J Ind Microbiol Biotechnol (2012) 39:869–876
DOI 10.1007/s10295-012-1087-z

High-level expression of a novel *Penicillium* endo-1,3(4)-β-D-glucanase with high specific activity in *Pichia pastoris*

Xiaoyan Chen · Kun Meng · Pengjun Shi · Yingguo Bai · Huiying Luo · Huoqing Huang · Tiezheng Yuan · Peilong Yang · Bin Yao

Received: 29 November 2011 / Accepted: 12 January 2012 / Published online: 22 February 2012

Abstract A novel endo-1,3(4)-β-D-glucanase gene (*bgl16C1*) from *Penicillium pinophilum* C1 was cloned and sequenced. The 945-bp full-length gene encoded a 315-residue polypeptide consisting of a putative signal peptide of 18 residues and a catalytic domain belonging to glycosyl hydrolase family 16. The deduced amino acid sequence showed the highest identity (82%) with the putative endo-1,3(4)-β-glucanase from *Talaromyces stipitatus* ATCC 10500 and 60% identity with the characterized β-1,3(4)-glucanase from *Paecilomyces* sp. FLH30. The gene was successfully overexpressed in *Pichia pastoris*. Recombinant Bgl16C1 constituted 95% of total secreted proteins (2.61 g l^{-1}) with activity of 28,721 U ml^{-1} in a 15-l fermentor. The purified recombinant Bgl16C1 had higher specific activity toward barley β-glucan (12,622 U mg^{-1}) than all known glucanases and also showed activity against lichenan and laminarin. The enzyme was optimally active at pH 5.0 and 55°C and exhibited good stability over a broad acid and alkaline pH range (>85% activity at pH 3.0–7.0 and even 30% at pH 11.0). All these favorable enzymatic properties make it attractive for potential applications in various industries.

Keywords *Penicillium pinophilum* C1 · Endo-1,3(4)-β-glucanase · *Pichia pastoris* · Overexpression · High specific activity

X. Chen · K. Meng · P. Shi · Y. Bai · H. Luo · H. Huang · T. Yuan · P. Yang · B. Yao (✉)
Key Laboratory for Feed Biotechnology of the Ministry of Agriculture, Feed Research Institute, Chinese Academy of Agricultural Sciences, No. 12 Zhongguancun South Street, Beijing 100081, People's Republic of China
e-mail: binyao@caas.net.cn; yaobin@mail.caas.net.cn

Abbreviations

CMC-Na	Carboxymethyl cellulose-sodium salt
TAIL–PCR	Thermal asymmetric interlaced polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
DNS	3,5-Dinitrosalicylic acid
RT–PCR	Reverse-transcription polymerase chain reaction
ORF	Open reading frame
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight

Introduction

β-Glucan consists of D-glucose units linked by β-1,3 and β-1,4 glycosidic bonds and is the most important cell wall constituent of barley, wheat, rice, and rye [4]. β-Glucanase can specifically hydrolyze the mixed-linkage (1 → 3) or (1 → 4) of β-D-glucan into short oligosaccharides [29]. According to the type of glycosidic bonds which they cleave, endoglucanases are classified into three subgroups: endo-1,3-β-glucanase (EC 3.2.1.39; laminarinase) that cleaves β-1,3-glycosyl linkages, endo-1,3-1,4-β-glucanase (EC 3.2.1.73; lichenase) that strictly cleaves β-1,4-glycosidic linkage adjacent to a 3-*O*-substituted glucose residue in mixed-linkage β-glucans but does not act on β-D-glucans containing only 1,3- or 1,4- bonds, and endo-1,3(4)-β-glucanase (EC 3.2.1.6) that catalyzes endohydrolysis of (1 → 3) or (1 → 4) linkage of β-D-glucan when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3 [12].

