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A comparative study of β-1, 4-endoglucanase (possessing β-1, 4-exoglucanase activity) from *Bacillus subtilis* LH expressed in *Pichia pastoris* GS115 and *Escherichia coli* Rosetta (DE3)

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ABSTRACT

β-1, 4-Endoglucanase (EG) from *Bacillus subtilis* LH was expressed in *Escherichia coli* Rosetta (DE3) and *Pichia pastoris* GS115, respectively. The CMCase activity of EG (EGE) from the cell lysate of DE3 reached 20,010 U/ml, and that of EG (EGP) from the supernatant of GS115 was only 2008 U/ml. EGE and EGP were bifunctional cellulases excluding β-1, 4-glucosidase (BGL). The CMCases of them, optimally active at 65 °C and pH 6.8, exhibited more than 80% residual activity at pH 5–10 and 60% activity at 40–70 °C and pH 5–9. EGE (EGP) mixed with BGL had more than 1.5-fold higher CMCase and filter paperase activities compared to EGE (EGP). *N*-glycosylation protected EGP from immobilized-papain attack and accounted for 30 kDa and a higher thermostability, whereas EGE was decomposed into a 33 kDa active truncated EG (EGT) and two 18 kDa fragments. EGE and EGP performed much better than EGT in denim biostoning.

1. Introduction

Cellulose, a β-1, 4-linked homopolymer of D-glucose, is the main structural material in natural environment. Cellulases, consisting of endo-1, 4-β-D-glucanases (EC 3.2.1.4, EG), exo-1, 4-β-D-glucanases (EC 3.2.1.91, CBH) and β-glucosidases (EC 3.2.1.21, BGL), play essential roles in the degradation of cellulosic materials and carbon cycle (Kim et al., 2008; Sánchez, 2009). Cellulase cocktails can significantly increase ethanol yield during the simultaneous saccharification and fermentation of cellulose (Khramtsov et al., 2011; Vasan et al., 2011; Wood et al., 1997). Wild-type strains of *B. subtilis* are not candidates for the commercial production of EG because of catabolite repression, end-product inhibition and protease attack (Hueck and Hillen, 1995; Wu et al., 1991; Zhang and Zhang, 2010). The heterologous expression of *B. subtilis* EG in *E. coli* have been carried out, but the expression level of the recombinant EG is very low (Li et al., 2009; Tang et al., 2009; Yang et al., 2010).

Enzymes from *Pichia pastoris* are very easily *N*-glycosylated by post-translational modification. The properties of glycosylated EGs are not distinct from those of deglycosylated EGs. However, thermal stabilities of recombinant EGs can be decreased or increased after glycosylation (Li et al., 2007). Boer et al. (2000) has reported that *N*-glycosylation of *Trichoderma reesei* Cel7A expressed in *P. pastoris* reduces the degradation rate on crystalline. It is well known that the native EG from *B. subtilis* do not be glycosylated, whereas there is little information whether the glycosylation of *B. subtilis* EG expressed in *P. pastoris* has an effect on the enzyme properties.

EGs are known to be key enzymes that produce aged appearances in denim (Heikinheimo et al., 2000). Acid EGs mainly from *T. reesei* are commercially used in denim biostoning because of their low price and high activities, whereas they cause backstaining of indigo dye and severely damage denim strength (Andreaus et al., 2000). To solve the above problems, on the one hand, acid EGs by limited protease digestions are decomposed into core catalytic domains (CD) that can deal with denim (Andreaus et al., 2000), but it is not very useful in industry. On the other hand, neutral EGs are excellent substitutes for acid EGs such as *Humicola insolens* EG V (Cavaco-Paulo, 1998). Cellulose-binding domains (CBD) of neutral EGs can improve the adsorption capacity on avicel and increase indigo dye removal and weight loss of denim (Wu et al., 2007), whereas *Penicillium verruculosum* Eg3 and *Melanocarpus albomyces* Cel45A without their CBDs do well in denim biostoning (Gusakov et al., 2000; Haakana et al., 2004). Accordingly, CBD may not be essential in the denim biostoning process. It has been demonstrated that indigo dye removal is related to Surface Hydrophobic Amino Acid Residues and Aromatic Amino Acids (SHAARAAA) (Gusakov et al., 2000). However, whether the denim

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biostoning efficiencies of CD and the intact EG are related to SHAARAAA have been not reported yet.

Several *B. subtilis* EGs have been purified and characterized (Li et al., 2009; Robson and Chambliss, 1984; Yang et al., 2010). Here, the expression level and properties of *B. subtilis* LH EG synthesized in *E. coli* Rosetta (DE3) (EGE) and *P. pastoris* GS115 (EGP) were compared. Furthermore, the reason for the denim biostoning efficiencies of EGP, EGE and the truncated active EG (EGT) was also clarified.

2. Methods

2.1. Cloning of EG gene from B. subtilis LH

The full-length EG gene from *B. subtilis* LH was amplified using two oligonucleotide primers, 5′-ATGATGCGAAGGAGGAAAAGATC-3′ and 5′-CTAATTKGGTTCTGWTCCCCAAATC-3′. PCR was conducted in a final volume of 20 μl containing 10 μl of 2 × ExTaq (TaKaRa) mix reaction buffer, 100 ng of template genome DNA, 2 μl of 0.2 μM each of forward and reverse primers and 5.7 μl of filter-sterilized water. The thermal cycling conditions included an initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1.5 min. Then a final extension step was carried out at 72 °C for 10 min. The amplified PCR product was purified and cloned into the pMD19-T Simple Vector (TaKaRa).

