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Cloning and Overexpression of a *Paenibacillus* β-Glucanase in *Pichia pastoris*: Purification and Characterization of the Recombinant Enzyme

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Abstract Isolation, expression, and characterization of a novel endo-β-1,3(4)-D-glucanase with high specific activity and homology to *Bacillus* lichenases is described. One clone was screened from a genomic library of *Paenibacillus* sp. F-40, using lichenan-containing plates. The nucleotide sequence of the clone contains an ORF consisting of 717 nucleotides, encoding a β-glucanase protein of 238 amino acids and 26 residues of a putative signal peptide at its N-terminus. The amino acid sequence showed the highest similarity of 87% to other β-1,3-1,4-glucanases of *Bacillus*. The gene fragment *Bgl* containing the mature glucanase protein was expressed in *Pichia pastoris* at high expression level in a 3-l high-cell-density fermenter. The purified recombinant enzyme Bgl showed activity against barley β-glucan, lichenan, and laminarin. The gene encodes an endo-β-1,3(4)-D-glucanase (E. C. 3.2.1.6). When lichenan was used as substrate, the optimal pH was 6.5, and the optimal temperature was 60°C. The K_m, V_{max}, and k_{cat} values for lichenan are 2.96 mg/ml, 6,951 μmol/min·mg, and 3,131 s^{-1}, respectively. For barley β-glucan the values are 3.73 mg/ml, 8,939 μmol/min·mg, and 4,026 s^{-1}, respectively. The recombinant Bgl had resistance to pepsin and trypsin. Other features of recombinant Bgl including temperature and pH stability, and sensitivity to some metal ions and chemical reagents were also characterized.

Key words: *Paenibacillus*, endo-β-1,3(4)-D-glucanase, gene cloning, expression, characterization, *Pichia pastoris*

β-Glucans, the principle endosperm cell-wall component of barley, rye, rice, and wheat, are linear polysaccharides comprising both β-1,3- and β-1,4-linked D-glucose. This mixed linkage also exists in lichenan, a similar polysaccharide from the lichen *Cetraria islandica* [30]. Endo-β-1,3-1,4-glucanase, also called lichenase (E. C. 3.2.1.73), can hydrolyze β-1,4-glycosyl linkages adjacent to β-1,3-linkages in mixed linkages of β-glucan, such as lichenan and barley β-glucan [2]. Laminarinase (endo-β-1,3-glucanohydrolase, E. C. 3.2.1.39) cleaves β-1,3-glucosyl linkages, such as those found in the algal polysaccharide laminarin. A related group of enzymes able to depolymerize both β-1,3-1,4-glucans and β-1,3-glucans have been classified as β-1,3(4)-glucanases (E. C. 3.2.1.6) [5].

Endo-β-1,3-1,4-glucanase from bacteria belongs to glycosyl hydrolase family 16 (GH16). Endo-β-1,3-1,4-glucanase genes have been cloned from several members of the *Bacillaceae* family such as *Bacillus subtilis* [17], *B. amyloliquefaciens* [9], *B. licheniformis* [5], *B. macerans* [5], *B. halodurans* [1], *B. circulans* [3], and *B. polymyxa* [13]. Those genes encoding β-1,3-1,4-glucanase from non-*Bacillus* species have also been cloned and expressed heterogeneously, such as *Clostridium thermocellum* [4], *Streptococcus bovis* [10], and *Fibrobacter succinogenes* [8]. Genes encoding both activities have been cloned from *Rhizopus arrhizus* [9], *Clostridium thermocellum* [6], *Cellvibrio mixtus* [3], *Rhodothermus marinus* [7], *Streptococcus bovis* [10], *Pyrococcus furiosus* [14], *Cochilobolus carbonum* [12], *Phaffia rhodozyma* [3], *Bacillus halodurans* [1], and *Phanerochaete chrysosporium* [1].

Lichenases have been applied as industrial enzymes in beer brewing and feed additives [2]. In animal feedstuff, especially for broiler chickens and piglets, the addition of enzymatic preparation containing lichenases improves the digestibility of barley-based diets and reduces sanitary problems such as sticky droppings [6]. However, new enzymes with excellent properties should be further isolated and high-level expressed.

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Paenibacillus sp. F-40 was isolated from soil in Xinjiang Province, China. It produces several polysaccharide-degrading enzymes such as xylanase and mannanase in low amount, and glucanase with high activity. Analysis of the 16S ribosomal RNA gene (GenBank accession No. DQ514297) shows 99% identity to *Paenibacillus macerans*. In this paper, cloning of a *Paenibacillus* gene coding for a β-glucanase, and its overexpression and characterization in *Pichia pastoris* are described.