Among them, endo-1,3-1,4-β-glucanases and endo-1,3(4)-β-glucanases have been widely applied as industrial enzymes in the brewing process to reduce the viscosity and filtration time of mash and in animal feed to improve the digestibility of β-glucan [2, 7, 38]. A number of bacterial glucanases have been reported from *Streptomyces* [33], *Fibrobacter succinogenes* [36], *Bacillus* [30, 37], and *Paenibacillus* [10, 39]. Fungi are another good microbial source of glucanases, such as *Rhizomucor miehei* [1], *Orpinomyces* sp. PC-2 [9], *Aspergillus japonicus* [14], *Paecilomyces* spp. [15, 16], and *Bispora* sp. MEY-1 [25].

Penicillium [20] has been proved to be an excellent producer of cellulases and hemicellulases for industrial application as well as the genera *Aspergillus* and *Trichoderma*. Several endo-β-1,4-glucanases from *Penicillium pinophilum* KMJ601 [19], *P. purpurogenum* [22], and *P. echinulatum* [32] have been reported, but all of them only have the ability to hydrolyze the β-(1 $\rightarrow$ 4) glycosidic bonds of glucan. In the present paper, we describe the molecular cloning, overexpression, and biochemical properties of a novel glycosyl hydrolase family 16 endo-1,3(4)-β-glucanase gene from *P. pinophilum* C1.

Materials and methods

Strains, plasmids, and chemicals

P. pinophilum C1 (China General Microbiological Culture Collection Center accession no. 4432) from acidic wastewater (pH 3.0) of a tin mine has been reported to be an excellent hemicellulase producer [5, 6] and was the donor strain of endo-1,3(4)-β-glucanase gene. *Escherichia coli* trans1-T1 and vector pEASY-T3 (TransGen, China) were employed for gene cloning. *Pichia pastoris* GS115 and plasmid pPIC9 (Invitrogen) were used for gene expression. Substrates such as barley β-glucan, laminarin, lichenan, birchwood xylan, and carboxymethyl cellulose-sodium salt (CMC-Na) were purchased from Sigma. The DNA purification kit, LA Taq DNA polymerase, and restriction endonucleases were purchased from TaKaRa (Tsu, Japan). T4 DNA ligase was supplied by Promega. All other chemicals were of analytical grade and commercially available.

Gene cloning

The genomic DNA of *P. pinophilum* C1 was extracted using Omega Fungal DNA kit after 72 h of cultivation in potato dextrose broth at 30°C and used as the template. According to the conserved motifs of glycosyl hydrolase family 16 β-glucanases CGTWPA and FCGD(Q/N)WAG, a set of degenerate primers GH16F and GH16R (Table 1) were designed and used to amplify the partial endo-1,3(4)-β-glucanase gene. The PCR product with correct size was purified and ligated into pEASY-T3 vector for sequencing. Based on the known partial nucleotide sequence, six nested specific primers (Table 1) were designed and used to obtain the full-length endo-1,3(4)-β-glucanase gene (*bgl16C1*) by using thermal asymmetric interlaced (TAIL)–PCR with TaKaRa Genome Walking kit.

To induce endo-1,3(4)-β-glucanase production, strain C1 was cultured in a medium containing 5 g l^{-1} $(NH_4)_2SO_4$, 1 g l^{-1} KH_2PO_4, 500 mg l^{-1} $MgSO_4{\cdot}7H_2O$, 200 mg l^{-1} $CaCl_2$, 10 mg l^{-1} $FeSO_4{\cdot}7H_2O$, and 30 g l^{-1} corn straw, pH 3.0 at 30°C for 3 days. Total messenger RNA (mRNA) was isolated using SV Total RNA isolation system (Promega) and reverse-transcribed to synthesize complementary DNA (cDNA) with a ReverTra Ace-α-® kit (Toyobo, Osaka, Japan). The cDNA of *bgl16C1* was amplified via reverse-transcription (RT)–PCR and ligated into pEASY-T3 vector for sequencing.

Sequence analysis

The nucleotide sequences were assembled and analyzed by Vector NTI advance 7.0 (Invitrogen). Alignment of nucleotide and deduced amino acid sequences was carried out with online Blastn and Blastp programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. The coding region was predicted online using the FGENESH program (http://linux1.softberry.com/berry.phtml/). SignalP 4.0 web server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. The putative *N*-glycosylation sites were predicted by the NetNGlyc 1.0 program (http://www.cbs.dtu.dk/services/NetNGlyc).