2.2. Expression of EG from B. subtilis LH in P. pastoris GS115

The deduced mature sequence was amplified using 5′-GAATTCGCAGGGACAAAAACGCCAG-3′ (EcoRI restriction site underlined) and 5′-GCGGCCGCCTAATTTGGTTCTGTTC-3′ (NotI restriction site underlined). The PCR reaction condition was carried out as described above, and the purified PCR product was cloned into the pMD19-T Simple Vector. The mature EG gene from the pMD19-EG was ligated between the EcoRI and NotI sites of the pPIC9k vector (Invitrogen) using T4 DNA ligase (TaKaRa). pPIC9K-EG was linearized by SalI (TaKaRa). Then transformation of *P. pastoris* GS115 (Invitrogen) was performed by electroporation. *P. pastoris* GS115/pPIC9k-EG was grown slowly on minimal methanol plates (MM) after 4 days of incubation at 28 °C and screened on YPD plates with 3 mg/ml G418. Ten selected transformants were grown in 10 ml of BMGY medium at 28 °C for 24 h at 250 rpm in order to screen the transformant with the maximal activity, and the cells were recovered and then resuspended to 10 ml of BMMY at 28 °C at 250 rpm. Subsequently methanol was added to a final concentration of 0.5% at every 24 h until 72 h. *P. pastoris* GS115/pPIC9k-EG with the maximal EG activity was grown to an OD600 of 3–5 in a 250 ml Erlenmeyer flask including 25 ml of BMGY medium for 24 h at 250 rpm and 28 °C. The cells were harvested at 3000 rpm and 4 °C for 10 min and transferred to 25 ml of BMMY at 28 °C and 250 rpm. EGP was induced as described above.

2.3. Expression of EG from B. subtilis LH in E. coli Rosetta (DE3)

PCR amplification was carried out using two primers, 5′-CCATGGGCAGGGACAAAAACGCCAG-3′ (NocI restriction site underlined) and 5′-CTCGAGCTAATTTGGTTCTGTTC-3′ (XhoI restriction site underlined). The PCR reaction condition was carried out as described above. The deduced mature EG gene from the pMD19-T Simple Vector was inserted between the NocI and XhoI (TaKaRa) sites of pET-28a(+) (Novagen) introducing a 6-His tag at the C-terminus of EG. pET-28a(+)-EG was transformed into *E. coli* Rosetta (DE3) (Novagen). *E. coli* Rosetta (DE3)/pET-28a(+)-EG was grown at 37 °C for 3 h at 200 rpm in Terrific Broth (TB) medium (pH 7.2, 50 μg/ml Kanamycin and 34 μg/ml Chloramphenicol) containing 0.12% $MgSO_4$ and 0.4% $(NH_4)_2SO_4$. Subsequently α-lactose was added to a final concentration of 1.7% at 25 °C for 15 h at 170 rpm. Then, the cells were harvested by centrifugation (10,000 rpm) for 10 min at 4 °C. The cell pellets were resuspended in 25 ml of 20 mM sodium phosphate buffer (pH 7.4). After ultrasonication, the cell debris was removed by centrifugation at 10,000 rpm for 30 min at 4 °C.

2.4. Purification of EGP and EGE

For purification of EGP, the supernatant of the culture broth underwent dialysis against 25 mM Tris–HCl (pH 8.5) at 4 °C, and then was changed to a new buffer at every 12 h until 24 h. Then the supernatant was precipitated by 5 times volume of acetone at −20 °C for 10 min and centrifugated at 10,000 rpm at 4 °C for 10 min. The precipitated proteins were obtained at 4 °C for 24 h until dry and then dissolved in 25 mM Tris–HCl (pH 8.5). A DEAE-Sepharose FF (Amersham Bioscience) column (20 × 210 mm, equilibrated with 25 mM Tris–HCl, pH 8.5) was loaded with the supernatant and washed with equilibration buffer at 1 ml/min until the A280 of the effluent was equal to the value of the equilibration buffer. Binding proteins were eluted by applying a buffer solution (0.08 M NaCl, 25 mM Tris–HCl, pH 8.5). The eluted EGP was pooled, dialyzed (20 mM sodium phosphate buffer, pH 7.0, 0.2 M NaCl) and concentrated. The concentrated EGP was injected into a Sephadex G-100 (Amersham Bioscience) column (15 × 500 mm) and eluted with 20 mM sodium phosphate buffer (pH 7.0, 0.2 M NaCl) at 1 ml/min. The purified EGP were pooled and stored at 4 °C.

For purification of EGE, the cell pellets were resuspended in sodium phosphate buffer (pH 7.4, 0.5 M NaCl). The cell lysate was centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant was injected into HiTrap IMAC HP (GE Healthcare Bio-Sciences AB) at 1 ml/min, and binding proteins were washed with 0.1 M imidazole in 20 mM sodium phosphate buffer (0.5 M NaCl, pH 7.4). The first eluted EGE was loaded into HiTrap IMAC HP as washed above. The second eluted EGE were pooled and dialyzed to remove imidazole.

2.5. EG activity assay and protein assays

EG activity was measured by determining the amount of reducing sugars released from CMC. Fifty microliters of diluted EG was incubated with 450 μl of 1.5% CMC (50 mM sodium phosphate buffer, pH 6.8) for 30 min at 50 °C. The reaction was stopped by adding 500 μl DNS (Miller et al., 1960). Then the mixture was boiled for 10 min and cooled on ice to stabilize color. The absorbance of the samples at 540 nm was measured, and the release of reducing sugars was determined by glucose standard curve. One EG activity unit was defined as the amount of enzyme that liberated 1 μg reducing sugar equivalent to glucose per minute. Protein concentration was determined according to the Bradford method using bovine serum albumin as the standard.

2.6. Characterization of both purified EGs

The substrate specificities of both purified recombinant EGs were determined by assaying different substrates: avicel (Sigma), whatman filter paper (Whatman), carboxymethyl cellulous (CMC) (Sigma), cellobiose (Sigma), *p*-nitrophenyl-β-D-cellobioside (pNPC) (Sigma), α-glucan (Sigma) and xylan (Merck). CMCase, filter paperase and avicelase activities were assayed by measuring the amount of reducing sugar as described above. The detection of β-1, 4-glucosidase was performed by using glucose oxidase and peroxidase in a coupled reaction (Schülein, 1997), while cellobiose as the hydrolyzed substrate. β-1, 4-Exoglucanase activity was measured by

hydrolysis of pNPC leading to the release of *p*-nitrophenol which was measured at 420 nm (Deshpande et al., 1984).