MATERIALS AND METHODS

Microorganisms, Plasmids, and Growth Conditions

Paenibacillus sp. F-40, which can synthesize and secrete β-glucanase, was isolated from soil in Xinjiang Province, China, and was grown in a 1-l wide-mouth shaker flask containing 300 ml enzyme-producing medium with 0.5% KH_2PO_4, 0.5% tryptone, 0.5% NaCl, and 0.5% lichenan (from *Cetraria islandica*, Sigma-Aldrich, MO, U.S.A.) at 30°C, 250 rpm, for 48 h. The *P. pastoris* expression system containing host strains *P. pastoris* GS115 (His^+ Mut^S) and plasmid vector pPIC9 was obtained from Invitrogen (Carlsbad, CA, U.S.A.). *Escherichia coli* JM109 (*e14⁻ (McrA⁻) recA1 endA1 gyrA96 thi-1 hsdR17*(r_{K-} m_{K+}) *supE44 relA1(lac-proAB)* [F' *traD36 proAB lacI^q ZM15*]) (Takara, Dalian, China) was utilized in this work.

Luria-Bertani (LB) medium containing 1% yeast extract, 0.5% tryptone, and 1% NaCl was used to cultivate *E. coli* JM109. RDB (Regeneration Dextrose Base), MM (Minimal Mathanol), MD (Minimal Dextrose), BMGY (Buffered Glycerol-complex Medium), BMMY (Buffered Methanol-complex Medium), BSM (Basal Salt Medium), and PTM1 trace salts solution were used for the growth and expression of recombinant protein in *P. pastoris* according to the manufacturer's instructions (http://www.invitrogen.com/content/sfs/manuals/pich_man.pdf).

Construction of a Genomic Library and Isolation of the β-Glucanase

Genomic DNA of *Paenibacillus* sp. F-40 was prepared from 100 ml of culture. The cell pellet was treated with lysase and proteinase K at the ratio of 1:10 for 1 h at 37°C followed by 30 min at 42°C. After addition of 2% SDS, the DNA was extracted by phenol and chloroform and precipated with 100% ethanol. The genomic DNA was partially digested with the restriction enzyme Sau3A I. The fractionated DNA fragments of 2–8 kb were ligated at the BamHI site in vector pUC19, which was prepared by the alkaline lysis method, [4, 22] followed by sequencing. *E. coli* JM109 transformants were plated on LB agar plates containing X-gal and IPTG. White colonies were transferred onto LB agar plates containing 0.5% lichenan and incubated at 37°C overnight. The plates were stained with 0.1% Congo red, followed by several washes of 0.1 M NaCl. The positive colonies with a small zone of hydrolysis showing glucanase activity were inoculated in LB liquid medium and incubated overnight.

Nucleotide Sequences Analysis

The nucleotide sequences of the positive clones were analyzed by the NCBI ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The presence of signal peptide in the deduced amino acid sequences was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP/). Phylogenetic analyses were conducted using CLUSTAL W (http://www.ebi.ac.uk/clustalw/). The structure-based sequence alignment was carried out by the JOY program (http://www-cryst.bioc.cam.ac.uk/~joy/) [27].

Construction of Recombinant Expression Vector

The DNA fragment encoding the mature protein Bg1 was amplified by PCR using *Pfu* DNA polymerase (Sangon, Shanghai, China) and two synthetic primers (glu1 & glu2). Primer 1, 5'-aaa**gaattc**ggttatgtgttctgggaacctc-3', contains an EcoRI site (in bold), and primer 2, 5'-acc**gcggccgc**tt-aattactcgtatatttaaccc-3', contains a NotI site (in bold). The amplified product carrying the mature β-glucanase-coding sequence was inserted at the EcoRI and NotI sites in vector pPIC9 to generate a recombinant plasmid, pPIC9-*Bg1*. The β-glucanase-coding sequence was in-frame with the α-factor lead sequence in the vector. In-frame fusion and authenticity of the *Bg1* gene on the expression plasmid was confirmed through nucleotide sequence analysis of the recombinant construct.

Expression and Fermentation of β-Glucanase in *P. pastoris*

The plasmid pPIC9-*Bgl* was linearized by BglII and integrated into the *P. pastoris* GS115 (His^+ Mut^S) genome at the *AOX1* locus through homologous recombination following the manufacturer's instructions (http://www.invitrogen.com/content/sfs/manuals/pich_man.pdf). The recombinant clones grown on RDB medium were transferred to MM and MD plates for further selection and inoculated into 50 ml BMGY and cultured at 30°C in a gyratory shaker at 250 rpm for 2 days. The cells were harvested by centrifugation at 3,000 rpm for 5 min at 4°C and resuspended in 10 ml of BMMY. Subsequently, cells were incubated at 30°C for 2 days with addition of 1% methanol after every 24 h to maintain induction conditions. Enzyme activity was determined by the method of enzyme activity assay described. The positive strain with the highest activity was cultivated in a 3-l fermenter containing BSM with PTM1 trace salts solution at 30°C and pH 5.0. Glycerol feeding was carried out for about 12 h until the cell density reached up to 180 g/l, and then 100% methanol was added at 4.8 ml/h·l for 6 days. The condensed air flow was kept

above 20% and the agitation rate was 1,000 rpm. Samples were collected every day. Cell wet weight and enzyme activity in the supernatant were determined. Biologically active β-glucanase was identified through zymography after fractionation on 12% SDS-PAGE, as described previously [19, 24].

