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Full Length Research Paper

Recombinant EXLX1 from *Bacillus subtilis* for enhancing enzymatic hydrolysis of corn stover with low cellulase loadings

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BsEXLX1 protein from *Bacillus subtilis* has been proposed to have a structure that is similar to plant expansin. In this study, the recombinant BsEXLX1 protein was successfully expressed and purified in *Escherichia coli* BL21 (DE3). When the purified BsEXLX1 which contained the thioredoxin (Trx) protein was incubated with low-dose cellulases either simultaneously or sequentially, it showed a significant synergistic activity in corn stover hydrolysis. Furthermore, an even greater increase in the synergistic activity was obtained when cellulose was pretreated with BsEXLX1 followed by cellulase hydrolysis, and the synergistic activity was found as high as 1.5-fold greater than that when cellulose was treated simultaneously with the same concentrations of BsEXLX1 and cellulases. These results provided a feasible way for the potential application of BsEXLX1 in the efficient saccharification of cellulose materials for bioethanol production.

Key word: *Bacillus subtilis*, BsEXLX1, cellulase, cellulose hydrolysis, bioethanol

INTRODUCTION

Bioethanol is one of the most important biofuel as a result of its potential use as an automotive fuel and its environmental, energy and socioeconomic advantages relative to fossil fuel consumption and greenhouse gases emissions reduction. Recently, lignocellulosic materials are thought to be the most appropriate feedstocks for ethanol production because they are abundant, cheap and renewable, most importantly they are not subject to the ethical concerns associated with the use of a potential food resource (Zhao et al., 2007). Currently, the performance of lignocellulosic biomass-to-ethanol processes includes pretreatment, enzymatic saccharification and fermentation. However, the large amounts of enzymes required for enzymatic conversion of cellulose to fermentable sugars impact severely on the cost effectiveness of this technology. In order to solve this problem, researchers have focused on enhancement of cellulase productivity and optimization of cellulose hydrolysis (Chang and Holtzapple, 2000). On the other hand, different additives, such as surfactants (Kristensen et al., 2007), bovine serum albumin(BSA) (Brethauer et al., 2011), expansin or expansin-like protein (Arantes and Saddler, 2010) were also used in enzymatic hydrolysis of lignocellulosic materials.

Previous study indicated that expansin has the ability to disrupt hydrogen bonds between complex polysaccharides in plant cell walls and enhance the accessibility of cell wall degrading enzymes (McQueen-Mason et al., 1992; Sampedro and Cosgrove, 2005).

The expansin superfamily is made up of five subcategories: four major plant subcategories (EXPA, EXPB, EXLA and EXLB) and all the other non-plant

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expansin-related proteins (EXLX) (Kende et al., 2004). Here, the BsEXLX1 protein from *B. subtilis*, which we selected, was found to possess structural features and wall extension activities characteristic of plant expansins (Kerff et al., 2008). Although the BsEXLX1 protein has been shown as the significant synergism with cellulases at low level on filter paper (Kim et al., 2009), however, this is the first time that using corn stover as the substrate to verify its functions is reported.

So, in our work, the gene of BsEXLX1 from *B. subtilis* was cloned and then heterologously expressed in *Esherichia coli*. The purified BsEXLX1 was finally used in enzymatic hydrolysis of acid-pretreatment corn stover through two different treatment methods. The results of this study showed different outcomes by different methods, and an ideal result had been obtained when cellulose was pretreated with BsEXLX1 followed by cellulase hydrolysis.

MATERIALS AND METHODS

Compositional analysis of corn stover

Corn stover was obtained from Mount Emei (Sichuan Province, China) in July 2010 (He et al., 2011). The collected corn stover was dried in an oven at 45°C to obtain constant weight, milled and sieved to 0.5-mm-diameter particles. The material was mixed to obtain a homogeneous sample and stored at room temperature in polyethylene bags. The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) fractions of corn stover were determined according to Van Soest's et al. (1991) method by Foss Fibertec 2010 (Sweden) (Yu et al., 2009; Wen et al., 2010). The hemicellulose content of diets was estimated as the numerical difference between NDF and ADF. The cellulose content was estimated as the numerical difference between ADF and ADL. The lignin content is presented as ADL.

