Process Biochemistry 45 (2010) 641–646

Contents lists available at ScienceDirect

Process Biochemistry

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

Thermostability of crude endoglucanase from *Aspergillus fumigatus* grown under solid state fermentation (SSF) and submerged fermentation (SmF)

Abdul A.N. Saqib *, Madiha Hassan, Nahida Farooq Khan, Shahjahan Baig

Food and Biotechnology Research Centre, PCSIR Labs Complex, Ferozepur Road, Lahore 54600, Pakistan

ARTICLE INFO

Article history:
Received 5 November 2009
Received in revised form 15 December 2009
Accepted 16 December 2009

Keywords:
Aspergillus fumigatus
Endoglucanase
Cellulase
Thermostability
Solid state fermentation (SSF)
Submerged fermentation (SmF)

ABSTRACT

Aspergillus fumigatus produced more protein (114 μg/ml vs 51 μg/ml) and a higher specific activity of the endoglucanase (9158 vs 6294) in the culture filtrates from solid state fermentation (SSF) than that from submerged fermentation (SmF) when grown on wheat straw. The activation energy (E_a) of substrate hydrolysis was less (33 kJ mol^{-1}) for the endoglucanase produced under the SSF (SSF-EG) than the enzyme produced under the SmF (SmF-EG), which was 51 kJ mol^{-1}. The SSF-EG was more thermostable as compared to the SmF-EG, evidenced by the larger enthalpy of activation of denaturation, ΔH^*_D, 152 kJ mol^{-1} vs 112 kJ mol^{-1} at 50 °C (similar results at higher temperatures). It was further evidenced by a steeper decline in the half lives ($T_{1/2}$) of the SmF-EG than the SSF-EG. The free energy changes of activation of denaturation of the enzymes, ΔG^*_D, were 107 kJ mol^{-1} and 106 kJ mol^{-1} for the SSF-EG and the SmF-EG, respectively, at 50 °C (similar results at higher temperatures). The entropies of activation of denaturation, ΔS^*_D, were positive for both of the enzymes. The melting temperature (T_m) of the enzyme was 5 °C more for the SSF-EG than the SmF-EG.

1. Introduction

Cellulases are the enzymes involved in the hydrolysis of cellulose, the major polysaccharide of plant cell walls [1]. They represent a large proportion of the industrial enzymes. They are used in textile, detergent, beverage, juice extraction, animal feed and pulp and paper industries. Currently they are the third largest industrial enzymes being sold worldwide. However, there is also a fast growing market for their use in the biofuel industry and they are predicted to become the largest volume industrial enzyme [1,2]. Three major types of enzymes are required to completely degrade cellulose. They include: (1) endoglucanases (EG) which cleave internal bonds in the amorphous regions of the cellulose chains, (2) cellobiohydrolases (CBH) which act on the ends of the chains liberating cellobiose moieties, and (3) β-glucosidases which hydrolyse cellobiose to glucose [3–5]. In addition to the well-known enzyme activities, a number of auxiliary proteins and mechanisms have also been suggested to take part in the overall hydrolysis of cellulose [6–11]. The precise nature and the role of each of the enzymes and other factors involved in the lignocellulose degradation are yet to be unraveled. That is why a true mechanistic model of the cellulose hydrolysis cannot be predicted [2].

Given that a number of enzyme activities are required for the complete hydrolysis of cellulose, crude cellulase preparations are considered advantageous over the purified enzymes for industrial use [3]. In fact, the cost of the industrial cellulases could be dramatically reduced if the crude cellulase from the fermentor is directly used [2]. Crude enzyme preparations have been used by many researchers to investigate the efficiencies of the preparations [5,11,12] and/or to study the kinetic and thermostability parameters [13–16].

Cellulases can be produced through both submerged fermentation (SmF) and solid state fermentation (SSF). The enzymes produced through the SSF can have many advantages over the SmF, such as higher yield and higher product stability [17,18]. However, SmF is technically easier to perform as compared to SSF [18]. For example SSF requires a longer lag time, different optimal conditions for spore germination and vegetative growth and larger inoculum size requirement [19]. It is also difficult to control the heat transfer in the large scale SSF bioreactors [20]. The problems associated with the SSF have been described in detail in the literature [21,22].

