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Beta glucosidase from *Bacillus polymyxa* is activated by glucose-6-phosphate

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ABSTRACT

Optimization of cellulose enzymatic hydrolysis is crucial for cost effective bioethanol production from lignocellulosic biomass. Enzymes involved in cellulose hydrolysis are often inhibited by their end-products, cellobiose and glucose. Efforts have been made to produce more efficient enzyme variants that are highly tolerant to product accumulation; however, further improvements are still necessary. Based on an alternative approach we initially investigated whether recently formed glucose could be phosphorylated into glucose-6-phosphate to circumvent glucose accumulation and avoid inhibition of beta-glucosidase from *Bacillus polymyxa* (BGLA). The kinetic properties and structural analysis of BGLA in the presence of glucose-6-phosphate (G6P) were investigated. Kinetic studies demonstrated that enzyme was not inhibited by G6P. In contrast, the presence of G6P activated the enzyme, prevented beta glucosidase feedback inhibition by glucose accumulation and improved protein stability. G6P binding was investigated by fluorescence quenching experiments and the respective association constant indicated high affinity binding of G6P to BGLA. Data reported here are of great impact for future design strategies for second-generation bioethanol production.

1. Introduction

Second-generation bioethanol is of great importance for biofuel production since it utilizes lignocellulosic residues as feed stock and, therefore, greater ethanol yields can be obtained from fixed planting areas [1]. Enzymatic cellulose hydrolysis can be accomplished by the synergic action of three enzymes named endoglucanases, cellobiohydrolases and beta glucosidases. Endoglucanases catalyze random cleavage of the internal bonds of the cellulose chain, while cellobiohydrolases attack the terminal ends, releasing cellobiose. Beta glucosidases act on cello-oligosaccharides and cellobiose, releasing glucose units for further fermentation to ethanol [2,3]. Cellobiohydrolases and endoglucanases are often inhibited by cellobiose [4–7], decreasing the overall lignocellulose-glucose conversion yields. Ideally, highly efficient beta glucosidases are desirable, in order to avoid cellobiose buildup and prevent feedback inhibition of endo and exoglucanases, increasing cellulose hydrolysis. However, rapid consumption of cellobiose by beta glucosidases leads to glucose accumulation, causing beta glucosidase product inhibition [8]. Therefore, beta glucosidases are currently considered one important bottleneck for second-generation bioethanol production [9]. There are diverse ways to produce ethanol from lignocellulosic material. One promising approach is known as Simultaneous Saccharification and Fermentation (SSF), in which cellulose hydrolysis and fermentation occur simultaneously [10]. The primary advantage of this method is that end-product inhibition is prevented, since subsequent fermentation avoids glucose buildup [11]. However, the major drawback of this method is the need to tune the reaction conditions (e.g. temperature and pH) precisely for both processes, simultaneously. Unfortunately, optimal temperature of catalysis for most cellulases varies between 50 and 60 °C, while fermentation often occurs around 25–30 °C, hampering this

Abbreviations: SSF, Simultaneous Saccharification and Fermentation; SHF, Separate Hydrolysis and Fermentation; G6P, glucose-6-phosphate; BGLA, beta-glucosidase; pNPG, p-nitrophenyl β-D-glucopyranoside.

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strategy. In the Separate Hydrolysis and Fermentation (SHF) method, the two processes occur separately and reaction conditions can be individually tailored. However, in this case, during the hydrolysis step, glucose is not consumed, and feedback inhibition of the hydrolytic enzymes can occur. Therefore, efforts have been made to create highly efficient and glucose resistant beta glucosidases [2,12]. Some glucose tolerant enzymes have already been identified [13–15], but their catalytic efficiency is still insufficient for their direct application in the SHF industrial process. Directed evolution studies have also been carried out. However, all studies have so far failed to produce a variant that fulfills all the requirements for an optimal performance during the intricate process of complete cellulose hydrolysis [16,17]. Continuous beta glucosidase supplementation during cellulose breakdown accelerates hydrolysis and represents the current strategy of choice for second-generation biofuel production [18]. However, enzyme supplementation significantly raises the production costs restricting even further the commercialization of second-generation bioethanol. In order to circumvent glucose accumulation using the SHF method, we initially hypothesized that recently formed glucose could be rapidly phosphorylated into glucose-6-phosphate (G6P) to avoid product inhibition caused by glucose accumulation. Therefore, we initially investigated whether G6P also behaves as an inhibitor of the glucosidase reaction. Surprisingly, our data demonstrated that G6P is not an enzyme inhibitor, but is in fact a beta glucosidase activator. This is the first report of beta glucosidase activation by G6P, and data reported here are of great impact for future optimization of cellulose hydrolysis during second-generation bioethanol production. Efforts are currently underway to determine the structural changes caused by G6P binding that produced the observed behavior.

2. Materials and methods

2.1. Material

Bacillus polymyxa strain ATCC842 was provided by Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. 4-Nitrophenyl β-D-glucopyranoside (pNPG) and D-glucose 6-phosphate (G6P) disodium salt hydrate were purchased from Sigma Aldrich.