Both purified EGs were incubated with 1.5% CMC (50 mM sodium phosphate buffer, pH 6.8) at different temperatures (25–80 °C) for 30 min, and the optimum temperature was assayed by DNS method. For the thermostability, they were preincubated at different temperatures (25–80 °C) and pH 6.8 for 30 min, and the residual CMCase activity was assayed by DNS method. The optimum pH was determined by incubating the mixture of both purified EGs and 1.5% CMC at 50 °C for 30 min in the presence of buffers: 50 mM citric acid buffer (pH 3–6), 50 mM phosphate buffer (pH 6–8), 50 mM Tris–HCl (pH 8–9) and 50 mM NaOH–glycin buffer (pH 9–10). For the pH stability, EGE was preincubated at different pH values (3–10) for 30 min at 40 °C, whereas EGP at 50 °C. Then the residual CMCase activity of the treated samples were assayed by DNS method.

The CMCase and filter paperase activities of cellulase cocktails of EGP (EGE) and BGL from Sunson Industry Group Co. Ltd. (China) were measured with CMC and filter paper as substrates by DNS method at pH 5 and 55 °C.

2.7. Deglycosylation of EGP and EGE

EGP and EGE were subjected to deglycosylation with endoglycosidase H (Endo H, New England Biolabs) according to the instructions of the manufacturer.

2.8. Sequencing of the truncated EG

EGE and EGP (50 mM phosphate buffer, pH 7.0) were limitedly digested by immobilized-papain at 37 °C overnight, respectively. CMC-SDS–PAGE was carried out to identify EGT. A 20 μl of reaction mixture, which was heated at 95 °C for 2–5 min and mixed with sample buffer, was loaded into 12% SDS–PAGE including 0.15% CMC. After electrophoresis, SDS–PAGE was stained with Coomassie blue G250. For activity staining, SDS–PAGE was soaked in solution A (50 mM sodium phosphate buffer containing 25% isopropanol, pH 6.8) for 2 h and solution B (50 mM sodium phosphate buffer, pH 6.8) for 30 min at 50 °C (Onsori et al., 2005). Subsequently SDS–PAGE was stained in 0.1% Congo red for 20–30 min and washed by 1 M NaCl until showing a yellow halo. The gel of the yellow halo was digged to sequence EGT by MALDI-TOF/MS.

2.9. Biostoning and 3D model of EGE, EGP and EGT

Denim cloth by treatments with EGE, EGP and EGT was performed in a 250 ml Erlenmeyer flask supplied with 10 glass beads (diameter 0.7 cm). At the solid/liquid ratio (1:10), about 2 g of 6 cm × 8 cm denim swatch was immersed into 20 ml of 50 mM sodium phosphate buffer (pH 6.8) at 200 rpm for 1 h at 50 °C. The dosages of EGP, EGE and EGT were 540 U per gram of fabric. The amount of indigo dye released into the buffer solution was determined by assaying absorbance at 370 nm (Wu et al., 2007).