Thermostable cellulases are potentially more beneficial in the hydrolysis of lignocellulosic substrates [23]. High stability is a desired quality of industrial enzymes because most of the industrial processes are run at elevated temperatures. For example, in biostoning of denim, the fabric is treated with cellulases, possessing strong endoglucanase activity, at 55 °C for 1 h [24]. This demonstrates the need to evaluate and assess the thermostability of the enzyme preparations. A number of studies have analyzed the

* Corresponding author. Tel.: +92 3317780266; fax: +92 42 9230705.
E-mail address: a.saqib@gmail.com (Abdul A.N. Saqib).

1359-5113/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2009.12.011

thermostability of cellulases produced either by SmF or SSF [3,25] but no report has been found on the comparison of crude endoglucanases produced by a fungus growing under the two fermentation conditions. In the present work we have compared the thermostabilities of the crude endoglucanase produced by *Aspergillus fumigatus* under the SSF and the SmF conditions. Various thermostability parameters, such as half life of the enzyme preparation ($T_{1/2}$), melting temperature (T_m), the activation energy of thermal denaturation ($E_{a(D)}$), enthalpy of activation of the thermal denaturation (ΔH_D^*), entropy of activation of thermal denaturation (ΔS_D^*) and the Gibbs free energy of activation of thermal denaturation (ΔG_D^*) were used to evaluate the thermostability of the endoglucanase preparations.

2. Materials and methods

All chemicals were purchased from the Sigma–Aldrich, St. Louis, MO (USA), unless mentioned otherwise.

2.1. Culture isolation/identification

The fungal strain was locally isolated from the soil containing decomposing leaves and wood chips. The fungus was identified as the *A. fumigatus* sp. by the First Fungal Culture Bank of Pakistan, Institute of Mycology and Plant Pathology (IMPP), University of the Punjab, Lahore, Pakistan (IMPP Reference: 922). The fungal strain was named *A. fumigatus* SMN1.

2.2. Culture maintenance and propagation

The culture was maintained on the Vogel's minimal medium (VM) agar overlaid with a Whatman No. 1 filter paper (FP) disc as the sole carbon source. The VM agar contained 2% purified agar (Oxoid Ltd, UK) dissolved in 1× Vogel's medium [26]. The filter paper, which is rich in cellulose, was used on a minimal medium in order to make sure that the fungus could grow using cellulose as the sole carbon source.

2.3. Preparation of the spore suspension

Ten milliliters sterilized distilled water was aseptically poured onto the Petri plate containing the fungus growing on the VM + FP agar. The spore suspension was gently mixed with the help of a sterile glass spreader for 1 min; with 60° rotation of the Petri plate every 20 s, in order to achieve a uniform spore suspension. The spore suspension was aseptically transferred to a sterile test tube for the subsequent inoculations.

2.4. Enzyme production

2.4.1. Submerged fermentation (SmF)

One percent (w/v) chopped wheat straw (5–10 mm length) was added, in quadruplicate, to the 50 ml of the Vogel's medium in 250 ml Erlenmeyer flasks which were then cotton plugged and autoclaved at 121 °C and 15 psi for 20 min. Upon cooling, 1 ml of the spore suspension was aseptically added to the medium in the flasks. One milliliter sterilized distilled water was used instead of the inoculum in the control flask. The inoculated flasks were incubated at 30 °C and 130 rpm for 1 week. At the end of the incubation period, the solid mass was removed from the culture filtrate by centrifuging it at 1467 × *g* (RCF) for 20 min. The resulting supernatant was a clear solution; hence no further filtration step was performed. The supernatant was stored at 4 °C for further use as the crude endoglucanase preparation.

2.4.2. Solid state fermentation (SSF)

Five grams chopped wheat straw (5–10 mm length) was added, in quadruplicate, into 50 ml of the Vogel's medium in 250 ml Erlenmeyer flasks. The liquid was absorbed by the wheat straw resulting in a damp solid substrate inside the flasks. After the addition of the Vogel's medium there was no free flowing liquid medium in the flasks. The flasks were then tightly plugged with cotton and autoclaved at 121 °C and 15 psi for 20 min. Upon cooling 1 ml of the spore suspension was aseptically added to the wheat straw in the flasks. One ml sterilized distilled water was used instead of the inoculum in the control flask. The flasks were incubated, with manual shaking once a day, at 30 °C for one week. At the end of the incubation period, 50 ml 0.05 M acetate buffer pH 4.8 was added to all of the flasks. The resulting suspension was centrifuged at 1467 × *g* (RCF) for 20 min. The centrifugation process resulted in 50 ml clear supernatant, therefore no further filtration step was performed. The supernatant was stored at 4 °C for further use as the crude endoglucanase preparation.