Expression of *bgl16C1* in *P. pastoris* and high-cell-density fermentation

The gene fragment coding for Bgl16C1 without the signal peptide was amplified from the cDNA of strain C1 with a set of expression primers Bgl16SF and Bgl16NR (Table 1) using *pfu* DNA polymerase (TransGen). After digestion with restriction endonucleases *Spe*I and *Not*I, the gene fragment was ligated into the pPIC9 vector in-frame with the α-factor signal peptide. The recombinant plasmid pPIC9-*bgl16C1* was linearized with *Bgl*II and transformed into *P. pastoris* GS115 competent cell using Gene Pulser Xcell electroporation system (Bio-Rad). Positive strains were cultured and induced with methanol in test-tubes, and the one with highest β-glucanase activity was picked out for further study.

A fed-batch-mode fermentation process was developed to produce recombinant Bgl16C1 in a 15-l fermentor containing 8 l growing medium. The whole procedure was

Table 1 Primers used in this study

Primer	Sequence (5′ → 3′)[a]	T_m (°C)[b]
GH16F	TGCGGTAYNTGGCCNGC	45.0–50.0
GH16R	CCGGCCCANTBNCCRCARAA	
Bgl16uSP1	GCTCCGGCTGATCTCACTTCTGGTAG	60.5
Bgl16uSP2	CTGGTAGCCCTAACCCAAGTACCTGG	60.5
Bgl16uSP3	CCTACAGTCATACTTGTCTAGAAAAC AATTAACTAATCATGTCC	60.2
Bgl16dSP1	CGGCGCTGTTGAATCCATCTCCGTAC	60.5
Bgl16dSP2	GAGGAAGTCGCATCAATACCGCAGCC	60.5
Bgl16dSP3	GCTGGTGTGCAGGGTCATGGCATTG-	60.5
Bgl16SF	GGGACTAGTCAAACCTACACTTTGTACGACGAC	54.0
Bgl16NR	GGGGCGGCCGCCTAGCTTGAGTAGACCTTGAGAGAG	55.6

[a] Y = C/T, R = A/G, B = T/G/C, N = A/T/G/C; restriction sites are underlined

[b] T_m: annealing temperature

carried out according to the Invitrogen *Pichia* expression manual. β-Glucanase activity in the culture supernatant was assayed at each 24-h interval during induction/expression.

Purification and SDS-PAGE analysis of recombinant Bgl16C1

The supernatant medium was collected by centrifugation (12,000×*g*, 4°C, 10 min) to remove cell debris and undissolved materials. Then, the cell-free culture supernatant was loaded onto a FPLC HiTrap Q Sepharose XL 5 ml column (GE Healthcare) that was equilibrated with 20 mM Tris–HCl (pH 8.0). Proteins were eluted using a linear gradient of NaCl (0–1.0 M) in the same buffer mentioned above at flow rate of 3.0 ml min^{-1}. Fractions containing the enzyme activity were pooled and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [21]. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a Bruker Daltonics Autoflex system (Bruker Daltonics) was used to determine the accurate molecular mass of Bgl16C1. The instrument settings were: delayed extraction time, 190 ns; detection mode, reflector mode; laser frequency, 50 HZ; accelerating voltage, 20 kV; sum shots, 100; matrix, α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid. To determine the protein concentration, a Bradford assay was used with bovine serine albumin as standard [3].

Enzyme activity assay

The endo-1,3(4)-β-glucanase activity was assayed using the 3,5-dinitrosalicylic acid (DNS) method [27] by measuring the amount of reducing sugar released. The standard assay system was composed of 100 µl appropriately diluted enzyme and 900 µl 100 mM Na_2HPO_4–citric acid (pH 5.0) containing 1.0% (w/v) barley β-glucan at 50°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol reducing sugar per minute. Each reaction was repeated three times, and each experiment had triplicate determinations.

