity. In acidified (0.1% (w/w) H_2SO_4) glycerol, *Tl*-EG retained similar activity (80%) to that obtained in glycerol alone, while *Tm*-EG retained only 35%. With acidified ethylene glycol under these conditions, both *Tl*-EG and *Tm*-EG retained 36% of their activity. The results therefore show that *Tl*-EG is more stable in both acidified glycerol and ethylene glycol than *Tm*-EG. A preliminary kinetic study showed that pure glycerol improved the thermal stability of *Tl*-EG but destabilized *T*m-EG, relative to the buffer solution. The half-lives of both *Tl*-EG and *Tm*-EG are 4.5 min in acidified glycerol, indicating that the effectiveness of these enzymes under typical pretreatment times of greater than 15 min will be considerably diminished. Attempts have been made to explain the differences in the results obtained between the two enzymes.

1. Introduction

Plant biomass (more commonly known as lignocellulosic biomass) is a global, renewable source of fermentable sugars and is composed primarily of complex polysaccharides (cellulose and hemicellulose), lignin, and proteins [1,2]. Bacterial, fungal, and algal fermentation can transform these sugars into a wide range of renewable platform chemicals, fuels, and value-added products. Biochemical saccharification of cellulose in biomass is achieved using mixtures of highly specific glycosyl hydrolase enzymes (cellulases) [3] that are produced naturally by a wide range of fungi and bacteria [4]. Cellulose hydrolysis by cellulases secreted from aerobic fungi (such as *Trichoderma reesei*) is the best studied cellulolytic system, and a minimum of three such cellulases from different functional classes are required for complete degradation of cellulose to glucose: (i) endoglucanases (endo-1,4-β-D-glucanases, EG) hydrolyze regions of low crystallinity, creating free chain ends; (ii) exoglucanases (exo-1,4-β-D-glucanases, CBH) cleave cellobiose units from either the reducing (CBH I) or non-reducing (CBH II) free chain-ends, and (iii) β-glucosidases (BG) hydrolyze cellobiose to glucose [5]. Most cellulases from aerobic fungi have a modular structure consisting of a catalytic domain and a carbohydrate-binding module (CBM) [6], a pH optima between 4 and 6, and a temperature optima between 50 and 70 °C [7]. Cellulases produced by aerobic bacteria are typically either secreted, like those produced by fungi, or associate with the outer membrane, while cellulases produced by anaerobic bacteria typically assemble into supramolecular complexes called "cellulosomes" [8].

There are numerous studies describing the identification and characterization of thermotolerant biomass-degrading enzymes from bacterial and fungal sources (reviewed in [9]). The majority

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of these studies have focused on microbes from extreme environments (so called "extremophiles"), such as the archaeabacteria *Pyrococcus furiosus* and *Sulfolobus solfataricus*, and the eubacteria, *Thermotoga maritima*. *T. maritima* is a hyperthermophilic, anaerobic, saccharolytic, and Gram-negative eubacteria isolated from marine hydrothermal vents, and the optimum environment for the organism includes a water temperature of 80 °C [10]. However, *T. maritima* is capable of growing in water at temperatures up to 90 °C [10]. EGs from *Thermotoga sp*. lack CBMs, which partially explains the inability of these organisms to grown on crystalline cellulose [9]. The *T. maritima* genome encodes the largest number of glycosyl hydrolases of any bacterial or archaeabacterial genome sequenced to date [11].

