Journal of Biotechnology 186 (2014) 148–155

Contents lists available at ScienceDirect

Journal of Biotechnology

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

Heterologous expression and functional characterization of a novel cellulose-disruptive protein *Le*EXP2 from *Lycopersicum esculentum*

Xuewei Liu[a,b], Cheng Liu[a], Yuanyuan Ma[a,*], Jiefang Hong[a], Minhua Zhang[a,c]

[a] *R&D Center for Petrochemical Technology, Tianjin University, Tianjin 300072, China*
[b] *Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China*
[c] *State Key Laboratory of Engine, Tianjin University, Tianjin 300072, China*

ARTICLE INFO

Article history:
Received 11 April 2014
Received in revised form 1 June 2014
Accepted 8 July 2014
Available online 17 July 2014

Keywords:
Expansin
Heterologous expression
Cellulose hydrolysis
Synergistic activity
Robustness
Binding characteristic

ABSTRACT

There has been little research reported on the synergism of plant expansins in cellulose hydrolysis because of the difficulty of heterologous expression. In this study, the expansin gene *LeExp2* of *Lycopersicum esculentum* was expressed for the first time in *Pichia pastoris* to identify its function in vitro. The optimal expression level was obtained by adding PMSF and a commercial protease inhibitor, and the maximum expression level was 70.9 mg/L. The affinity-purified *Le*EXP2 displayed cellulose-weakening activity and synergism with cellulase, and the reducing sugar yield in the reaction mixture with *Le*EXP2 was 1.4–9.8-fold that of control. The optimum pH and temperature for reducing-sugar liberation by *Le*EXP2 and endoglucanase were pH 4.8 and 50 °C. Cu^{2+} and Fe^{2+} notably increased the synergistic activity of *Le*EXP2, and Co^{2+} enhanced the activity of endoglucanase but failed to improve synergism. *Le*EXP2 exhibited a high level of robustness to reagents and heat, indicating its potential application for cellulose decomposition. *Le*EXP2 preferentially bound to cellulose over lignin, and the binding capability to given substrates was not related to the crystallinity. This is the first study to characterize the functional role of *Le*EXP2 in cellulose degradation.

1. Introduction

Lignocellulosic biomass has attracted considerable attention as a promising renewable energy feedstock. However, the enzymatic conversion of lignocellulose into sugars is inherently difficult because of the tightly bound hydrogen-bonding network of cellulose (Himmel et al., 2007). As a result, either physical or chemical pretreatment is required to overcome its recalcitrance, but pretreatment produces some toxic byproducts (Huang et al., 2009a). Efforts have been made to develop and employ accessory proteins that have non-hydrolytic disruptive activity on crystalline cellulose. Recent studies have confirmed the synergism of cellulase with non-hydrolytic proteins such as plant expansins (Baker et al., 2000; Cosgrove, 2001), bacterial expansins (Kang et al., 2013; Kim et al., 2009), fungal expansins (Quiroz-Castañeda et al., 2011) and grass pollen group-2/3 allergens (GR2) (Cosgrove, 2007), all of which facilitate the hydrolysis of cellulose.

Expansins, which are secreted by plant cells, play an important role in physiological processes, and belong to a multi-gene family that includes α and β expansin-like A, expansin-like B and the other non-plant expansin-like group X (Sampedro and Cosgrove, 2005). However, to date, only a few plant expansins, such as cucumber Ex29/Ex30 and maize EXPB1, can increase the efficiency of enzymatic hydrolysis in a synergistic manner (Baker et al., 2000; Cosgrove, 2001).

Interestingly, more expansin-like proteins from bacteria and fungi have been reported to be involved in cellulose hydrolysis than plant expansins. One reason is that the isolation of one member of the expansin family is troublesome because there are multiple members of the family. Another reason is that the heterologous expression of expansin is difficult and unsuccessful to date (Kerff et al., 2008), and proteins expressed in bacteria often form inclusion bodies (Jungbauer and Kaar, 2007). A complex purification process and unsuccessful expression thus prevent detailed investigation of the synergism of expansins and cellulases. The expression of recombinant proteins in *Pichia pastoris* offers several advantages such as high-level secretion of foreign proteins and post-translational processing. Expansin-like proteins *Bs*EXLX1 from *Bacillus subtilis* and *Tr*Swo1 from *Trichoderma reesei* have been

* Corresponding author. Tel.: +86 22 87401536; fax: +86 22 27406119.
E-mail address: myy@tju.edu.cn (Y. Ma).

http://dx.doi.org/10.1016/j.jbiotec.2014.07.013
0168-1656/

successfully expressed in *P. pastoris* (Wang et al., 2014). Hence, *P. pastoris* is a good candidate for the production of expansins.

The binding characteristic of several expansin-like proteins to biomass (Georgelis et al., 2012; Lin et al., 2013) and structural-functional relationships of *Bs*EXLX1 (Kerff et al., 2008; Kim et al., 2009, 2013) have been reported, providing valuable informations for the understanding of synergistic action in cellulose hydrolysis. However, some microbial expansins can cause the extension of cell walls in vitro and weaken filter paper networks, but they do not show synergistic activity in the cellulose hydrolysis (Georgelis et al., 2014). Accordingly, it is necessary to identify the synergistic function of plant expansins for their application in cellulose hydrolysis.

In this study, gene database searches identified *Le*EXP2 from *Lycopersicum esculentum* (Genebank accession no. AAC64201.1) with high sequence similarity (83.0%) to *Cs*EXPA1 (AAB37746.1) from *Cucumis sativus*, which displays a synergism in cellulose hydrolysis, suggesting that *Le*EXP2 is a potential cellulase synergist. We first tried to express and optimize the production of *Le*EXP2 in *P. pastoris* to disclose a new function in hydrolysis. The binding characteristic and robustness were also investigated to evaluate its capability as a cellulase synergist.