Purification of the Recombinant β-Glucanase

Mycelia were removed from the supernatant by centrifugation. Enzymes were precipated by addition of ammonium sulfate at a saturation of 80% and centrifuged, followed by resuspension in 20 mM Tris/HCl buffer, pH 8.0 (buffer A). A 15-ml dialysate in buffer A was applied to the HiTrap Q Sepharose XL FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with the same buffer. Proteins were eluted with a NaCl step gradient in buffer A at a flow rate of 0.5 ml/min. Fractions with enzyme activities were collected and concentrated. The protein concentration was assayed by the Bradford method [6].

Enzyme Activity Assays

The standard assay for β-glucanase was carried out in 500 μl McIlvaine buffer (pH 6.5) containing 450 μl 1% substrate and 50 μl appropriately diluted enzyme at 60°C for 10 min. The reducing sugars released were detected by the DNS method with glucose as a standard [38, 40]. One unit of enzyme activity is defined as the amount of enzyme required to release 1 μmol reducing sugars per minute.

Characterization of the Recombinant Protein Bg1

The optimal pH was determined by incubating the purified recombinant Bg1 with lichenan in McIlvaine buffers (0.1 M citric acid and 0.2 M Na_2HPO_4, pH 2.0–8.0) and NaOH/glycine buffer (pH 8.0–12.0), respectively. The effect of pH on Bg1 stability was estimated from pH 3.0 to 9.0 by using the same buffer system. The optimal temperature was determined by performing the standard assay at temperature of 10–90°C. Thermal stability was measured by assessing the residual enzyme activity after incubation of the enzyme at 50, 60, and 70°C for 30 min, respectively. The remaining activity was measured as the standard described.

To determine the effects of metal ions and chemical reagents on the hydrolysis reaction of Bg1, the enzyme solutions were incubated in the assay buffer containing 1 mM of metal ions and tested compounds, respectively. The activity of the enzyme was then measured.

The K_m, V_{max}, and k_{cat} values for the recombinant Bg1 were defined by Lineweaver-Burk plotting using activity assay at 60°C in McIlvaine buffer (pH 6.5) with 1–20 mg/ml lichenan and barley β-glucan as substrates.

Hydrolysis Experiment

The substrate [0.5% (w/v)] in McIlvaine buffer (pH 6.5) including lichenan from *C. islandica*, barley β-glucan, laminarin from *L. digitata*, xylan from oat spelt and birchwood, or carboxymethylcellulose (CM-cellulose) (Sigma-Aldrich, MO, U.S.A.), respectively, was hydrolyzed by the purified β-glucanase at 60°C for 10 min and the specific hydrolysis activity was determined.

The substrates were incubated with 25 μg purified Bg1 for 4 h at 60°C in McIlvaine buffer (pH 6.5). Following hydrolysis, the enzyme was abolished using the Nanosep Centrifugal 10 K Device (Pall, MI, U.S.A.). The products were analyzed by High-Performance Anion-Exchange Chromotography (HPAEC) with a Dionex model 2500 HPAEC system (Dionex, CA, U.S.A.) equipped with a CarboPac PA100 column (4×250 mm), which was previously equilibrated in 0.01 M NaOH at a flow rate of 1.0 ml/min. The analysis was performed by the wash step for 50 min consisting of 0.01 M NaOH and 0.05 M sodium acetate. The separations were detected using an ED50 electrochemical detector equipped with a disposable gold working electrode and Ag/AgCl reference electrode. The waveform used was a quadruple potential pulsed amperometry waveform (E_1= +0.1 V from 0 to 0.4 ms; E_2=−0.2 V from 0.41 to 0.42 ms; E_3=+0.6 V from 0.42 to 0.43 ms; E_4=−0.1 V from 0.44 to 0.5 ms). The chromatographic data were further processed.

Nucleotide Sequences Accession Number

The nucleotide sequences for the *Paenibacillus* sp. 16S rDNA gene and glucanase gene have been deposited in the GenBank under accession Nos. DQ514297 and DQ437528, respectively.

RESULTS

Cloning and Sequencing of *Paenibacillus* sp. F-40 β-Glucanase Gene

A genomic library was constructed by ligating 2–8 kb Sau3AI fragments of *Paenibacillus* sp. F-40 DNA into the BamHI site of vector pUC19. Positive clones were detected by plating the bacterial colonies onto LB plate containing 0.5% lichenan in duplicates. Among over 5000 transformants screened, one clone, pUCP-2287, was found to produce a clear halo on 0.5% lichenan, and the glucanase activity of 3.42 U/ml was detected in the supernatant when the clone was cultured in LB medium.