The cost of enzymes for saccharification of cellulose in lignocellulosic biomass is a major component of the overall process cost [12,13] and the production of recombinant cellulolytic enzymes in transgenic plants provides an opportunity to substantially reduce saccharification costs [13]. Further, transgenic expression of cellulolytic enzymes in existing biofuel crops, such as sugarcane [14] and corn [15], provide opportunities to simplify consolidated bioprocessing of plant biomass [16]. However, the plant cell wall is a resilient, heterogeneous barrier, and pretreatment is essential for the efficient enzymatic hydrolysis of biomass to fermentable sugars [17,18]. Lignocellulosic biomass pretreatments fall into three broad categories (chemical, mechanical, and biological) and the primary goal of any such pretreatment is to improve the accessibility of hydrolytic enzymes to cell wall polysaccharides. Current industrial-scale biomass pretreatments are most likely to be chemical because of shorter processing times, higher fermentable sugar yields, and lower energy requirements [19]. Chemical biomass pretreatments typically operate at temperatures from 150 to 290 °C [20]. While there have been significant advances in the identification of thermostable cellulolytic enzymes [21], it remains extremely unlikely that any enzyme naturally occurring in bacteria or fungi could remain functional after exposure to the temperatures typically employed during chemical pretreatment of lignocellulosic biomass.

Chemical pretreatments of lignocellulosic biomass at temperatures from 60 to 130 °C [22–24] open up hitherto unavailable options for the delivery of thermostable and non-thermostable cellulases into a commercial biomass hydrolysis process. One of these options is the delivery of cellulases into the process prior to biomass pretreatment, either by direct addition of purified cellulase or by transgenic expression of cellulase in plants [25]. Polyols are alcohols containing multiple hydroxyl groups and include glycerol and ethylene glycol. We have recently demonstrated that the pilot-scale pretreatment of sugar cane bagasse under acidic conditions using glycerol as the co-solvent at temperatures from 90 to 130 °C produced solid residues with 40–100% glucan digestibility [23]. In our study, the initial glycerol concentration was >80% but was reduced to ~50% by the end of pretreatment because of the direct injection of steam into the reactor to maintain reaction temperature [23]. Further, we have demonstrated that pretreatment of sugar cane bagasse using high concentrations (>98%) of mixtures of acidified ethylene carbonate and ethylene glycol as co-solvents at atmospheric pressure and temperatures from 60 to 90 °C produced solid residues with 40–93% glucan digestibility [24]. Pretreatment of sugar cane bagasse with acidified glycerol or mixtures of ethylene glycol and ethylene carbonate generates residues that are easily digested by commercial cellulase mixtures and simple, synthetic cellulase mixtures [26]. Therefore, we tested the thermal stability and acid tolerance of EGs from *T. maritima* (*Tm*-EG) and *Trichoderma longibrachiatum* (*Tl*-EG) under conditions that mimicked those found during acidified glycerol and ethylene carbonate/ethylene glycol pretreatment of sugar cane bagasse.

2. Materials and methods

2.1. Enzyme preparation

Glycerol (≥99.5%), ethylene glycol (≥99.0%) and sulfuric acid were supplied by Merck (NJ, USA). Native *Tl*-EG and recombinant *Tm*-EG were supplied by Megazyme (Bray, Ireland) as ammonium sulfate suspensions at protein concentrations of 13.82 mg mL^{-1} and 2.04 mg mL^{-1}, respectively. *Tl*-EG belongs to glycosyl hydrolase class 7 and has a molecular mass of 57.25 kDa, a temperature optimum of 70 °C, and a pH optimum of 4.75 (Megazyme, Bray, Ireland). *Tm*-EG belongs to glycosyl hydrolase class 5 and has a molecular mass of 38.2 kDa, a temperature optimum of 80 °C, and a pH optimum of 6.00 (Megazyme, Bray, Ireland). Working stocks of *Tl*-EG and *Tm*-EG were prepared by dilution to concentrations of 0.92 mg mL^{-1} and 0.20 mg mL^{-1}, respectively, with 100 mM acetate buffer pH 4.75 (*Tl*-EG) and 100 mM phosphate buffer pH 6.00 (*Tm*-EG). The dilution buffers both contained 0.02% (w/v) NaN_3 (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% (v/v) Tween® 20 (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Thermal deactivation of endoglucanase