Construction of BsEXLX1 expressed plasmid

All DNA manipulation, including plasmid preparation from *E. coli*, restriction enzyme digestion, ligation, *E. coli* transformation, agarose gel electrophoresis and Western blotting were performed according to standard protocols. Plasmid pMD19-T (Takara, China) and *E. coli* DH5α were used for plasmid cloning and amplification of the target gene. Plasmid pET32a-c (+) (Novagen) and *E. coli* BL21 (DE3) were used for the expression of the cloned gene. Restriction enzymes and T4 DNA ligase were purchased from Fermentas (Lithuania) and Takara, respectively.

The target gene was obtained by PCR amplification using *Bacillus subtilis* genomic DNA as template. The genomic DNA of *B. subtilis* was kindly provided by Sichuan Key Laboratory of Molecular Biology and Biotechnology (Chengdu, China). The following primers, which were synthesized by BGI (Shenzhen, China) were used to amplify the BsEXLX1 gene: P1 (5'-*GCGGGGTACC*GACGACGACGACAAGATGAAAAAGATCA-3') and P2 (5'-*CGAGTGCGGCCGC*TTATTCAGGAAACTG-3'), containing *Kpn* I and *Not* I restriction sites, respectively (underlined). A 699 bp BsEXLX1 fragment amplified with primers P1 and P2 from the chromosomal DNA of *B. subtilis* was directly cloned into pMD19-T vector, and then digested with *Kpn* I and *Not* I. Finally, the 699 bp target product was recovered from the gel, and then cloned into the pET32a-c (+) to give plasmid pBsEXLX. The recombinant plasmid was further confirmed by restriction enzyme digestion and sequencing (Invitrogen, China). A 6×hexahistidine (His)-tag in pBsEXLX1 allows easy purification of the fusion protein by metal affinity chromatography.

Expression and purification of the BsEXLX1 protein

E. coli BL21 (DE3) transformants containing recombinant plasmid were grown in Luria- Bertani (LB) broth (Tryptone 1.0%, yeast extract 0.5%, NaCl 1.%) containing 100 μg/ml ampicillin until the absorbance of the media at 600 nm was 0.6 to 0.7. Expression was then induced by adding 1.0 mM isopropyl-β-D-thiogalactoside (IPTG) to the media and incubating at 30°C for 3 h. The cells were then harvested by centrifugation at 8,000 rpm for 10 min at 4°C, the cell pellet was resuspended in a lysis buffer (20 mM sodium phosphate and 0.5 M sodium chloride, pH 7.4) and then disrupted by sonicate for 15 min in a sonicator (Sonics & Materials, U.S.). Next, the suspension was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was then used as the soluble fraction of the protein, while the pellet was resuspended in a lysis buffer and used as the insoluble fraction. Both fractions were analyzed by 12.0% (w/w) sodium dodecyl sulphate-polyacrylamide gel electrophresis (SDS-PAGE). The soluble fraction was loaded onto a His-trap FF crude column (GE Healthcare, U.S.), which had been equilibrated with a lysis buffer. The column was washed with 10 column volumes of buffer containing 20 mM imidazole. Then, the His-tagged target protein was collected with a gradient elution using AKTA prime system (GE Healthcare, U.S.). The eluted protein was desalted with 20.0 mM sodium phosphate buffer and quantified using a protein assay kit (Bio-Rad Laboratories, U.S.). Furthermore, the recombinant BsEXLX1 protein was detected with Western blot experiment by mouse monoclonal antibody (Cwbio, China) and eECL Western blot kit (Cwbio, China).

Acid pretreatment

Corn stover samples were pretreated with 1.5% sulfuric acid (1:10 solid: liquid proportion) (He et al., 2011). The mixture was autoclaved at 121°C for 1 h. After cooling, the treated biomasses were washed with deionized water several times. Then, the biomass was dried at 45°C in order to fix the moisture for enzymatic hydrolysis. All reagents used in this study were of analytical grade.

Synergism analysis for BsEXLX1 and cellulases in cellulose hydrolysis

Different amount of purified BsEXLX1 or BSA and a commercial cellulase mixture (2.7 filter paper activity units/g cellulase) from the Ningxia xiasheng Co. Ltd. (Ningxia, China) diluted to a final concentration of 0.1 FPU/g cellulose were incubated simultaneously or sequentially with 5.0% (w/v) of pretreated corn stover in 50.0 mM citrate buffer solution (pH 4.8) for 48 h. Triplicate samples were then evaluated every 12 h to assay the released glucose levels. When treated sequentially, the substrate was first incubated with BsEXLX1 alone at 50°C on the rotary shaker at 150 rpm for 48 h and then washed extensively with 50.0 mM citrate buffer solution (pH 4.8) to remove BsEXLX1 before corn stover was further incubated with cellulases.