2.5. CMCase assay

The CMCase assays to estimate the endoglucanase activity of the enzyme sample were performed using dinitrosalicylic acid (DNS) reagent [27] to estimate the reducing sugars as described previously [4]. One hundred microliters of the enzyme solution was mixed, in a test tube, with 900 μl of 1% (w/v) carboxymethylcellulose (CMC) solution made in 0.05 M acetate buffer pH 5, and incubated at 40 °C for 1/2 hour. Three milliliters DNS reagent was added to the test tubes which were subsequently placed in a boiling water bath for 5 min. After boiling, the tubes were cooled down to room temperature and the absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol glucose equivalents/minute under the reaction conditions.

2.6. Effect of temperature on substrate hydrolysis

2.6.1. Optimum temperature and activation energy (E_a)

The effect of temperature on the cellulase activity was determined by performing the CMCase assay at various temperatures ranging from 25 °C to 80 °C. The optimum temperature and the activation energy (E_a) of the cellulase to convert its substrate into the product, under the given experimental conditions, were determined by plotting the data as an Arrhenius plot, as described previously [28].

2.6.2. Temperature coefficient (Q_{10})

The Q_{10}, which is the factor by which the rate of a reaction changes for every 10° rise in temperature (°C or K), was calculated by the Dixon and Webb equation [29]:

$$\ln Q_{10} = \frac{E_a \times 10}{RT^2} \quad (1)$$

where E_a is the activation energy of the enzyme (J mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and T is the absolute temperature (K).

2.7. Thermostability analysis

One hundred microliters of the enzyme samples were incubated, in the absence of the substrate, at 50 °C, 60 °C, 70 °C and 80 °C for various lengths of time ranging from 0 min to 120 min. At the end of incubations the test tubes containing the enzyme were immediately cooled down by placing then onto ice and subsequently kept at 4 °C for overnight so that the enzyme's secondary and tertiary structures become stable after the heat treatment and any reversible unfolding reverts back to the native forms [7]. The residual enzyme activity was then determined and plotted as described previously [30] to determine various thermostability parameters as follows.

2.7.1. Half life of the enzyme solution ($T_{1/2}$)

The $T_{1/2}$ of the endoglucanase preparations was estimated by the following formula [7,30]:

$$T_{1/2} = \frac{\ln 2}{k_d} = \frac{0.693}{k_d} \quad (2)$$

where k_d is the first order rate constant of thermal inactivation of the enzyme activity, obtained through the slops of the plots described above.

2.7.2. Thermodynamics of the irreversible thermal denaturation of the cellulase

The enthalpy of activation of thermal denaturation (ΔH_D^*), which is the amount of energy required to bring the enzyme to the activated state for the subsequent denaturation at a given temperature, Gibbs free energy of activation of the thermal denaturation (ΔG_D^*) and the entropy of activation of the denaturation (ΔS_D^*) were calculated through the following equations [30,31]:

$$\Delta H_D^* = E_{a(D)} - RT \quad (3)$$

$$\Delta G_D^* = -RT \times \ln\left(\frac{k_d \times h}{k_B \times T}\right) \quad (4)$$

$$\Delta S_D^* = \frac{\Delta H_D^* - \Delta G_D^*}{T} \quad (5)$$

where $E_{a(D)}$ is the activation energy for the thermal denaturation of the enzyme, R is the universal gas constant = 8.314 J K^{-1} mol^{-1}, T is the absolute temperature (K), k_d is the first order rate of thermal inactivation of the enzyme, h is the Planck's constant = 6.63 × 10^{-34} J s and k_B is the Boltzmann constant = 1.38 × 10^{-23} J K^{-1}.

2.7.3. Melting temperature (T_m)

The residual activities of the enzyme samples incubated at various temperatures (50 °C, 60 °C, 70 °C and 80 °C) for 30 min were plotted according to Rangarajan et al. [32] to determine the T_m for 30 min. The residual activity refers to the activity of the enzyme that remains behind after the heat treatment. The T_m corresponds to the temperature at which the enzyme activity drops down to 50% of the initial activity.

2.8. Statistical analysis

Appropriate statistics were applied using the GraphPad InStat software. Student's *t*-test was used to compare means.