Plasmid DNA was isolated from pUCP-2287 and sequenced. The sequencing result showed that the clone contained a 2,075 bp DNA fragment (Fig. 1). One complete open reading frame was found at 645–1,361 bp, preceded by the deduced Shine-Dalgarno (SD) ribosome-binding site (GGAGG sequence, 636–640 bp). The translation product was predicted to be 238 amino acids long with molecular weight of 24 kDa. A typical signal peptide sequence and a cleavage site between Ala26 and Gly27 were found at the N terminal. Within the 5'-noncoding region of the

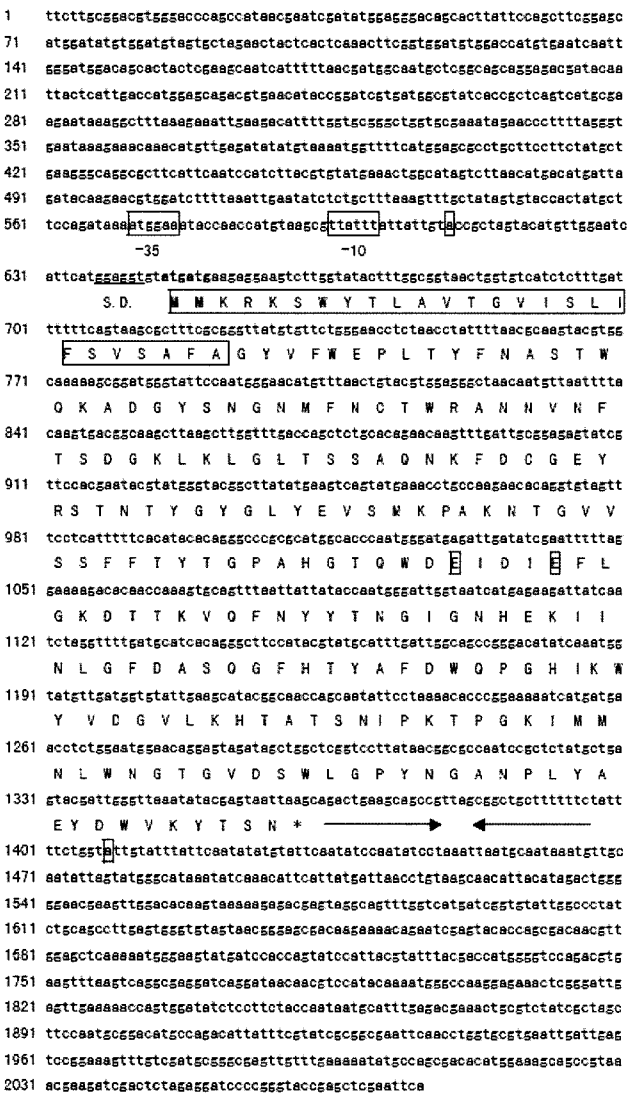

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the β-glucanase gene.

The presumptive Shine-Dalgarno ribosome-binding site preceding the start codon is underlined and indicated. The putative −35 and −10 promoter regions are boxed and marked. The palindromic sequence of a possible transcriptional terminator is underlined by facing arrows. The predicted signal peptide sequence is boxed.

clone, a canonical −10 promoter sequence (TTATTT) was located at 595 bp, and −35 sequence (ATGGAA) at 571 bp (Fig. 1). These sequences might function as an efficient transcriptional site recognized by *E. coli* and *Paenibacillus* sp. RNA polymerase.

Database searches of the gene and deduced amino acid sequence were performed by BLAST. The encoded protein belongs to the family GH16 glycoside hydrolases. It was homologous to several *Bacillus* β-1,3-1,4-glucanases, such as *P. polymyxa* and *P. macerans* (87% identity in amino acid sequence and 78% in nucleotide sequence) and *B. licheniformis* (68% in 212 amino acids overlap). The

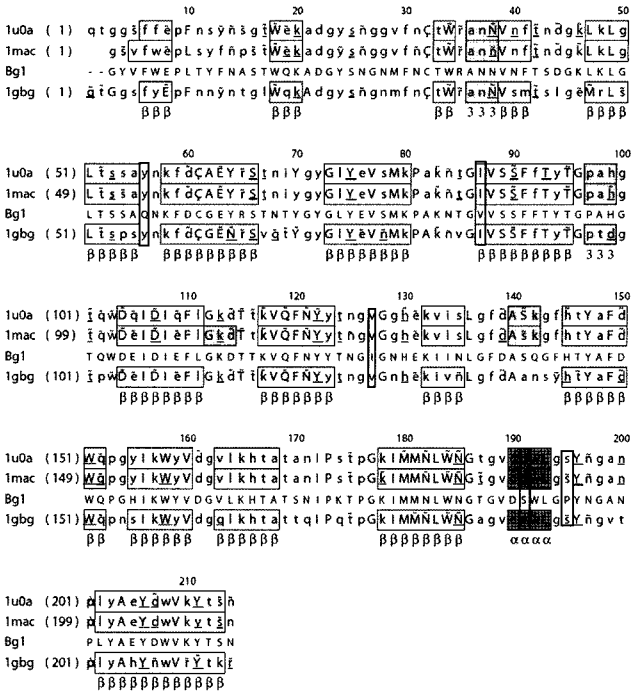

Fig. 2. The structure-based sequence alignment of Bg1 and related sequences of known structure.