Biochemical characterization of purified recombinant Bgl16C1

The optimal pH of purified recombinant Bgl16C1 was determined at 50°C in 100 mM Na_2HPO_4–citric acid (pH 2.0–6.0) containing 1.0% barley β-glucan for 10 min. The optimal temperature was determined at 0–60°C in 100 mM Na_2HPO_4–citric acid (pH 5.0) for 10 min. To determine its pH stability, purified Bgl16C1 was preincubated in buffers of various pHs at 37°C for 1 h, and the residual enzyme activity was assayed under standard conditions (pH 5.0, 50°C, 10 min). The buffers used were 100 mM Na_2HPO_4–citric acid (pH 2.0–8.0), 100 mM Tris–HCl (pH 8.0–9.0), and 100 mM glycine–NaOH (pH 9.0–12.0). For thermostability assay, the enzyme was preincubated at 50°C, 55°C, and 60°C for various time periods, and the remaining activity was assayed under standard conditions.

The effects of various metal ions and chemical reagents on the endo-1,3(4)-β-glucanase activity of Bgl16C1 were investigated at 50°C for 10 min in 100 mM Na_2HPO_4–citric acid (pH 5.0) containing 5 mM or 10 mM of Na^+, K^+, Ca^{2+}, Li^+, Co^{2+}, Ni^{2+}, Cu^{2+}, Mg^{2+}, Fe^{3+}, Mn^{2+}, Pb^{2+}, Ag^+, SDS, ethylenediamine tetraacetic acid (EDTA), or β-mercaptoethanol (β-ME). The experiment without any additive was carried out as a control.

Substrate specificity and kinetic parameters

Substrate specificity of Bgl16C1 was assayed at 50°C for 10 min in 100 mM Na_2HPO_4–citric acid (pH 5.0) containing 1.0% (w/v) barley β-glucan, lichenan, laminarin, birchwood xylan, or CMC-Na. The kinetic parameters K_m and V_{max} of Bgl16C1 were examined in 100 mM

Na_2HPO_4–citric acid (pH 5.0) containing 1–10 mg ml^{-1} barley β-glucan at 50°C for 5 min. The resulting data were plotted according to the Lineweaver–Burk method [24].

Nucleotide sequence accession number

The nucleotide sequence of the endo-1,3(4)-β-glucanase gene (*bgl16C1*) from *P. pinophilum* C1 was deposited in the GenBank database under accession no. JN903529.

Results

Gene cloning and sequence analysis

A gene fragment of 589 bp was cloned from the genomic DNA of *P. pinophilum* C1. By using TAIL–PCR, the 3′ and 5′ flanking fragments were obtained and assembled with the known sequence mentioned above. Sequence analysis indicated that the full-length endo-1,3(4)-β-glucanase gene (*bgl16C1*) consisted of 1,113 base pairs.

When using corn straw as sole carbon source, strain C1 exhibited 0.97 U ml^{-1} of β-glucanase activity in the culture supernatant after 96 h induction at 30°C. The cDNA sequence of *bgl16C1* was obtained via RT–PCR. The open reading frame (ORF) of 945 bp was identified, interrupted by three introns of 59, 52, and 57 bp, respectively. SignalP analysis indicated the presence of a putative signal peptide at residues 1–18. The calculated molecular mass of the mature protein was 31.5 kDa. No putative *N*-glycosylation site (Asn-Xaa-Thr/Ser-Zaa, where Zaa is not Pro) was identified.

Homology analysis indicated that the deduced Bgl16C1 shared the highest identity with putative endo-1,3(4)-β-glucanases from *Talaromyces stipitatus* ATCC 10500 (82%; XP_002484108) and *Penicillium marneffei* ATCC 18224 (71%; XP_002150155), and 60% identity with characterized β-1,3(4)-glucanase from *Paecilomyces* sp. FLH30 (ADZ46179) [16] and 58% identity with β-1,3-1,4-glucanase from *Paecilomyces* sp. J18 (ADK55597) [15]. The deduced Bgl16C1 contained a catalytic domain of glycosyl hydrolase family 16 and no carbohydrate binding domain. The conserved motif EIDIIE of most fungal glucanases of family 16 was identified (Fig. 1), and Glu120 and Glu125 were the putative nucleophile and acid/base catalytic residues, respectively.

