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Dynamic synergistic effect on *Trichoderma reesei* cellulases by novel β-glucosidases from Taiwanese fungi

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ABSTRACT

Dynamic synergistic effects in cellulosic bioconversion have been revealed between *Trichoderma reesei* cellulases and β-glucosidases (BGLs) from six Taiwanese fungi. A high level of synergy (8.9-fold) was observed with the addition of *Chaetomella raphigera* BGL to *T. reesei* cellulases. In addition, the *C. raphigera* BGL possessed the highest activity (V_{max}/K_m = 46.6 U/mg mM) and lowest glucose inhibition (*Ki* = 4.6 mM) with the substrate 4-nitrophenyl β-D-glucopyranoside. For the natural cellobiose substrate, however, the previously isolated *Aspergillus niger* BGL Novo-188 had the highest V_{max}/K_m (0.72 U/mg mM) and lowest *Ki* (59.5 mM). The demonstrated dynamic synergistic effects between some BGLs and the *T. reesei* cellulase system suggest that BGLs not only prevent the inhibition by cellobiose, but also enhance activities of endo- and exo-cellulases in cellulosic bioconversion. Comparisons of kinetic parameters and synergism analyses between BGLs and *T. reesei* cellulases can be used for further optimization of the cellulosic bioconversion process.

1. Introduction

The potential importance of cellulose hydrolysis in the context of conversion of plant biomass to fuels and chemicals is well recognized. Cellulose hydrolysis by cellulases produced by numerous microorganisms has been widely employed for producing sustainable bio-based products and bioenergy to replace depleting fossil fuels (Lynd et al., 2005; Zhang et al., 2006). Currently, the utilization of cellulosic biomass for bioethanol poses significant technical and economic challenges, and its success largely depends on the development of highly efficient and cost-effective biocatalysts for conversion of pretreated biomass to fermentable sugars. One of the most efficient and successful ways of finding new biocatalysts or enzymes is to screen a large number of diverse microorganisms seeking different characteristics with improved versatility.

The effective conversion of cellulose to fermentable sugars requires three classes of enzymes: (1) endoglucanases (EC 3.2.1.4) which randomly cut cellulose chains to yield glucose and cello-oligosaccharides, (2) exoglucanases (EC 3.2.1.91) which exolytically attack the reducing or non-reducing end of celluloses to yield cellobiose, and (3) β-glucosidases (EC 3.2.1.21) which hydrolyze cellobiose and cello-oligosaccharides to form glucose. Cellulases from the fungal genus *Trichoderma* have received considerable attention due to their ability to effectively hydrolyze crystalline cellulose. The biomass-degrading fungus *Trichoderma reesei* contains a cellulase mixture consisting of many catalytically active proteins (Martinez et al., 2008), of which at least two exoglucanases or cellobiohydrolases (i.e., Cel7A [CBH1] and Cel6A [CBH2]), five endoglucanases (EG1 to EG5), several β-glucosidases, and hemicellulases have been identified by two-dimensional (2-D) gel electrophoresis in combination with mass spectrometry analysis (Vinzant et al., 2001). CBH1, CBH2, and EG2 are the three main components of the *T. reesei* cellulases system, representing 60%, 20%, and 12% of total cellulase proteins, respectively. As a result, the main activity of *T. reesei* on insoluble cellulosic substrates is to produce cellobiose. Therefore, to completely hydrolyze cellulose to glucose in this hydrolytic system, a supplement of β-glucosidase is required.

Ubiquitous BGLs exist in all the living kingdoms, from bacteria to highly evolved mammals. They comprise the main part of the cellulase enzyme system in bacteria and fungi and are responsible for the rate-limiting step in the hydrolysis of short-chain oligosaccharides and cellobiose. Based on amino acid sequence similarities,

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Nomenclature

EG	endoglucanase
CBH	cellobiohydrolase
BGL	β-glucosidase
CB	cellobiose
G1, G2	glucose
*V*max, K_m, *Ki*	kinetic constants and glucose inhibition constant for BGL
*V*mt, *K*mt	apparent kinetic constants for *T. reesei* with BGL
*V*glu, *V*CB	enzymatic rate for glucose and cellobiose
Novo-188	Novozyme 188
D2	*Chaetomella raphigera*
DNS	dinitrosalicylic acid
pNPG	4-nitrophenyl β-D-glucopyranoside

BGLs differing in their specificities for the aglycone part (aryl-, alkyl-, or amino-) linked to the glycosyl group has been classified into either family 1 or family 3 of glycosyl hydrolase families. Family 1 enzymes include most bacterial, archaeal, plant and animal BGLs, whereas family 3 includes some bacterial and all yeast and fungal enzymes (Bhatia et al., 2002).