The light grey box indicates the beta strand; the dark grey box indicates the alpha helix. The residues in this alignment are represented as follows: uppercase, solvent inaccessible; lower case, solvent accessible; italic, positive phi torsion angle; a tide over the letter, hydrogen bond to other sidechain; bold, hydrogen bond to main-chain amide; underline, hydrogen bond to main-chain carbonyl. The alignment was carried out by the JOY program [25] (http://www-cryst.bioc.cam.ac.uk/~joy/) with the following sequences: 1u0a, PDB ID of β-1,3-1,4-glucanase H (A16M) engineered from *Bacillus macerans* and *B. licheniformis* [11]; 1mac, PDB ID of β-1,3-1,4-glucanase from *B. macerans* [15]; 1gbg, PDB ID of β-1,3-1,4-glucanase from *B. licheniformis* [16].

structure-based sequence alignment among Bg1 and β-1,3-1,4-glucanases from *P. macerans* and *B. licheniformis* was carried out (Fig. 2). Bg1 showed an antiparallel jellyroll protein structure, the same as *Bacillus* β-1,3-1,4-glucanases. The conserved motif "EIDIEF" existing in most GH-16 β-1,3-1,4-glucanases was also found in Bg1. Two glutamic acid residues, Glu103 and Glu107 (shadow and boxed in Fig. 1) in the mature enzyme, are a putative nucleophile and acid/base catalyst, respectively [18, 20].

Expression and Fermentation of Recombinant Bg1 in *P. pastoris*

The gene encoding the mature protein Bg1 was amplified by PCR and inserted into vector pPIC9 to generate an in-frame fusion of the leader peptide of the yeast α-mating factor and the mature form of the *Bg1*. The resultant recombinant plasmid was linearized and transformed into *P. pastoris*.

The transformant GS115/pPIC9-Bgl-9 showed the highest β-glucanase activity among 103 positive clones.

Table 1. Purification of recombinant Bgl expressed in *P. pastoris*.

	Volume (ml)	Total activity (U)	Protein concentrtion (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant	20	91,074	1.48	3,076	100	1
HiTrap Q Sepharose XL	10	27,003	0.45	6,001	29.6	1.95

Bgl production was induced by methanol feed during fermentation. Samples of 1 ml from the bioreactor were collected every 12 h for analysis. The SDS-PAGE result showed that high recombinant Bgl concentration was present in the medium along with increasing induction time. The secretion level of Bgl evaluated from the glucanase activity was also increased. The enzymatic activity kept increasing up to the end of fermentation. After 144 h induction, the glucanase activity reached 4,554 U/ml and the maximal secretion yield of Bgl was approximately 0.8 g/l, approximately 65% of total protein in the medium. The recombinant Bgl showed a molecular weight of about 35 kDa, heavier than the theoretical molecular weight. It might be due to protein modifications, such as glycosylation, because of the four *N*-glycosylation sites existing in the Bgl amino acid sequence.

Purification of Recombinant Bgl from *P. pastoris* and Deglycosylation

The recombinant enzyme was purified to electrophoretic homogeneity by ammonium sulfate precipitation and anion-exchange chromatography. The specific activities of Bgl against barley β-glucan at each purification step are indicated in Table 1. The specific activity of the Bgl was 6,001 U/mg after 1.95-fold purification, with a final yield of 29.6%. The purified enzyme was shown on SDS-PAGE as a single band. After treatment with endo-β-*N*-acetylglucosaminidase H (endo-H), cleaving within the chitobiose core of high mannose and some hybrid oligosaccharides from *N*-linked glycoproteins, the molecular weight of the protein was 27 kDa (Fig. 3), which was different from the theoretical molecular weight.