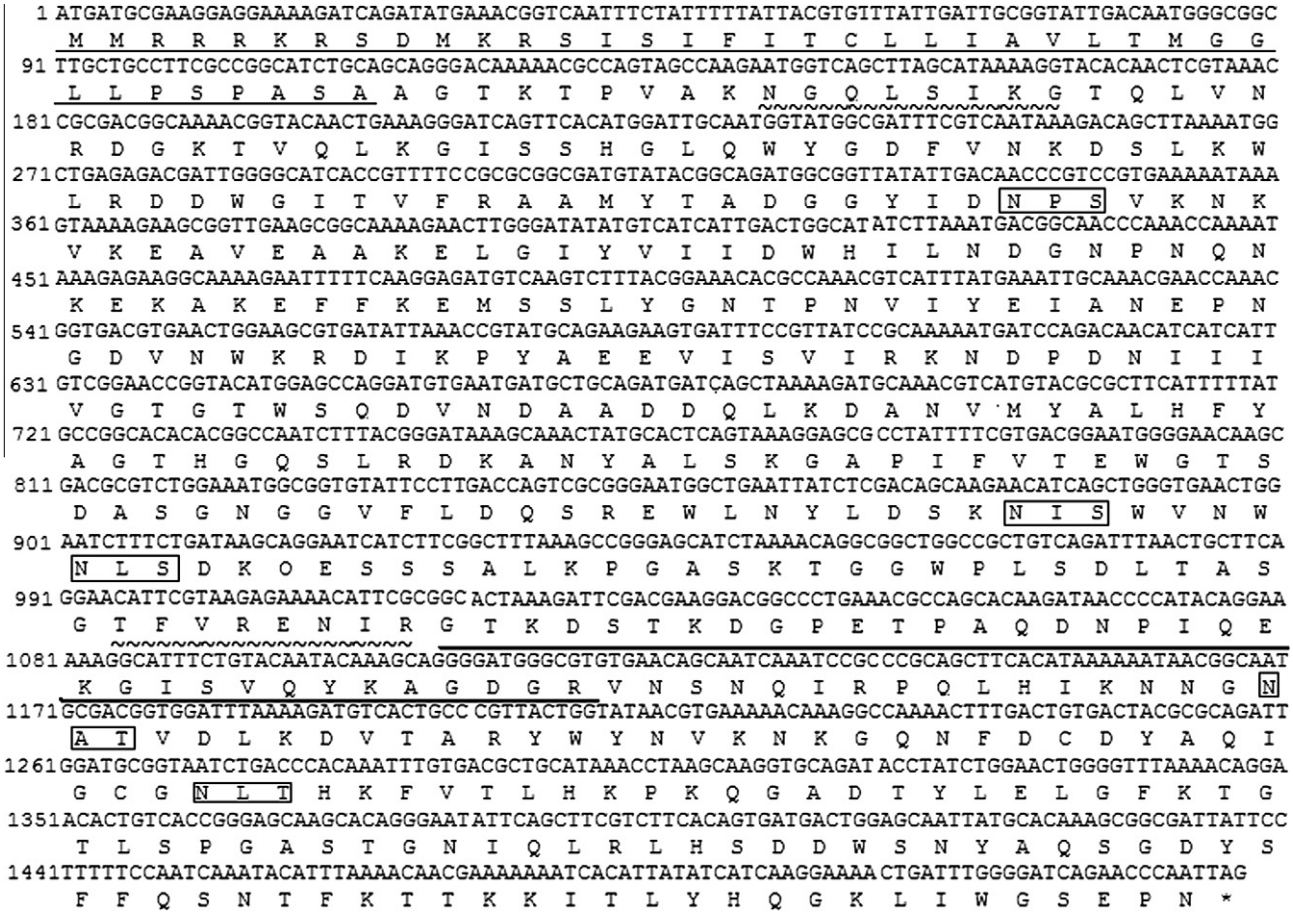

Fig. 1. Nucleotide sequence and deduced amino acid sequence of EG from *B. subtilis* LH. The putative signal peptide (38 amino acids) is underlined. The possible cleavage site is caused by immobilized-papain and underlined with bold lines. The wavy lines indicate two terminals of EGE. Potential N-glycosylation sites (N-X-S/T) are boxed. Asterisk indicates the transcriptional stop codon.

Denim dry weights were determined before and after enzymatic treatment.

3D structure of EGE was constructed by homology modeling using SWISS-MODEL workspace (Arnold et al., 2006). EG surface residues were displayed and analyzed by Swiss-PdbViewer (http://www.expasy.org/spdbv/). The template of about 72% sequence identity with the intact EG was used for modeling the 3D structure of EGE, EGP and EGT.

3. Results and discussion

3.1. Analysis of the EG gene from B. subtilis LH

As shown in Fig. 1, the amplified 1527 bp nucleotides from *B. subtilis* LH encodes 508 amino acids including a putative signal peptide (38 amino acids) and the deduced mature amino acids are made of 470 amino acids from 39th to 508th amino acid according to the SignalP program (http://www.cbs.dtu.dk/services/SignalP/). By amino acid sequence alignment, EG from *B. subtilis* LH shared 98% maximum identity with that from *B. subtilis* DLG (Robson and Chambliss, 1987). In fact, EG from *Bacillus* sp. had a high identity such as *B. subtilis* LH EG sharing 94% identity with *B. amyloliquefaciens* DL-3 EG (Lee et al., 2008).

In the light of 3D structures and function of glycoside hydrolases, more than 60 families of glycoside hydrolases consists of two different conservative structure domains that are CD and CBD, and cellulases belong to glycoside hydrolase family 5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 60 and 61 (Henrissat and Davies, 1997). As CDART program of NCBI analyzed, EG from *B. subtilis* LH belongs to glycoside hydrolase family 5 and the amino acid sequence includes CD (from Ala38 to Arg339) and CBD (from Val373 to Gly484).

3.2. Expression of both EGs

To express EGP, 10 GS115 transformants were selected on YPD containing 3 mg/ml G418, and they were Mut^s. The recombinant strains were induced by methanol under the control of *AOX1* promoter. The maximal activity reached 1886 U/ml in 25 ml BMMY supernatant. However, the maximal activity of the cell lysate from *E. coli* Rosetta DE3/pET-28a(+)-EG was 20,010 U/ml without concentration. *P. pastoris* and *E. coli* have become the most widely used heterogonous expression systems with easy culture, clear genetics and high expression efficiency. The report demonstrated that the yields of enzymes produced by *P. pastoris* were significantly higher than those by *E. coli* (Fan et al., 2007), however, in this study, the CMCase activity of EG from the cell lysate of *E. coli* was 10-fold higher than that from the supernatant of *P. pastoris*. Forty-two percent of EGE formed soluble expression in *E. coli* Rosetta (DE3) at 25 °C and 170 rpm. The culture conditions of low temperature and shaking speed favored correct folding of EGE and avoided tedious refolding (data not shown). Although the culture conditions of *P. pastoris* GS115/pPIC9K-EG were optimized, the maximal activity of its supernatant only reached 2008 U/ml at 25 °C, pH 6.0 and 250 rpm. Compared to EGE, the expression level of EGP was almost not affected by culture conditions. The reason why overexpression of recombinant proteins from *P. pastoris* did not appear could be owing to constraints in transcription, translation and post-translation controls, hence EG from *B. subtilis* possibly existed in barriers to efficient expression in *P. pastoris* GS115 such as codon bias, gene copy number, correct conformation, disulfide bond and glycosylation. In addition, *E. coli* Rosetta (DE3) had a shorter generation time than *P. pastoris* GS115. Accordingly, in this work, *E. coli* Rosetta (DE3) was more appropriate than *P. pastoris* GS115 for the expression of EG from *B. subtilis* LH.

3.3. Purification of both recombinant EGs

To purify EGP, the supernatant of the culture broth was subjected to desalting, concentration of acetone, DEAE-Sepharose FF anion-exchange chromatography and Sephadex G-100 gel filtration. Although EGP was purified to homogeneity at least 95% purity (Fig. 2), its yield was only 10.5% (Table 1). The purified EGP showed a single band on SDS–PAGE corresponding to the molecular weight of about 80 kDa distinct from the calculated molecular weight of 52 kDa. The purity of EGE eluted from the first HiTrap IMAC HP with 0.10 mM imidazole was about 90% (Fig. 2). After purification of the second HiTrap IMAC HP, EGE was purified to homogeneity, which exhibited a single band on SDS–PAGE with the molecular weight of 50 kDa (Fig. 2). As shown in Table 1, the overall yield and specific activity of EGP were significantly lower than that of EGE. The data demonstrated that a one-step purification of EGE by metal chelate affinity chromatography was more efficient than a multiple-step purification of EGP by ion-exchange chromatography, concentration and gel filtration.