Synergism occurs when the activity exhibited by mixtures of components (such as cellulases) is larger than the sum of each individually evaluated activity of these components. In the past decades, several groups have reported on synergisms between *Trichoderma* endo- and exocellulases (Medve et al., 1998; Nidetzky et al., 1994; Valjamae et al., 1999, 2003), however, none of them demonstrated effects of BGLs in the system encompassing CBHs and EGs. In this study, we have examined, for the first time, potential kinetic and dynamic synergism between BGLs and EGs/CBHs. Six fungi with high BGL activities have been isolated from screening of 42 fungi collected in Taiwan, and these BGLs were used for the synergism studies. As shown in Fig. 1, the *T. reesei* cellulases mainly hydrolyze cellulose to cellobiose (CB); only a minor fraction is hydrolyzed to glucose (G1). By adding extra BGLs, large amounts of glucose (G2) are produced, hence greatly improves the degree of hydrolysis of cellulose to glucose (G_{tot}). We furthermore determined kinetic parameters of BGLs in the hydrolysis of different substrates in the absence and presence of glucose and identified BGLs by mass spectrometry.

2. Methods

2.1. Preparation of fungi and enzymes

All fungi, except *Penicillium citrinum* YS40-5, which was isolated from rice straw compost (Ng et al., 2010), were collected previously by Prof. Yu-Ming Ju, Institute of Plant and Microbial Biology, Academia Sinica, Taipei, Taiwan. *T. reesei* cellulases (Celluclast 1.5L) and Novozyme 188, a commercial *Aspergillus niger* β-glucosidase, were purchased from Sigma. The minimal requirement (MR) medium consisted of 1 g soy-peptone, 1.4 g $(NH_4)_2SO_4$, 2.0 g KH_2PO_4, 0.34 g $CaCl_2{\cdot}2H_2O$, 0.30 g $MgSO_4{\cdot}7H_2O$, 5 mg $FeSO_4{\cdot}7H_2O$, 1.6 mg $MnSO_4{\cdot}H_2O$, 1.4 mg $ZnSO_4{\cdot}7H_2O$ and 2.0 mg $CoCl_2{\cdot}6H_2O$ per liter of H_2O, was adjusted to pH 6.5 with 0.1 N NaOH. All materials were autoclaved for 15 min at 121 °C. The fungal cultures were maintained on PDA medium for 5 days at 30 °C for sporulation. Five discs of PDA with a diameter of 0.8 cm containing approximately 10^6–10^7 spores per disc were inoculated into a 250-mL Erlenmeyer flask containing 2 mM cellobiose in 100 mL MR medium at 30 °C under shaking at 125 rpm. The time course of BGL activity was then analyzed daily. BGLs of the fungi, for which high enzymatic activities were measured, were subsequently concentrated ~80-fold by using Amicon® Ultra centrifugal filter devices (10k NMWL, Millipore, MA, USA).

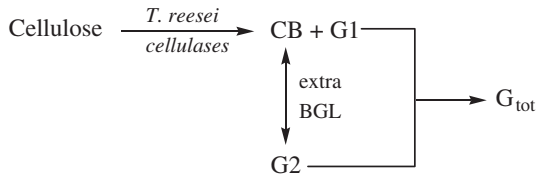

Fig. 1. Dynamic synergism between extra BGL and *Trichoderma reesei* cellulases on cellulosic bioconversion.

2.2. BGL assays

4-Nitrophenyl β-D-glucopyranoside (pNPG) from Sigma was used as the substrate for measuring BGL activity. The assay was performed by mixing 100 μl of 1 mM pNPG and 100 μl of enzyme solution in 50 mM sodium phosphate buffer (pH 7.0). After incubation for 15 min at 55 °C, the reaction was quenched by adding 600 μl of 1 M sodium carbonate. Subsequently, the absorbance of 4-nitrophenol in the reaction mixture was measured at 405 nm with a spectrophotometer. One unit (U) of BGL activity was defined as 1 μmol 4-nitrophenol released per minute. Protein concentrations were determined by Bradford assay using the Bio-Rad Protein Assay Kit) with bovine serum albumin as the standard.

2.3. Native PAGE analysis with MUG-zymogram

For the detection of in-gel BGL activity, samples were analyzed by native PAGE using 10% and 4% polyacrylamide as separation and stacking gels, respectively. Gels were run in Tris–glycine buffer (pH 8.3) at a constant current of 20 mA per slab for 3 h at 4 °C, washed with distilled water, and overlaid with 0.5 mM 4-methylumbelliferyl β-D-glucopyranoside (MUG, Sigma–Aldrich) in 0.1 M succinate buffer (pH 5.8), followed by incubation for 15 min at 55 °C. The presence of a fluorescent reaction product was then detected by visualization at 365 nm for 5 min. Gels were stained with Coomassie brilliant blue R-250 after being photographed under UV light. The gel bands with enzyme activities were excised for subsequent MS/MS analysis.

2.4. Tandem mass spectrometry and protein identification

A nanoLC-MS/MS analysis was performed on an integrated system (QSTAR XL) comprising a LC Packings NanoLC system with an autosampler and a QSTAR XL Q-Tof mass spectrometer (Applied Biosystems, Lincoln, CA) fitted with nano-LC sprayer. Injected samples were first trapped and desalted on a LC-Packings PepMap™ C18 μ-Precolumn™ Cartridge (5 μm, 5 mm × 30 μm I.D.; Dionex, Sunnyvale, CA). Subsequently, peptides were eluted from the precolumn and separated on an analytical LC-Packings PepMap C18 column (3 μm, 15 cm × 75 μm I.D.) connected in-line to the mass spectrometer, at a flow rate of 200 nl/min using a 40-min gradient of 5–60% acetonitrile in 0.1% formic acid.