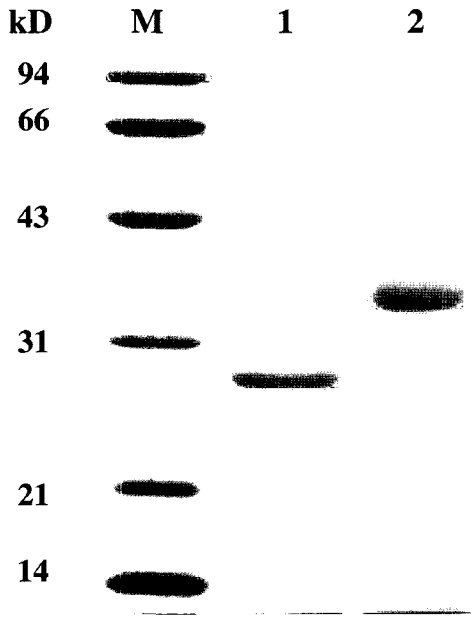

Fig. 3. SDS-PAGE analysis of purified and *N*-deglycosylated recombinant Bgl by endo-β-*N*-acetylglucosaminidase H.
M: standard protein molecular weight; 1: the *N*-deglycosylated Bgl and endo-H (29 kDa); 2: purified Bgl.

Enzyme Characterization

The characteristics of the recombinant Bgl were further determined using the lichenan as substrate. The optimal pH for enzyme activity was 6.5 (Fig. 4A). The effect of pH on enzyme stability was studied by incubating the enzyme in buffers of different pH at 37°C for 30 min. The enzyne was stable at a wide pH range. More than 80% of the total activity was retained after treatment in pH 4–9 (Fig. 4B). The optimal temperature was about 60°C (Fig. 4C). The enzyme was stable at high temperatures. More than 80% and 70% of enzyme activity was retained after treating the enzyme at 50°C and 60°C for 30 min, respectively, and even 50% of the activity was retained at 70°C for 10 min (Fig. 4D). Bgl had resistance to proteinase. After treatment by pepsin and trypsin at 37°C for 60 min, the enzyme activity remained about 60%.

The enzyme activity in the presence of metal ions and chemical reagents was also assayed (see Table 2). The activity was enhanced 57.66% by Mn^{2+} and 20.19% by Ag^{+}. The addition of other metal ions and chemicals had no or little effect on Bgl activity. The activity of the recombinant Bgl was not inhibited by SDS addition.

Kinetic parameters were determined for the overall hydrolytic reaction on barley β-glucan and lichenan. The reactions were carried out at the optimal pH 6.5 at 60°C. K_m, V_{max}, and k_{cat} values for barley β-glucan were 3.73 mg/ml, 8,939 μmol/min·mg, and 4,026 s^{-1}, respectively. For lichenan, the values were 2.96 mg/ml, 6,951 μmol/min·mg, and 3,131 s^{-1}, respectively.

Substrate Specificity and Hydrolysis Product of the Recombinant Bgl

The specific enzyme activities of the purified Bgl on different substrates were measured at 60°C in McIlvaine buffer (pH 6.5) for 10 min (Table 3). Bgl had the highest specificity for the soluble β-1,3-1,4-linked glucans, such as barley β-glucan and lichenan. A 10-fold lower activity was