Expression and purification of Bgl16C1

The gene fragment of *bgl16C1* without the signal peptide coding sequence was amplified by Bgl16SF and Bgl16NR from the genomic DNA of strain C1. The resulting sequence was digested and ligated into vector pPIC9. The recombinant plasmid pPIC9-*bgl16C1* was integrated into the chromosome of *P. pastoris* GS115. After induction with methanol for 72 h, the culture supernatant was subjected to endo-1,3(4)-β-glucanase activity assay. One recombinant cultured in test-tube showing the highest activity of 543 U ml^{-1} was picked out for high-cell-density fermentation in a 15-l fermentor. Before the induction phase, no endo-1,3(4)-β-glucanase was detected in the culture supernatant. With methanol induction, the weight of total secreted proteins and the β-glucanase activity increased together, reaching maximal values of 2.61 ± 0.11 g l^{-1} and 28,721 ± 664 U ml^{-1}, respectively, after 120 h of induction (Fig. 2).

Recombinant Bgl16C1 was purified from the culture supernatant by ion-exchange chromatography. SDS-PAGE showed only one protein band, corresponding to molecular mass of 42.0 kDa (Fig. 2). The accurate molecular mass of Bgl16C1 was determined to be 35.6 kDa by MALDI-TOF, which was higher than its calculated value (31.5 kDa) but lower than that of SDS-PAGE result. The result suggested that posttranslational modifications occurred in Bgl16C1 during heterologous expression in *P. pastoris* and these posttranslation modifications had effects on the migration rate of Bgl16C1 on SDS-PAGE.

Characterization of purified recombinant Bgl16C1

The optimal pH for active of recombinant Bgl16C1 was 5.0 at 50°C, and it exhibited >60% peak activity at pH 4.0–5.5 (Fig. 3a). At pH 5.0, Bgl16C1 was optimally active at 55°C and displayed as much as 8% of maximum activity at 0°C (Fig. 3b). After 1 h preincubation at pH 3.0–7.0 without substrate, Bgl16C1 retained >85% activity; the enzyme retained even 30% activity after 1 h incubation at pH 11.0 (Fig. 3c). Bgl16C1 was thermostable at 50°C, retaining about 90% activity after 1 h incubation at 50°C without substrate. Its half-life at 55°C was 30 min, and it lost almost all activity within 5 min at 60°C (Fig. 3d).

The effects of metal ions and chemical reagents on the activity of Bgl16C1 were tested at concentrations of 5 and 10 mM. Most cations had slight or no effect on the activity of Bgl16C1 (Table 2). β-ME and Fe^{3+} enhanced the β-glucanase activity at concentration of 10 mM by 1.4- and 1.3-fold, respectively. EDTA slightly enhanced the activity of Bgl16C1. Ag^{+} and SDS were strong inhibitors, and Cu^{2+}, Mn^{2+}, and Pb^{2+} moderately inhibited the β-glucanase activity.

Substrate specificity and kinetic parameters

Purified recombinant Bgl16C1 showed highest activity toward barley β-glucan (12,622 ± 265 U mg^{-1}) followed by lichenan (8,517 ± 316 U mg^{-1}) and laminarin (1,596 ± 33 U mg^{-1}). No activity against birchwood xylan and