3.4. Characterization of both purified recombinant EGs

The relative activities for CMC, pNPC, avicel and filter paper were 100%, 21%, 16% and 0.11%, respectively, and both purified

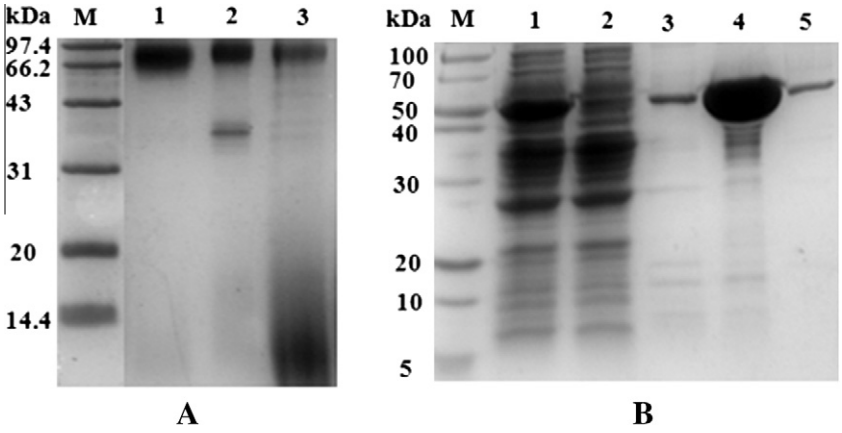

Fig. 2. SDS–PAGE of both purified recombinant EGs. M: protein molecular weight marker; A: SDS–PAGE of the purified EGP, (1) EGP of Sephadex G-100 gel filtration, (2) EGP of DEAE-Sepharose FF ion exchange chromatography, (3) supernatant of culture broth; B: SDS–PAGE of purified EGE, (1) the supernatant of the crude cell lysate, (2) the effluent fractions of loading sample, (3) 0.05 mM imidazole eluate fractions, (4) the fraction of the first elution with 0.10 mM imidazole eluate fractions, and (5) the fraction of the second elution with 0.10 mM imidazole.

Table 1
Purification of EGP and EGE.

Purification steps	Total activity (U)	Total protein (mg)	Purification (fold)	Yield (%)
P. pastoris GS115/pPIC9K-EG				
The supernatant	3772,160	552	1.0	100
DEAE-Sepharose FF	727,381	31.2	3.4	19.3
Sephadex G-100	394,839	0.6	96.3	10.5
E. coli Rosetta (DE3)/pET28a(+)-EG				
Crude cell lysate	600,300	18.5	1.0	100
Fist HiTrap IMAC HP	476,820	0.9	22.1	72.8
Second HiTrap IMAC HP	421,889	0.5	35.1	70.3

enzymes did not degrade cellobiose, α-glucan and xylan. The data revealed that EGE and EGP were bifunctional cellulases excluding BGL. There was a similar report stating that a novel alkaline EG from an alkaliphilic *Bacillus* sp. also decomposed pNPC, CMC and avicel but not xylan (Endo et al., 2001). However, in this work, the results were obviously distinct from *B. amyloliquefaciens* DL-3 EG that could hydrolyze cellobiose and xylan (Lee et al., 2008). A substitute of a surface amino acid residue produced a new substrate specificity (Norledge et al., 2001), so it was possibly caused by 6% inconsistency of amino acid sequences of *B. subtilis* LH and *B. amyloliquefaciens* DL-3 EGs.

EGP and EGE showed optimal activity at 65 °C and more than 60% activity was observed between 40 and 70 °C (Fig. 3A). A fast decrease in activity below 70 °C was seen in Fig. 3A. The optimum pH was 6.8 and both enzymes possessed more than 60% activity at pH 5–9 (Fig. 3B). More than 80% residual activity at 5.0–10.0 was shown in Fig. 3C and a rapid decrease below pH 5.0 was observed, indicating that EGE and EGP were alkalistable. EGE showed a fast decrease in stability above 45 °C and retained 10% residual activity at 65 °C (Fig. 3D). Furthermore, no residual activity was detected above 70 °C. In contrast, EGP possessed about 75% and 40% residual activities at 65 °Cand 80 °C, respectively (Fig. 3D). Although the thermostability of EGP increased from 40 to 50 °C (Fig. 3D), there

Table 2
The specific activities of EGE, EGP and cellulase cocktails on CMC and filter paper.

Enzyme	CMC (U/μmol)	Filter paper (U/μmol)
BGL	ND	ND
EGE or EGP	33,088,010	33,301
EGE(EGP) + BGL	50,089,007	53,827

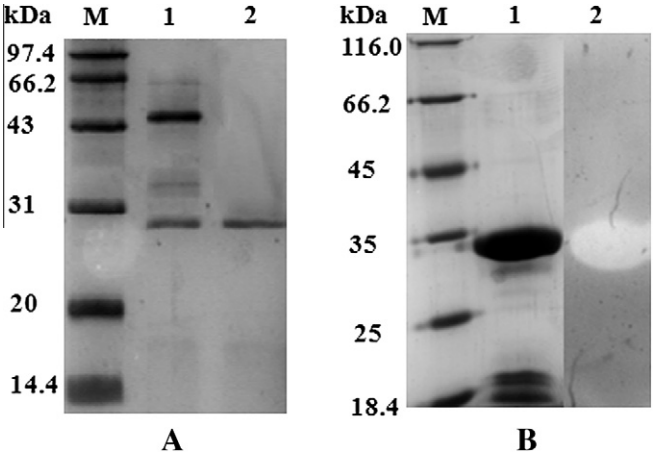

Fig. 4. Identification of the deglycosylated recombinant EG and active truncated EG. M: the molecular weight marker; A: SDS–PAGE of EGP, (1) EGP was digested by Endo H, (2) Endo H; B: Identification of the active truncated EG, (1) SDS–PAGE staining of Coomassie Brilliant blue of the active truncated EG and two small inactive components, and (2) SDS–PAGE activity staining of EGT.