Aliquots (5 μL) of EG working stocks were mixed with 195 μL of either aqueous buffer at the optimum pH for enzyme activity (described in Section 2.1) or polyol containing H_2SO_4 (0, 0.001, 0.01, 0.1, 0.5, and 1%, w/w) in a 96 well PCR microplate and incubated in a Peltier thermal cycler (PTC-200, MJ Research) for 5 min at 4, 60, 70, 80, 90, and 100 °C. The concentrations of glycerol and ethylene glycol in the thermal deactivation reactions ranged from 96.0–97.0% (w/w) and 95.6–96.5% (w/w), respectively. The concentrations of *Tl*-EG and *Tm*-EG in the thermal deactivation reactions were 23.0 μg mL^{-1} and 5.1 μg mL^{-1}, respectively. After incubation, the plates were chilled on ice and sampled immediately for analysis of residual EG activity as described in Section 2.4.

2.3. Kinetics of thermal deactivation of endoglucanase

Tl-EG and *Tm*-EG working stock solutions were diluted to 23.0 μg mL^{-1} and 5.1 μg mL^{-1}, respectively, with either buffer at the optimum pH for enzyme activity (described in Section 2.1), glycerol (96.5%, w/w), or acidified glycerol (96.5%, w/w) containing 0.5% (w/w) H_2SO_4. Aliquots (20 μL) of each mixture were incubated at 80 °C in a Peltier thermal cycler (PTC-200, MJ Research) for 1 h. At designated times, aliquots were removed from the thermal cycler, quenched on ice, and assayed for residual EG activity as described in Section 2.4.

2.4. Endoglucanase activity assay

Prior to analysis for EG enzyme activity, *Tl*-EG and *Tm*-EG solutions were diluted to 1.53 μg mL^{-1} and 0.51 μg mL^{-1}, respectively, with 100 mM acetate buffer pH 4.75 and 100 mM phosphate buffer pH 6.00. The maximum polyol concentration after dilution was 10% (v/v) and we confirmed that the pH of the mixture after dilution was either 4.75 (*Tl*-EG) or 6.00 (*Tm*-EG) (*data not shown*). EG enzyme activity was measured using 0.5% (w/v) carboxymethylcellulose (CMC) as the substrate in either 100 mM acetate buffer pH 4.75 (*Tl*-EG) or 100 mM phosphate buffer pH 6.00 (*Tm*-EG). *A. niger* β-glucosidase (0.8 U/mL, Megazyme) was added to prevent feedback inhibition from cellobiose. Aliquots (10 μL) of diluted EG were mixed with substrate (50 μL) and incubated for 2 h at 40 °C in a Peltier thermal cycler (PTC-200, MJ Research). Reactions were stopped by incubation for 10 min at 95 °C and liberated glucose was measured using glucose oxidase/peroxidase chemistry, as described previously [14]. Control reaction mixtures were prepared and incubated for 10 min at 95 °C to allow the measurement of enzyme-dependent hydrolysis of CMC. Protein concentration was measured using the Bradford Assay (Bio-Rad Protein Assay, Bio-Rad), relative to a BSA standard curve. Statistical significance between endoglucanase assay data obtained from different treatments was determined using the Student's *t*-test and *P* values of <0.05 were considered significant.

3. Results

3.1. Effect of elevated temperature on residual endoglucanase activity in aqueous buffer

Tl-EG and *Tm*-EG were exposed to temperatures ranging from 60 to 100 °C in aqueous buffer at the optimal pH for each enzyme and the residual specific activity was determined using carboxymethylcellulose (CMC) as a substrate (Fig. 1a). Relative residual activities were determined by comparison between the activities of the heated enzymes with those of enzymes incubated at 4 °C (Fig. 1b). The specific activities of *Tl*-EG and *Tm*-EG in aqueous buffer at optimal pH and 4 °C were 331.9 ± 7.7 μmol glucose min^{-1} mg^{-1} protein and 139.6 ± 1.6 μmol glucose min^{-1} mg^{-1} protein, respectively.