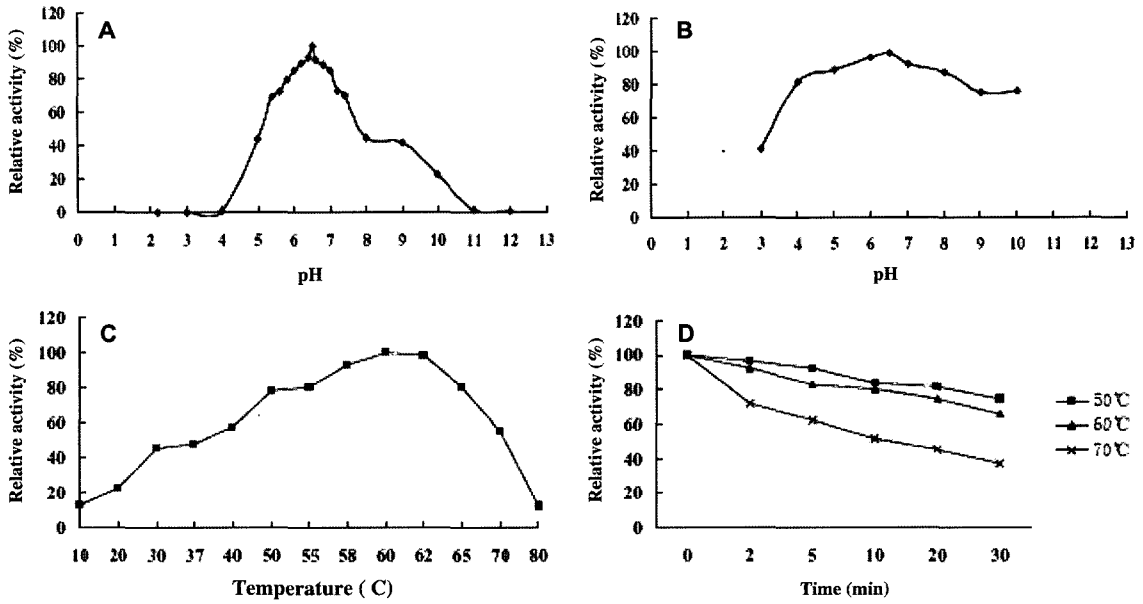

Fig. 4. Characterization of recombinant Bg1.
A. Effects of pH on activity, assay was done at 37°C for 10 min in buffers (pH 2–12) with 1% lichenan; **B.** pH stability of recombinant Bg1, assay was measured at 60°C after incubating the enzyme at 37°C for 30 min in buffers (pH 3–10); **C.** Effects of temperature on activity, enzymatic activity was measured in McIlvaine buffer (pH 6.5) for 10 min, using 1% lichenan as substrate; **D.** Thermal stability of recombinant Bg1, the enzyme was preincubated at 50, 60, 70°C in McIlvaine buffer (pH 6.5) for 30 min, residue activity measured at 60°C.

observed on β-1,3-linked laminarin. The β-1,4-linked substrates, including xylan from oat spelt and birchwood, and CM-cellulose were not hydrolyzed.

The hydrolyzed products of Bg1 were analyzed by HPAEC. Incubation of barley β-glucan (Fig. 5A), lichenan (Fig. 5B), and laminarin (Fig. 5C) with the enzyme resulted in the production of reducing sugars. A series of oligosaccharides, including glucose, cellobiose, and cellotriose were produced by glucan and lichenan hydrolysis. Glucose and laminaribiose were included in the final products of laminarin hydrolysis, indicating the enzyme can hydrolyze mainly β-1,3-linked laminarin.

Based on these results, the gene *Bg1* encodes an endo-type β-1,3(4)-D-glucanase (E.C. 3.2.1.6).

DISCUSSION

In this study, we described the cloning of a multifunctional β-glucanase secreted by *Paenibacillus* sp. F-40. A genomic library was constructed by using *E. coli* and vector pUC19 and screened in selective media. The β-glucanase could be synthesized and secreted to form a transparent zone on the LB plate containing lichenan. The supernatant of the cultivated clone pUCP-2287 showed the glucanase activity of 3.42 U/ml. The expression and secretion was under the control of the promoter, and signal peptide existed in the clone, indicating that the promoter and signal peptide of the *Bg1* gene can be functional in *E. coli*. However, the expression level was too low to stain on an SDS-PAGE gel (data not shown).

The cloning of the mature protein encoding sequence to vector pPIC9 led to high-level expression of *Bg1*. The recombinant protein Bg1 was higher than theoretically in molecular weight, which might be due to the glycosylation modification of the enzyme. There are four deduced *N*-glycosylation sites (Asn-X-Ser/Thr, X means any amino acid residue other than proline [7]) in sequence of Bg1 (Fig. 1). When treated with endo-H, the enzyme had a molecular weight of 27 kDa, which is heavier than that of theory. The

Table 2. Effects of metal ions and chemical reagents on Bg1 activity.

Metal ions and chemical reagents	Control	Na^+	K^+	Ca^{2+}	Cr^{3+}	Mg^{2+}	$Fe2^+$	Cu^{2+}	Ni^{2+}
Relativity activity (%)	100	94.23	84.86	92.79	95.16	80.46	83.27	82.38	95.88
Metal ions and chemical reagents	Pb^{2+}	Ag^+	Li^+	Zn^{2+}	Mn^{2+}	Co^{2+}	EDTA	SDS	
Relativity activity (%)	80.3	120.19	104.29	104.50	157.66	108.76	107.87	94.54	

Note: One mM of the ions and chemical reagents contained in the assay buffer.

Table 3. Substrate specificity of the purified Bgl.

Substrate	Main linkage (monomer)	Specific activity (IU/mg)	Relative activity (%)
Barley β-glucan	1,3-1,4-β-(glucose)	6,001±23	100
Lichenan	1,3-1,4-β-(glucose)	5,886±16	98.09
Laminarin	1,3-β-(glucose)	713±10	11.88
Oat spelt xylan	1,4-β-(xylose)	1.14±0.02	0.02
Birchwood xylan	1,4-β-(xylose)	0.6±0.01	0.01
CM-Cellulose	1,4-β-(glucose)	0	0

O-glycosylation and other modifications might be the reason.

The recombinant β-glucanase Bgl was able to hydrolyze both β-1,3-linked laminarin and β-1,3-1,4-linked barley β-glucan and lichenan, but not CM-cellulose and xylan (Table 3). The results suggest that Bgl is an endo-β-1,3(4)-glucanase (E.C. 3.2.1.6) instead of lichenase. Genes encoding both activities have been cloned [1, 3, 9, 10, 12, 14, 21, 33, 36, 37]. Comparing with other enzymes, Bgl is similar to the enzymes from *Cellvibro mixtus* [33], *Clostridium thermocellum* [36], and *Rhodothermus marinus* [37], which have 5–10 times higher activity on mixed linkage than on β-1,3 linkage.