Table 3
Denim fabric biostoning efficiencies by EG and the intact EG (EGE or EGP) treatment.

Preparation	Dosage (U/g)	Weight loss (%)	A_{370nm}
Buffer only	0	0.38	0.292
EGP	540	0.60	0.392
EGE	540	0.62	0.390
EGT	540	0.46	0.350

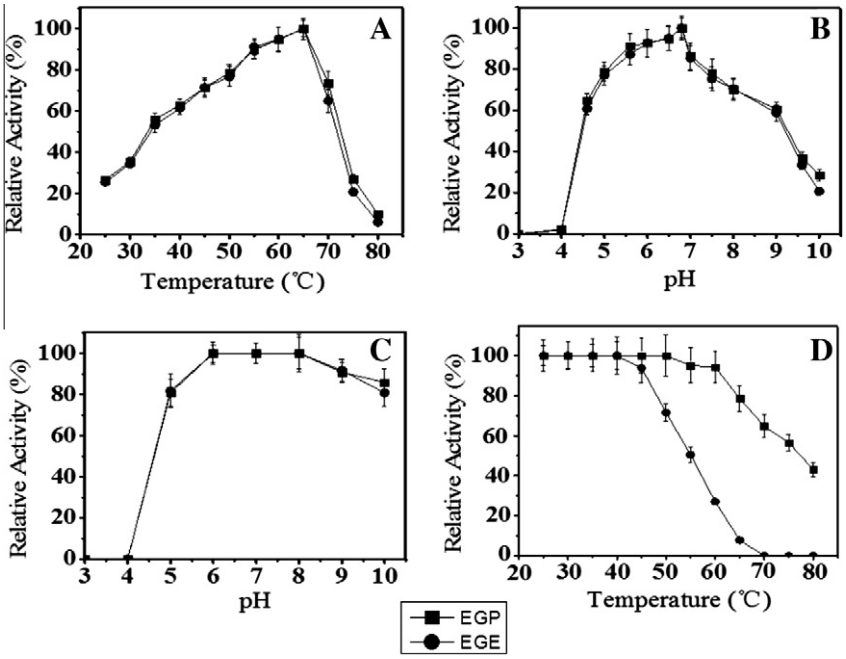

Fig. 3. Effect of temperature and pH on activity and stability of EGP and EGE. (A) Optimum temperature; (B) optimum pH; (C) pH stability (EGE was preincubated at different pH values (3–10) for 30 min at 40 °C, whereas EGP at 50 °C); and (D) thermal stability.

Table 4
The percentage of EGE and EGT SHAARAAA.

Enzyme	Total residues	Quantity of surface amino acid residues								%
		Tyr	Phe	Trp	Val	Leu	Ile	Pro	Met	
EGE	479*	11	7	3	14	16	10	13	1	15.7
EGT	295	5	1	0	8	9	4	8	0	11.9

%: SHAARAAA from the corresponding total residues.
479*: total EGE amino acid residues including additional Met, two residues and six His Tag.

were no significant differences in activity, pH stability and substrate specificities.

Cellulase cocktails can synergistically and efficiently decompose cellulosic materials (Khramtsov et al., 2011; Vasan et al., 2011). CelD-BGL produced more than 1.5-fold higher activities for CMC and PASC than CelD (Lee et al., 2011). Similarly, in the present study, the mixture of EGE (or EGP) and BGL got 1.5-fold higher activities for filter paper and CMC than 1 μmol EGE (EGP) at 55 °C and pH 5 (Table 2). Therefore, the mixture could be a candidate for cellulase cocktails to produce glucose.

3.5. Analysis of deglycosylation

Since EGP had a higher thermostability and larger molecular weight, EGP was possibly glycosylated. As shown in Fig. 4, the deglycosylation of EGP generated a peptide of about 50 kDa similar to the calculated molecular weight, indicating that *N*-glycosylation of EGP accounted for 30 kDa. According to a further examination of the amino acid sequence of *B. subtilis* LH EG (Fig. 1), there appeared to be five potential sites for *N*-glycosylation (N-X-S/T, NPS 114–116, NIS 294–296, NLS 301–303, NAT 390–392, NLT 424–426). Three sites were located within CD, but two within CBD. Compared to EGP, EGE was not glycosylated in *E. coli* Rosetta (DE3), because its molecular weight did not change before and after Endo H treatment (data not shown). Their differences were only caused by the thermostability as analyzed above, indicating that *N*-glycosylation could maintain the spatial structure of EGP encountering a high temperature. The long outer chains of glycosylation potentially hamper the folding, physical stability and function of a foreign protein through the secretory pathways (Lin and Cregg, 2000). However, in this study, *N*-glycosylation did not affect the optimum pH and temperature, the pH stability and the substrate specificities, but enhanced the thermal stability of EGP.

3.6. Analysis of the active truncated EG

EGE was mainly decomposed into a 33 kDa EGT and two 18 kDa small fragments (Fig. 4), which demonstrated the true domains within the enzyme. On the contrary, EGP was not cleaved by immobilized-papain (data not shown). By MALDI-TOF/MS and database search, Fig. 1 clearly showed that terminal sequences of EGT were NGQLSIKGTQLVNR (N-terminal sequence) and TGGWPLSDLTASGTFVRENIR (C-terminal sequence), revealing that the amino acid sequence of EGT consisted with CD. On the basis of the sequencing results of EGT and EGE, the possible cleavage site was located in some place of the linker region (GTKDSTKDGPETPAQDNPIQEKGISVQYKAGDGR). Although EGE was degraded by immobilized-papain instead of other proteases, the result was consistent with the fact that the more hydrophilic linker region was easily attacked by protease (Lo et al., 1988). Glycosylation took main responsibility for the difference between EGE and EGP. Therefore, glycosylation interfering with immobilized-papain attack played an important role to keep the structural stability of EGP in this work.

