Journal of Biotechnology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Expression optimization and biochemical properties of two glycosyl hydrolase family 3 beta-glucosidases

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ARTICLE INFO

Article history:
Received 29 October 2014
Received in revised form 26 March 2015
Accepted 18 April 2015
Available online xxx

Keywords:
β-Glucosidase
Saccharomycopsis fibuligera
Trichoderma reesei
Pichia pastoris
Response surface method
Biochemical property

ABSTRACT

The β-glucosidases from *Saccharomycopsis fibuligera* (SfBGL1) and *Trichoderma reesei* (TrBGL1) were cloned and expressed in *Pichia pastoris*. Methanol concentration and pH significantly affected the production. The combined effects of the two factors were optimized by using the response surface method, resulting in a 137% and 84% increase in rTrBGL1 and rSfBGL1 yield compared to single-factor experiment. Structure and biochemical properties of the two enzyme were investigated and compared. They belong to glycosyl hydrolase family 3 and exhibit significant hydrolysis activity and low-level transglycosylation activity. The two enzymes show similar substrate affinity and ion-tolerance, and both of them can be activated by Cr^{6+}, Mn^{2+} and Fe^{2+}. The rSfBGL1 has greater catalytic speed, higher specific activity and acid-tolerance than rTrBGL1, but rTrBGL1 is more thermostable and has higher optimal temperature than rSfBGL1. This study provides a useful and quick optimal method for recombinant enzyme production and makes a valuable comparison of biochemical properties, which opens important avenues of exploration for relationship between structure and function and further practical applications.

1. Introduction

Cellulose is the most abundant organic polymer on Earth, which can be hydrolyzed into glucose for further utilization, but the high cost of cellulase presents a significant barrier to commercialization (Merino and Cherry, 2007). β-Glucosidase is a component of cellulase system. As a rate-limiting regulator, it plays an important role in degradation of cello-oligosaccharides, which influences the liberation of glucose from cellulose (Barnett et al., 1991; Chen et al., 2008). Nevertheless, they are poorly produced by native celluase-producing strains. Besides cellulose degradation, β-glucosidases have been widely used in agricultural, medical and diversified industrial fields (Bhatia et al., 2002; Husain, 2010). β-Glucosidases belong to a large family of glycoside hydrolase (GH), and many of them have dual activities of hydrolysis and transglycosylation, which catalyze synthesis of poly- or oligo-saccharide (Bohlin et al., 2013; Ketudat Cairns and Esen, 2010). Hence, it is very necessary to investigate the specific functional property of several key members of this family for rational utilization.

Trichoderma reesei has been employed in commercial cellulase preparations. It can secret high amounts of cellulolytic enzymes, but the β-glucosidase yield is low compared with that from other filamentous fungi (Barnett et al., 1991; Zhang et al., 2010). The β-glucosidases I (TrBGL1) encoded by *bgl1* gene is an extracellular, cellulose-inducible β-glucosidase (Mach et al., 1995). The enzyme has been tried to be expressed in filamentous fungi and *Saccharomyces cerevisiae* to improve expression levels (Cummings and Fowler, 1996; Zhang et al., 2010). The β-glucosidase gene *bgl1* from *Saccharomycopsis fibuligera* (*Sfbgl1*) has drawn increasing attention as a potential donor of the β-glucosidase gene for the improvement of production (Shen et al., 2008; van Rooyen et al., 2005; Zhang et al., 2012), and the expression level of *Sfbgl1* gene in *S. cerevisiae* is higher than that of other fungal β-glucosidase genes (van Rooyen et al., 2005).

Although *Sfbgl1* and *Trbgl1* have been successfully heterologously expressed, the expression levels still leave room for

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http://dx.doi.org/10.1016/j.jbiotec.2015.04.016
0168-1656/

improvement. Various β-glucosidases have been tried to be used as supplement in cellulase mixture to enhance the saccharification of cellulose, however the low productivity and unknown biochemical property of TrBGL1or SfBGL1 limit the application for the purpose. Therefore, productivity improvement is a consideration of the most importance. An appropriate expression system and an effective optimal method are necessary for efficient protein production. *Pichia pastoris* possesses many advantages such as post-translational modification, easy manipulation, and higher protein expression levels. Therefore, it is worth to try to express bioactive β-glucosidases in *P. pastoris* host and optimize the conditions for high productivity.

Presently, the biochemical properties of TrBGL1 and SfBGL1 are little reported, and the potential differences of structure and biochemical properties between the two β-glucosidases are not elucidated. Post-translational modifications by host usually make their biochemical characteristics different from the native enzyme. However, functional properties are important for their usage as gene resources, enzyme modification and industrial application. Consequently, in the present study, the *Trbgl1* (GenBank accession no. TRU09580) and *Sfbgl1* (HQ891006) genes were cloned and over-expressed in *P. pastoris*, and the production conditions were quickly optimized by the response surface methods (RSM). The structural models and biochemical properties of purified proteins were revealed and compared for the first time.