2. Materials and methods

2.1. Bioinformatics analysis and heterogeneous expression of *LeEXP2*

Bioinformatics analysis of *Le*EXP2 was conducted as described in supplementary data. The gene encoding *Le*EXP2 (AF096776) from *L. esculentum* was amplified and inserted into pPICZαA vector (Supplementary Fig. 1). *Le*EXP2 gene was then integrated into *P. pastoris* X33 genomic DNA by electroporation, and positive transformants were verified by PCR as shown by Supplementary Fig. 2. Induction experiments with Buffered Glycerol/Methanol-complex (BMGY/BMMY) media were carried out (Supplementary Material). To study the effect of protease inhibitors on *Le*EXP2 expression, *P. pastoris* strain L4–1 was cultured in pH 6.0 BMMY medium containing 1.0% (v/v) methanol supplemented with 0.5 and 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 and 3 mM EDTA (ethylenediaminetetraacetic acid), and 1/5, 2/5, 3/5, and 4/5 tablet of commercial protease inhibitor cOmplete ULTRA Tablets (EDTA-free Roche, later called 1/5, 2/5, 3/5, and 4/5 CUT). The protease inhibitors were supplemented on day 0, and the control group did not contain any protease inhibitor. The supernatant was prepared daily to detect the total protein concentration by sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot. Western blot was carried out by the method reported in a previous study (Wang et al., 2014). The integrated optical density (IOD) of target bands on western blot images was analyzed with Gel-Pro 4.5 software, and the IOD values of each band were summed to yield total IOD values. The total proteins concentration in culture supernatants and the total IOD values of target bands were combined to quantify the expression level.

2.2. Purification of recombinant LeEXP2 and deglycosylation assay

The cultures were taken after 4 days, and *Le*EXP2 was purified via His-tag affinity chromatography as described previously(Wang et al., 2014) with minor revision (Supplementary Material). Purified *Le*EXP2 was exchanged by ultrafiltration into 0.05 M citrate buffer (50 mM citric acid, 1 mM NaOH, pH 4.8) and ultra-sterile water for functional investigation and deglycosylation analysis, respectively. Deglycosylation was performed by digesting proteins in denaturing and non-denaturing reaction mixture with PNGase F (NEB, USA) and Protein Deglycosylation Mix (NEB, P6039) according to the instruction manual. The digested proteins were assessed by western blotting to check the electrophoretic mobility shift. Proteins were quantified by the Bradford assay.

2.3. Swelling assay and a measure of synergism

Whatman filter paper No. 1 (Whatman, Florham Park, NJ) was incubated in buffer solution with (0.75 or 1.0 μg/mg filter paper) or without *Le*EXP2 at 50 °C for 18 h with agitation at 150 rpm, and was then dried in a vacuum drying-oven at 45 °C for 24 h. Samples were coated with goldpalladium, and photomicrographs were then taken using a scanning electron microscope (Hitachi S-4800, Tokyo, Japan) at a voltage of 3.0 kV.

Five milligrams of filter paper, *Le*EXP2 (0–1.5 μg per mg of cellulose), and commercial endoglucanase (EC 3.2.1.4, Megazyme) (0–0.016 U per mg of cellulose) were added to a final volume of 200 μL of 0.05 M citrate buffer (pH 4.8). Buffer solution containing only filter paper was used for blank correction, and samples only containing *Le*EXP2 or endoglucanase were used as control. Hydrolysis reactions were conducted as previously described (Wang et al., 2014). All experiments were carried out in triplicate and expressed as the mean ± the standard deviation. The synergistic activity is calculated as follows:

$$\text{Synergistic activity}(\%) = \left\{ \left[\frac{\text{reducing sugar released by LeEXP2 and endoglucanase}}{\text{reducing sugar released by endoglucanase alone}} \right] - 1 \right\} \times 100$$

2.4. Effect of reagents on the synergism of LeEXP2 in cellulose hydrolysis

Metal ions (0, 1, and 10 mM) were added to reaction mixture containing 1 μg *Le*EXP2 and 0.008 U endoglucanase per mg filter paper, and control reactions without *Le*EXP2 were performed as described above. *Le*EXP2 was pre-incubated in citrate buffer containing urea (1 M or 2 M), SDS (0.5% or 1.0%) and ethanol (10.0% or 20.0%) at 25 °C for 240 s to investigate the effect of denaturants on synergistic activity. These denaturing detergents were subsequently removed from the proteins via repeated ultrafiltration using 3 kDa ultrafiltration membranes (Pall Corporation, USA). The treated *Le*EXP2 was then immediately used for hydrolysis assays.

2.5. Analysis of the binding activity of LeEXP2

Filter paper (Whatman No. 1), Avicel PH101 and xylan from birchwood (Sigma–Aldrich) were purchased as binding matrices. Phosphoric acid-swollen cellulose (PASC) was prepared by pretreating Avicel PH101 with 85.0% (w/w) phosphoric acid. The ACID was produced to remove mainly hemicellulose by incubating corn cobs in dilute sulfuric acid, and the ACID/ALKALI was prepared to generate cellulose-rich corn cob by using sulfuric acid and NaOH to remove major hemicellulose and lignin as previously described (Huang et al., 2009b). An excess of purified *Le*EXP2 (60 μg) was incubated with 10 mg of the above substrates in 400 μL 0.05 M citrate buffer (pH 4.8) at 25 °C for 1 h with shaking at 250 rpm, and the supernatant was then collected for protein quantification. The amount of bound protein was calculated by subtracting the amount of the unbound protein from that of total protein.