EGT possessed the higher CMCase and lower avicelase activities than EGP and EGE in good agreement with Hefford et al. (1992). In addition, EGT gained a better thermal stability than the intact EG (data not shown). The amino acid sequence comparison between *B. subtilis* LH and thermophilic *B. subtilis* EG (Yang et al., 2010) confirmed that the N-terminal CD had higher identity (97.34%, 301 amino acids) than the C-terminal CBD (90.37%, 134 amino acids), indicating that EGT was more heat-tolerant than EGP and EGE.

3.7. Biostoning

As shown in Table 3, indigo dye removal and weight loss of denim by treatment of EGT obtained an increase of 19.9% and 21.1%, respectively, however, those by that of EGE and EGP got an increase of about 33.6% and 60%, respectively, indicating that EGE and EGP coped better than EGT with denim. In addition, the data also demonstrated that glycosylation did not affect denim biostoning efficiency.

Since EGE and EGP possessed the almost completely same amino acid sequence, the 3D protein model of EGE was only constructed. The structure was divided into two small 3D models. EGT (CD) is typical of the $(\alpha/\beta)_8$ barrel structure, and CBD is folded in a β-sandwich fashion (data not shown). EGE got 15.7% of SHAARAAA, whereas EGT only acquired 11.9% (Table 4).

Indigo dye consists of aromatic rings and the heterocycle NH and =O groups, indicating that it possesses hydrophilic and hydrophobic properties at the same time. Amino acid residues combined indigo owing to hydrophobic interactions and formation of hydrogen bonds especially for Tyr and Phe, and denim biostoning efficiencies were correlated with the percentage of SHAARAAA (Gusakov et al., 2000). 3D model (data not shown) and denim biostoning data clearly demonstrated that EGE and EGP with the higher percentage of SHAARAAA were more efficient than EGT with a lower that in denim biostoning in this study. Wu et al. (2007) reported that the intact EG from *Volvariella volvacea* was much better than the truncated EG in denim biostoning. Therefore, the higher avicel-adsorption capacity of EG with CBD leaded to the higher hydrolytic activity for filter paper and increased indigo dye removal and denim weight loss (Wu et al., 2007). However, 3D models of EG from *V. volvacea* demonstrated that the intact EG possessed the higher percentage of SHAARAAA than EGT (data not shown). In addition, some native EGs without CBDs also had good biostoning efficiencies such as *P. verruculosum* Eg3 and M. albomyces Cel45A (Gusakov et al., 2000; Haakana et al., 2004). Thus, it was the most possible that CBD was not necessarily required for EG in biostoning of denim. In fact, the cellulose structure of denim coated with indigo dye is obviously different from avicel and filter paper. As a result, efficient indigo dye removal and moderate weight loss of denim were caused by the higher percentage of SHAARAAA regardless of whether EG exited in CBD.

4. Conclusion

The results showed that EG from *B. subtilis* LH was more efficiently expressed in *E. coli* Rosetta (DE3) than in *P. pastoris*

GS115. Cellulase cocktails of EGE (EGP) and BGL could become a potential application in the production of glucose. The present study demonstrated that *N*-glycosylation enhanced the thermal stability and protected EGP from immobilized-papain attack. EGP and EGE possessed the similar range of the pH stability and the same of the optimum pH and temperature. The high percentage of SHAARAAA of EGE and EGP enhanced indigo dye removal and weight loss of denim in biostoning process.

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References

Andreaus, J., Campos, R., Gübitz, G., Cavaco-Paulo, A., 2000. Influence of cellulases on indigo backstaining. Text. Res. J. 70, 628–632.

Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201.

Boer, H., Teeri, T.T., Koivula, A., 2000. Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastories* using two different promoters. Biotechnol. Bioeng. 69, 486–494.

Cavaco-Paulo, A., 1998. Mechanism of cellulase action in textile processes. Carbohyd. Polym. 37, 273–277.

Deshpande, M.V., Eriksson, K.E., Pettersson, L.G., 1984. An assay for selective determination of exo-1,4,-β-glucanases in a mixture of cellulolytic enzymes. Anal. Biochem. 138, 481–487.

Endo, K., Hakamada, Y., Takizawa, S., Kubota, H., Sumitomo, N., Kobayashi, T., Ito, S., 2001. A novel alkaline endoglucanase from an alkaliphilic *Bacillus* isolate: enzymatic properties, and nucleotide and deduced amino acid sequences. Appl. Microbiol. Biotechnol. 57, 109–116.

Fan, Y.H., Zhang, Y.J., Yang, X.Y., Pei, X.Q., Guo, S.J., Pei, Yan., 2007. Expression of a *Beauveria bassiana* chitinase (Bbchit1) in *Escherichia coli* and *Pichia pastoris*. Protein Expres. Purif. 56, 93–99.

Gusakov, A.V., Sinitsyn, A.P., Berlin, A.G., Markov, A.V., Ankudimova, N.V., 2000. Surface hydrophobic amino acid residues in cellulase molecules as a structural factor responsible for their high denim-washing performance. Enzyme Microb. Tech. 27, 664–671.

Haakana, H., Miettinen-Oinonen, A., Joutsjoki, V., Mäntylä, A., Suonminen, P., Vehmaanperä, J., 2004. of cellulase genes from *Melanocarpus albomyces* and their efficient expression in *Trichoderma reesei*. Enzyme Microb. Tech. 34, 159–167.