Analytical methods

The amount of released glucose was determined by ions chromatography (871 Advanced BioScan, Metrohm AG, Switzerland) (He et al., 2011; Masarin et al., 2011). Sodium hydroxide (0.1 M) was

Table 1. Corn stover composition before and after acid hydrolysis treatment.

Component	Dry matter (%, w/w)	
	Before	After
Cellulose	32.90	51.1
Hemicellulose	28.13	4.33
Acid-insoluble lignin	6.35	18.35

used as mobile phase at a flow rate of 1 ml/min. Sugars were quantified by comparing their peak areas with standard sugar of known concentrations. Prior to ion chromatography injection, all samples (derived from hydrolysate) were boiled for 10 min, and centrifuged at 8,000 rpm for 10 min; the supernatants were collected and then filtered through a 0.2 µm membrane (Millipore, China).

As the BsEXLX1 has no hydrolysis activity, the synergistic activity is calculated as follows when BsEXLX1 was added either simultaneously or sequentially with corn stover:

$$synergistic\ activity = \left(\frac{glucose\ released\ by\ BsEXLX1\ and\ cellulase}{glucose\ released\ by\ cellulase\ alone} - 1 \right) \times 100\ \%$$

RESULTS AND DISCUSSION

Compositional analysis of corn stover

The chemical composition of corn stover varies according to its growth location, season, harvesting method, as well as analysis procedure (National Renewable Energy Laboratory). The chemical components for corn stover were analyzed before and after acid pretreatment (Table 1). Based on the Foss Fibertec 2010 analysis, the corn stover contains cellulose (32.90%, w/w), hemicelluloses (28.13%, w/w) and acid-insoluble lignin (6.35%, w/w). After acid hydrolysis, the pretreated corn stover contained the followed proportions: cellulose (51.10%, w/w), hemicelluloses (4.33%, w/w) and acid-insoluble lignin (18.35%, w/w). Thus, cellulose and lignin in corn stover increased significantly, and hemicelluloses decreased simultaneously. These results indicate that almost all the hemicelluloses were completely hydrolyzed with the acid treatment, which was convenient for the cellulase or BsEXLX1 to attack the tested corn stover.

Expression and purification of BsEXLX1

The specific primers P1 and P2 were used to amplify the DNA of BsEXLX1, which was finally cloned into pET32a-c (+) to construct the recombinant expression vector, pBsEXLX1. Sequence analysis indicated that the target gene of BsEXLX1 was 699 nucleotides in length. The BsEXLX1 fusion protein was highly expressed in *E. coli* BL21 cells after induction by adding 1.0 mM IPTG for 3 h at 30°C. SDS–PAGE analysis of cell lysates revealed a major protein band, which corresponds to the size of BsEXLX1 with a 6×His tag at its N-terminus (Figure 1, lanes 5, 6), and the Trx protein which was expressed by BL21/pET32a had an apparent molecular weight of 20.0 kDa (Figure 1, lane 2). As shown in Figure 1, the recombinant BsEXLX1 protein was expressed both in the form of insoluble fraction and soluble fraction at high level in *E. coli*. In this study, we collected the soluble fraction to purify the recombinant protein with a single protein band (Figure 1, lane 7). And western blot analysis illustrated the exact outcome (Figure 1, lane 8). Furthermore, the purified protein BsEXLX1 which contained the Trx protein was used to detect straightly without digestion with enterokinase.

Synergism activity of BsEXLX1 and cellulase

Expansins were important because of their unique ‘loosening’ effect on the cellulosic network within plant cell walls during growth since they were first identified in the early 1990s (McQueen-Mason et al., 1992; Cosgrove, 2005). And recently, with the appraisal of more and more expansins and expansin-like proteins, these non-hydrolytic proteins were given enough attention for the efficient bioconversion of cellulosic biomass (Han and Chen, 2007; Kim et al., 2009). EXLX1, which was secreted by *B. subtilis*, has been researched for the cellulose-binding and cellulose-weakening activities towards filter paper (Kim et al., 2009). However, it is recognized that filter paper assays provide a poor estimate of the ability of cellulase preparations to hydrolyze the complex lignocellulosic substrates typically encountered in biomass conversion research and development (Berlin et al., 2006). So, the synergistic activity was performed by different amount of BsEXLX1 and low-dose cellulases either simultaneously or sequentially using corn stover as substrate.