2. Materials and methods

2.1. Bioinformatics analysis and construction of expression vectors

Bioinformatics analysis of TrBGL1 and SfBGL1 was carried out as described in the Supplementary Material. *TrBGL1* and *SfBGL1* genes were cloned into pPICZαC and pPICZαA vectors, resulting in pPICZαC-TrBGL1 and pPICZαA-SfBGL1 plasmids, respectively (Fig. S1). *TrBGL1* and *SfBGL1* genes were then integrated into *P. pastoris* Gs115 genomic DNA by electroporation, and positive transformants were verified by PCR (Supplementary Material).

2.2. Selection of high-level expression colonies and expression optimization

In order to determine the productivity of the selected colonies, induction experiments with pH 6.0 of buffered glycerol/methanol-complex (BMGY/BMMY) media were carried out as shown by Supplementary Material. The effect of initial pH 4–7 of media on the production of target proteins was also investigated. BMMY containing 0.5% and 1.0% methanol were used for the production of rSfBGL1 (recombinant SfBGL1) and rTrBGL1 (recombinant TrBGL1), respectively. The pH 5.0 of BMGY/BMMY was used to investigate the effect of methanol concentration on production, and methanol was added to a final concentration of 0.25%, 0.5%, 1%, 1.5% and 2%, respectively. The culture was taken daily to determine cell growth and expression levels of rBGLs (recombinant β-glucosidases). The culture supernatants were collected for SDS-PAGE analysis and activity assay of β-glucosidase. The activity was expressed as units per milliliter culture media.

2.3. Enzyme assay

The β-glucosidase activity was determined by measuring the hydrolysis of p-nitrophenyl-beta-D-glucopyranoside (pNPG, Sigma). The activity assay was conducted in a reaction mixture (1 ml) consisting of 100 μL of an appropriately diluted supernatant sample or purified rBGLs, 50 mM sodium acetate buffer (pH 5.0) and 5 mM pNPG. The reaction was conducted at 40 °C for 10 min, and then terminated by adding 1 ml of 1 M Na_2CO_3. After 5 min, the absorbance of the resulting mixture was measured at 405 nm, and the concentration of released pNP (p-nitrophenol) was calculated. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol PNP per minute under the conditions of the assay. Relative expression levels of recombinant enzyme in culture supernatants were evaluated by the units of β-glucosidase activity per milliliter of culture (u/ml). All enzyme assays were performed in triplicate.

2.4. Optimization of β-glucosidase production by response surface method

Response surface method was used to investigate the combined effect of pH and temperature on the production. A two-factors, five-levels central composite design (CCD) was used. The cultures were incubated at 28 °C with shaking at 250 rpm for 12 days. The culture supernatants from day 10 were used to measure the activity of rBGLs. Enzyme activity value per ml of supernatant liquid (u/ml) was used as the response value. Design Expert™ version 7.0.3 (Stat-Ease, Inc., Minneapolis, USA) was used for experimental design and data analysis (Dong et al., 2012). The experimental results are usually fitted via the response surface regression procedure based on the following second-order polynomial equation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_1 x_2 + \beta_4 x_1^2 + \beta_5 x_2^2 \quad (1)$$

where Y is the predicted response, β_1 and β_2 are linear coefficients, β_4 and β_5 are quadratic coefficients, β_3 is interaction coefficients and β_0 is the constant coefficient. Purification and deglycosylation of β-glucosidases

The rBGLs with C-terminal hexahistidine (His 6) tag were purified using Ni-NTA His·Bind resins system (Merk, 70666) according to the manufacturer's instructions with slight modification as previously described (Wang et al., 2014; Liu et al., 2014). Purified proteins were exchanged by ultrafiltration into 50 mM NaAc buffer (pH 4.8) and then quantified by Bradford's assay for functional investigation. N-linked and O-linked glycosylation sites were predicted, and the N-linked glycosylation of recombinant proteins was confirmed by digestion of purified protein with PNGase F (NEB, P0704S) as described in Supplementary Material. Deglycosylation of protein was assessed by a shift in electrophoretic mobility on a 10% SDS-PAGE gel.

2.5. Measurement of kinetic parameters

The Michaelis–Menten constant (K_m) and the maximal reaction velocities (V_{max}) were determined at 40 °C using pNPG (0.04–0.18 mM) as substrate by Lineweaver–Burk plot. The reaction was performed as described in Section 2.3. The turnover number (k_{cat}) is the number of moles of substrate converted to product each second per mole of enzyme, and described as following formula:

$$k_{cat} = \frac{V_{max}}{E_{total}}$$

The specific activity, a quantity that is used to monitor enzyme purification, is defined as units per microgram of protein. Effect of metal ions and chaotropic agents on activity

The residual activity in presence of various metal ions and reagents was determined after pre-incubating purified rBGLs in 50 mM sodium acetate buffer (pH 5.0) containing different concentration of salts and detergents at 20 °C for 1 h. A reaction mixture with pNPG but without β-glucosidase was used as a negative control. The absorbance value of control with each chemical was subtracted from that of reaction containing that chemical, resulting in a final activity value. The relative activity was defined as the

Please cite this article in press as: Ma, Y., et al., Expression optimization and biochemical properties of two glycosyl hydrolase family 3 beta-glucosidases. J. Biotechnol. (2015), http://dx.doi.org/10.1016/j.jbiotec.2015.04.016