Multiple alignments of β-1,3-glucanases, β-1,3-1,4-glucanases, and some E. C. 3.2.1.6 enzymes showed that three additional amino acids surrounding the active sites

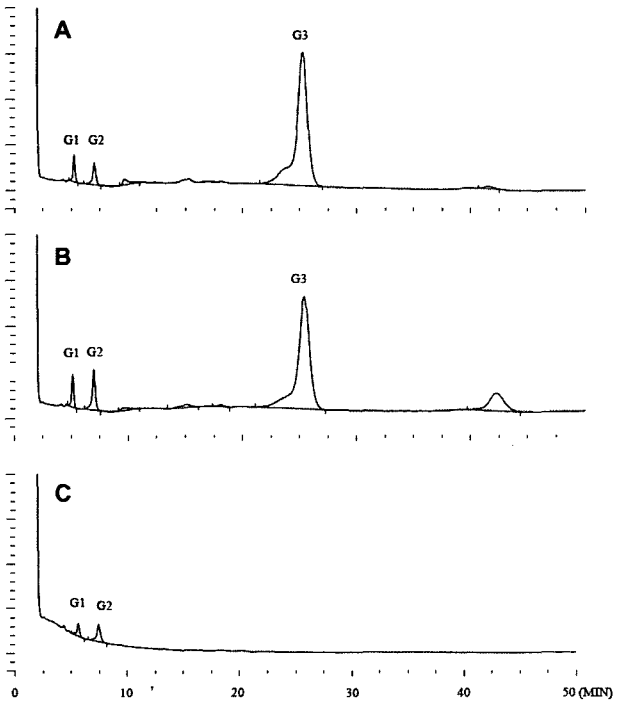

Fig. 5. HPAEC analysis of recombinant Bgl action on lichenan (**A**), barley β-glucan (**B**), and laminarin (**C**).
A 0.5% solution of each kind of substrate incubated with 25 μg recombinant Bgl for 4 h. G1, G2, G3 indicate glucose oligomers of different polymerization degree, as the standards.

are helpful to β-1,3-linkages hydrolysis and substrate binding [37]. However, the amino acid sequence of Bgl is homologous to *P. polymyxa* [13] and *P. macerance* β-1,3-1,4-glucanase [31], in which the three amino acids do not exist. The structure of Bgl was predicted and compared with lichenase of *P. macerans*, *B. licheniformis*, and hybrid enzyme H(A16-M) [11] (Fig. 2). Bgl is predicted to have an antiparallel jellyroll protein structure, the same as *Bacillus* β-1,3-1,4-glucanases. There were five unique amino acid residues existing in the Bgl sequence, including Q54, V85, I125, S189, and P193, instead of Y, I, V, D, and S in *P. macerans* and *B. licheniformis*, respectively (Fig. 2, boxed). Alignment of β-1,3-glucanases (E.C.3.2.1.39) showed that the conserved motif in accordance to S189 includes N (33%), Y (14%), Q (10%), W (10%), and other polar and nonpolar amino acids residues. The according residues of S189 in *Bacillus* β-1,3-1,4-glucanases mainly contain D (44%) and E (42%), which are both acidic amino acids. The two acidic amino acids may be a H^+ donor helpful to restrict substrate specificity, whereas S189 cannot. S instead of D or E was also found in endo-β-1,3(4)-glucanase of *C. acetobutylicum* [28] and *Streptococcus bovis* [10].

Gaiser *et al.* [11] and Piotukh *et al.* [31] reported sixteen residues involved in substrate binding. However, the above five amino acid residues in Bgl were not included in the line. The structure predicted by SWISS-MODEL (http://swissmodel. expasy.org/) showed that all the residues lie in a substrate binding cleft in a sandwich-like structure, except for Q54, which is at a turnover of two β-sheets (data not shown). These residues might have different substrate binding and catalytic functions compared with that in β-1,3-1,4-glucanases. However, the functions of the amino acids should be further determined by site-directed mutation.

High specific activity and efficient hydrolysis are necessary for an industrial process. The recombinant Bgl has a high specific activity of about 6,001 IU/mg on β-1,3-1,4-glucans, only lower than β-1,3-1,4-glucanases from *Fibrobacter succinogenes* [40] and *B. licheniformis* [25]. The characteristics of both crude enzyme preparations of the *E. coli* clone (data not shown) and purified protein Bgl of recombinant *P. pastoris* were determined. The optimal temperature of Bgl was 60°C, which was a little higher than crude enzyme from *E. coli*, probably because of glycosylation [39]; and

the enzyme activity retained about 50% at 37–40°C. However, in buffers of pH 4–9, the enzymes showed excellent stability. Both retained about 60% of enzyme activity after treatment with pepsin and trypsin. The pH stability and resistance to proteinase of the enzymes from *E. coli* and *P. patoris* are identical despite of glycosylation. These properties might contribute to production, storage, and utilizations in feed additives and beer brewing.

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