As shown in Figure 2A and B, a synergistic effect was observed when different concentrations of BsEXLX1 (100, 200, 300, 400 or 500 µg) were added simultaneously or sequentially with cellulases (0.1 FPU/g cellulose). The synergistic activity increased obviously at first until 300 µg of BsEXLX1 was incubated for 48 h, and its activity decreased when the higher concentrations of BsEXLX1 (400 and 500 µg) were used at both conditions. However, higher synergistic activity was observed when BsEXLX1 and cellulase were sequentially added (Figure 2A and B). It indicated that a competition for binding sites

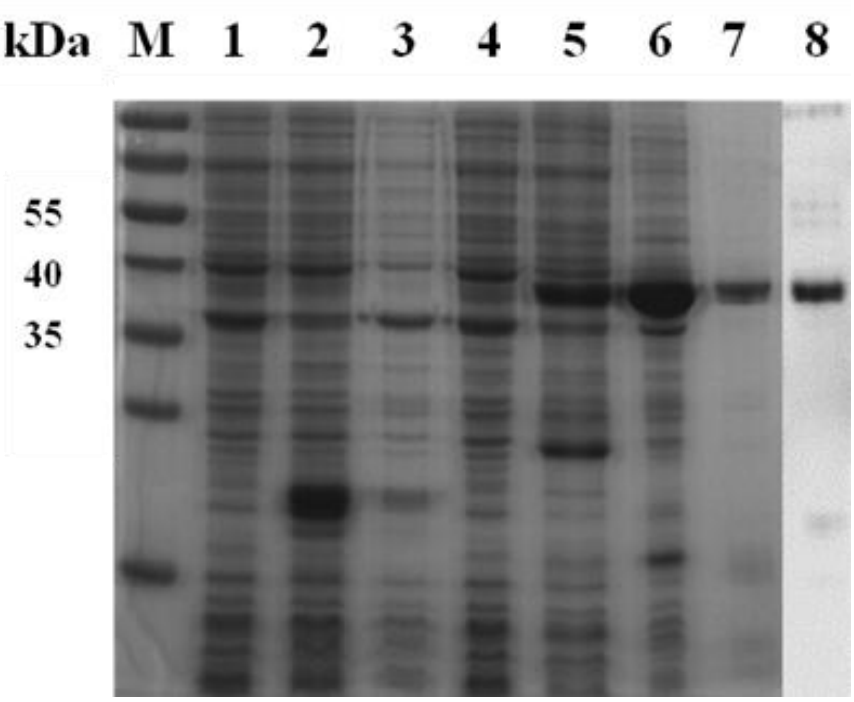

Figure 1. SDS-PAGE analysis of the recombinant BsEXLX1 protein. Lane 1, uninduced BL21/pET32a; lane 2, the supernatant of the induced BL21/pET32a culture; lane 3, the pellet of the BL21/pET32a culture; lane 4, uninduced BL21/pBsEXLX1; lane 5, the supernatant of the induced BL21/pBsEXLX1 culture; lane 6, the pellet of the BL21/pBsEXLX1 culture; lane 7, purified BsEXLX1 recombinant protein; lane 8, Western blotting analysis of purified BsEXLX1.

which resulted in the observed less distinctive synergistic effect (Kim et al., 2009). So, the competition between the BsEXLX1 and cellulase may be reduced by removing the bound BsEXLX1, which will have a positive effect on synergistic activity (Zhou et al., 2011).

The profiling of glucose produced from treated corn stover was also performed by using 300 µg of BsEXLX1 and low-dose cellulases (0.1 FPU/g cellulose) simultaneously and sequentially. Actually, incubation of the corn stover with BsEXLX1 (300 µg/g cellulose) alone could not produce any glucose after 48 h incubation in both treatment. However, when the substrate was incubated with cellulase alone, cellulase + BsEXLX1, cellulase + BSA, the extent of glucose production with time was significantly increased after 12 h incubation and the highest concentration of glucose was reached till 48 h in both treatments. Although, the substrate which was incubated with cellulase + BSA showed higher concentration of glucose than cellulase alone, the group of cellulase + BsEXLX1 showed the highest concentration of glucose (Figure 2C and D). The glucose yield from treated corn stover was 1.25- and 1.33-fold greater than cellulase alone in simultaneous and sequential treatments, respectively. These results show that the sequentially treatment with the BsEXLX1 and cellulase not only had no negative effect on the glucose yield, but could increase it to a certain degree.