2.2. Cloning, expression and purification of B. polymyxa beta glucosidase (BGLA)

Synthetic oligonucleotide primers amplified the *B. polymyxa BGLA gene* (1320 bp) from purified genomic DNA using standard PCR conditions. The fragment was embedded into a pET28a(+) expression vector *and t*he resultant plasmid pET28a(+):: *BGLA* was transformed into *Escherichia coli* Rosetta (DE3) cells. The transformed cells were inoculated into LB liquid media containing 50 μg/mL kanamycin and 37.5 μg/mL chloramphenicol and grown at 37 °C until reach the OD600 nm of 0.6. Cells were induced with 0.05 mM IPTG and incubated at 17 °C for 24 h. Control experiments were performed where *E. coli* Rosetta (DE3) cells were transformed with an empty pET28a(+) vector. For purification, all procedures were carried out at 4 °C. Cells were suspended in 50 mL of 50 mM Tris–HCl pH 8.0; 300 mM NaCl and 1.5 mM imidazole (buffer A), containing phenylmethylsulfonyl fluoride (PMSF), lysozyme (20 mg/mL) and DNAse I (5 μg/mL). Cells were disrupted by sonication and stirred on ice for 30 min. The cell debris was removed by centrifugation and the supernatant was applied to Ni–NTA Sepharose pre-equilibrated with buffer A. Weakly bound proteins were removed by resin washing with buffer B (50 mM Tris–HCl pH 8.0; 300 mM NaCl; 40 mM imidazole) and proteins were eluted in buffer C (500 mM imidazole; 50 mM Tris–HCl pH 8.0). Active fractions were pooled, dialyzed against buffer A, mixed to 50% glycerol and stored at −20 °C.

2.3. Measurement of enzyme activity

Measurement of enzyme activity was performed using either cellobiose or pNPG as substrates. All reactions were monitored non-continuously and enzyme activities were calculated from the initial rates (<10% completion). Cellobiose hydrolysis was monitored by glucose release detection using glucose oxidase assay kit (Sigma Aldrich) according to the manufactures protocol. Briefly, 150 μL reaction mixtures contained variable concentrations of cellobiose and G6P, BGLA (140 nM) and 50 mM Hepes, pH 7.0. Reactions proceeded for 2.5 min at 17 °C and were stopped by heating up the sample at 95 °C for 5 min. For the enzyme assays carried out with pNPG, reactions were performed in 150 μL reaction volume in the presence of BGLA (140 nM), variable concentrations of pNPG and G6P in 50 mM Hepes, pH 7.0 at 17 °C. After 2.5 min, the reaction was stopped with 150 μL of 1 M Na_2CO_3, pH 10, and the absorbance was measured at 405 nm. The product concentration was estimated using a *p*-nitrophenol extinction coefficient of 18,000 M^{-1} cm^{-1}.

2.4. Kinetic treatment for BGLA nonessential activation by G6P

Beta glucosidase catalyzed reaction occurs in the absence of G6P, therefore the observed increase in reaction rates is classified as a case of nonessential activation [19]. Kinetically, nonessential activation is analogous to nonlinear inhibition, but, changes are in the opposite direction: wherever there was an inhibition, there is now an activation [20].

In this study, enzymatic assays were performed as described in Section 2.3 and kinetic data were fitted using Sigma Plot 12.0. BGLA nonessential activation was analyzed by Lineweaver–Burk plots in the presence of eight different fixed G6P concentrations using seven different pNPG concentrations in the range 0.5–8.0 mM. Activator binding constant (K_b) was calculated from secondary replots of 1/Δ slope and 1/Δ intercept *versus* 1/[G6P] obtained from individual Lineweaver–Burk plots. The *x* and *y* intercepts from 1/Δ slope replot correspond to $-\beta/\alpha K_b$ and $\beta V_{max}/K_m(\beta-\alpha)$, respectively. The y intercept of the 1/Δ intercept replot is equal to $\beta V_{max}/(\beta-1)$. The α and β constants refer to fold change in K_m and k_{cat}, respectively, obtained in the presence of the nonessential activator.

2.5. Glucose inhibition studies

In order to investigate BGLA glucose inhibition, substrate saturation curves were obtained in the presence of 200 mM glucose and increasing concentrations of G6P (from 0 to 25 mM). Control experiments were performed in the absence of glucose or G6P as well as in the presence of 25 mM G6P. Individual substrate saturation kinetic data were fitted to Eq. (1):

$$v = VS/(K_m + S) \quad (1)$$

where, v is the initial velocity rate (μmol/min/mg of BGLA), S is the substrate concentration (mM), K_m is the Michaelis–Menten constant and V is the maximal velocity (μmol/min/mg of BGLA).

2.6. Tryptophan fluorescence quenching of BGLA

All tryptophan fluorescence experiments were performed in 50 mM Hepes pH 7.0 at 20 °C using a Multidimensional Fluorescence Spectrofluorimeter (ISS Inc., Illinois, USA) coupled to a Peltier temperature control system with water circulation.