Hefford, M.A., Laderoute, K., Willick, G.E., Yaguchi, M., Seligy, V.L., 1992. Bipartite organization of the *Bacillus subtilis* endo-β-1, 4-glucanase revealed by C-terminal mutations. Protein Eng. 5, 433–439.

Heikinheimo, L., Buchert, J., Miettinen-Oinonen, A., Suominen, P., 2000. Treating denim fabrics with *Trichoderma reesei* cellulases. Text. Res. J. 70, 969–973.

Henrissat, B., Davies, G., 1997. Structural and sequence-based classification of glycoside hydrolases. Curr. Opin. Struc. Biol., 637–644.

Hueck, C.J., Hillen, W., 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram-positive bacteria? Mol. Microbiol. 15, 395–401.

Kim, T.H., Taylor, F., Hicks, K.B., 2008. Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. Bioresour. Technol. 99, 5694–5702.

Khramtsov, N., McDade, L., Amerik, A., Yu, E., Divatia, K., Tikhonov, A., Minto, M., Mubalamate, G.K., Markovic, Z., Martinez, M.R., Henck, S., 2011. Industrial yeast strain engineered to ferment ethanol from lignocellulosic biomass. Bioresour. Technol. 102, 8310–8313.

Lee, H.L., Chang, C.K., Teng, K.H., Liang, P.H., 2011. Construction and characterization of different fusion proteins between cellulases and β-glucosidase to improve glucose production and thermostability. Bioresour. Technol. 102, 3973–3976.

Lee, Y.J., Kim, B.K., Lee, B.H., Jo, K.I., Lee, N.K., Chung, C.H., Lee, Y.C., Lee, J.W., 2008. Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. Bioresour. Technol. 99, 378–386.

Li, P., Anumanthan, A., Gao, X.G., Ilangovan, K., Suzara, V.V., Düzgünues, N., Renugopalakrishnan, V., 2007. Expression of recombinant proteins in *Pichia Pastoris*. Appl. Biochem. Biotechnol. 142, 105–124.

Li, W., H, X.J., Zhou, Y., Ma, Q.Y., Chen, Y.L., 2009. Simultaneous cloning and expression of two cellulase genes from *Bacillus subtilis* newly isolated from Golden Takin (*Budorcas taxicolor Bedfordi*). Biochem. Biophys. Res. Co. 383, 397–400.

Lin, J.L., Cregg, C.M., 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev. 24, 45–66.

Lo, A.C., Mackay, R.M., Seligy, V.L., Willick, G., 1988. *Bacillus subtilis* β-1, 4-endoglucanase products from intact and truncated genes are secreted into the extracellular medium by *Escherichia coli*. Appl. Environ. Microb. 54, 2287–2292.

Miller, G.L., Blum, R., Glennon, W.E., Burton, A.L., 1960. Measurement of carboxymethylcellulase activity. Anal. Biochem., 127–132.

Norledge, B.V., Lambeir, A.M., Abagyan, R.A., Rottmann, A., Fernandez, A.M., Filimonov, V.V., Peter, M.G., Wierenga, R.K., 2001. Modeling, mutagenesis, and structural studies on the fully conserved phosphate-binding loop (Loop 8) of triosephosphate isomerase: toward a new substrate specificity. Proteins 42, 383–389.

Onsori, H., Zamani, M.R., Motallebi, M., Zarghami, N., 2005. Identification of over producer strain of endo-β-1, 4-glucanase in *Aspergillus* species: characterization of crude carboxymethyl cellulase. Afr. J. Biotechnol. 4, 26–30.

Robson, L., Chambliss, G.H., 1984. Characterization of the cellulolytic activity of a *Bacillus* isolate. Appl. Environ. Microb. 47, 1039–1046.

Robson, L.M., Chambliss, G., 1987. Endo-P-1, 4-glucanase gene of *Bacillus subtilis* DLG. J. Bacteriol. 169, 2017–2025.

Sánchez, C., 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol. Adv. 27, 185–194.

Schülein, M., 1997. Enzymatic properties of cellulases from *Humicola insolens*. J. Biotechnol. 57, 71–81.

Tang, B., Pan, H., Zhang, Q., Ding, L., 2009. Cloning and expression of cellulase gene EG1 from *Rhizopus stolonifer* var. reflexus TP-02 in *Escherichia coli*. Bioresour. Technol. 100, 6129–6132.

Vasan, P.T., Piriya, P.S., Prabhu, D.I., Vennison, S.J., 2011. Cellulosic ethanol production by *Zymomonas mobilis* harboring an endoglucanase gene from *Enterobacter cloacae*. Bioresour. Technol. 102, 2585–2589.

Wood, B.E., Beall, D.S., Ingram, L.O., 1997. Production of recombinant bacterial endoglucanase as a co-product with ethanol during fermentation using derivatives of *Escherichia coli* KO11. Biotechnol. Bioeng. 55, 547–555.

Wu, S.F., Ding, S.J., Zhou, R., Li, Z.Z., 2007. Comparative characterization of a recombinant *Volvariella volvacea* endoglucanase I (EG1) with its truncated catalytic core (EG1-CM), and their impact on the bio-treatment of cellulose-based fabrics. J. Biotechnol. 130, 364–369.

Wu, X.C., Lee, W., Tran, L., Wong, S.L., 1991. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J. Bacteriol. 173, 4952–4958.

Yang, D.L., Weng, H.B., Wang, M., Xu, W.H., Li, Y.Z., Yang, H.L., 2010. Cloning and expression of a novel thermostable cellulase from newly isolated *Bacillus subtilis* strain I15. Mol. Biol. Rep. 37, 1923–1929.

Zhang, X.Z., Zhang, Y.H.P., 2010. One-step production of biocommodities from lignocellulosic biomass by recombinant celluloytic *Bacillus subtilis*: opportunities and challenges. Eng. Life Sci. 10, 398–406.