Our results demonstrate that the BsEXLX1 protein which contained the Trx protein could enhance enzymatic hydrolysis of corn stover with low-dose cellulases loading. On the other hand, the influence of the Trx protein on the activity of BsEXLX1 also needs to be confirmed in the future.

Conclusion

In this study, the recombinant BsEXLX1 from *B. subtilis* was successfully expressed in *E. coli* BL21 (DE3). And the active protein which contained the Trx protein was purified from the soluble fraction. Although the influence of the Trx protein on the activity of BsEXLX1 was needed to be confirmed, a significant synergistic activity in cellulose hydrolysis was observed when BsEXLX1 (300 µg/g cellulose) was incubated with low-dose cellulases (0.1 FPU/g cellulose), which was higher than BSA at the same condition. Specifically, an even greater increase in the glucose yield was obtained when cellulose was pretreated with BsEXLX1 followed by cellulase hydrolysis. Our results provide a feasible way for the potential application of BsEXLX1 in the efficient saccharification of cellulose materials.

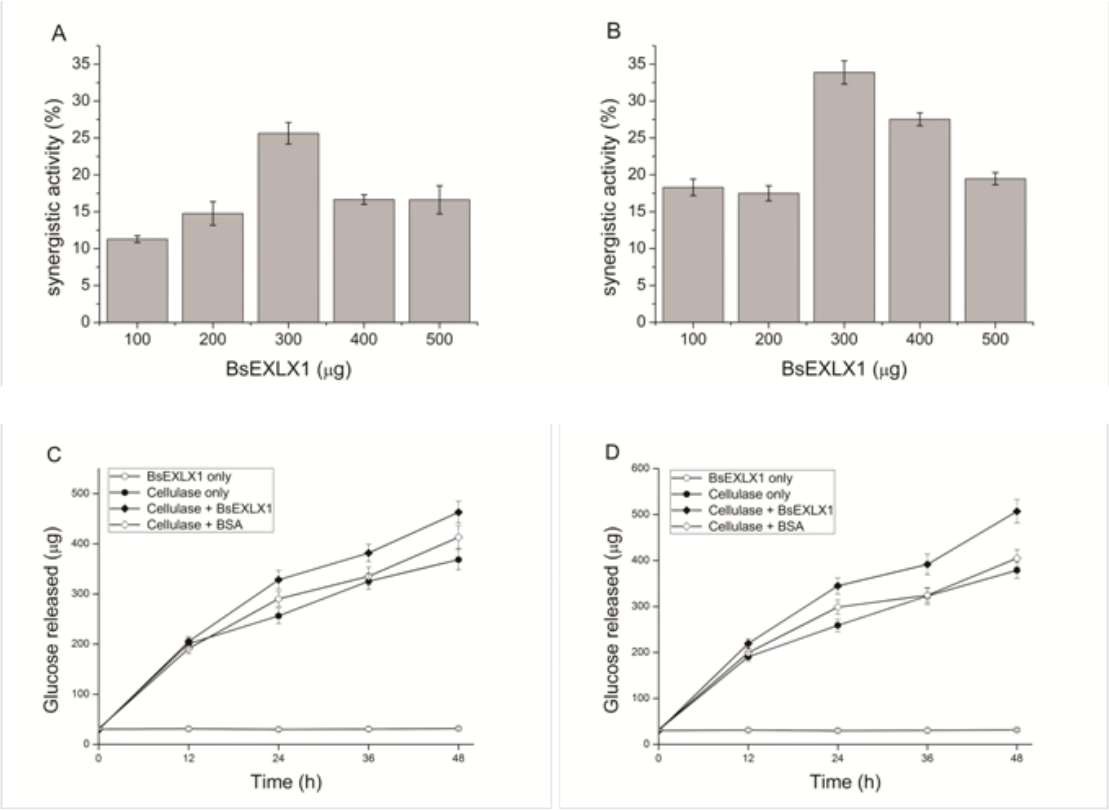

Figure 2. Synergism activity of BsEXLX1 and cellulases incubated simultaneously and sequentially. A and C: Synergistic activity of purified BsEXLX1 and cellulases incubated simultaneously; B and D, Synergistic activity of purified BsEXLX1 and cellulases incubated sequentially; 0.1 FPU of cellulases with or without different concentration of BsEXLX1 or BSA in 50 mM citrate buffer solution (pH 4.8) at 50°C was used. All experiments were performed in triplicate, and the data points and error bars indicate the means ± standard deviations.

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