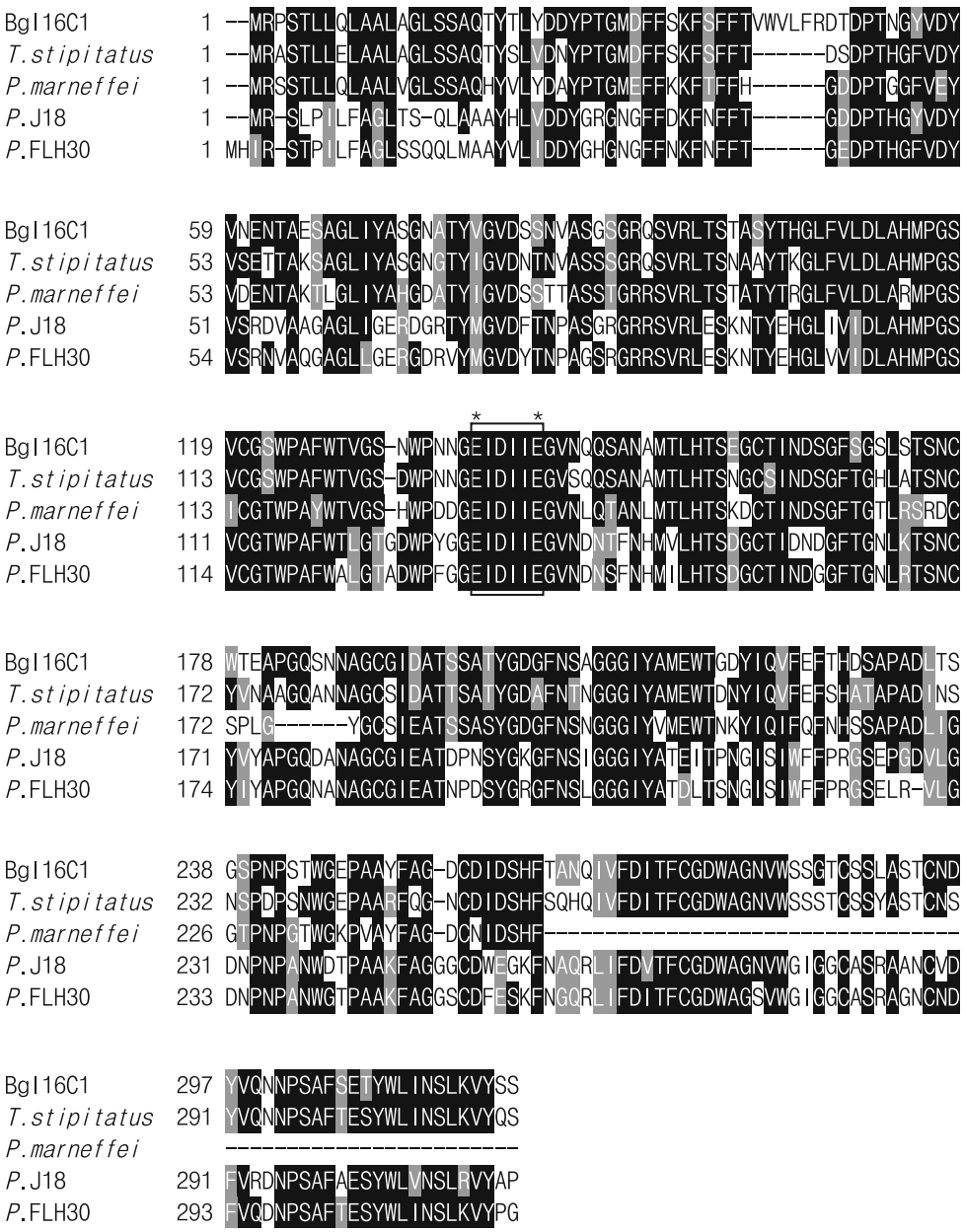

Fig. 1 Amino acid sequence alignment of Bgl16C1 with other endo-β-1,3(4)-glucanases from *T. stipitatus* (XP_002484108), *P. marneffei* (XP_002150155), *Paecilomyces* sp. FLH30 (ADZ46179), and *Paecilomyces* sp. J18 (ADK55597). Identical and similar amino acids are indicated by *solid black* and *gray boxes*, respectively. The conserved active-site motif is indicated by a *black frame*. The two catalytic glutamate residues are indicated by *asterisks*

CMC-Na was detected. When using barley β-glucan as the substrate, the K_m and V_{max} values were determined to be 7.581 mg ml^{-1} and 26,558 μmol min^{-1} mg^{-1}, respectively.

Discussion

In the present study, an endo-1,3(4)-β-glucanase gene (*bgl16C1*) was cloned from a *Penicillium* strain and successfully overexpressed in *P. pastoris*. The deduced Bgl16C1 consisted of two functional domains—one N-terminal leader sequence and one catalytic domain of glycosyl hydrolase family 16, which contains the conserved motif EIDIIE as most fungal GH16 glucanases. The recombinant Bgl16C1 had similar substrate specificity to its close homolog—the endo-1,3(4)-β-glucanase from *Paecilomyces* sp. FLH30 [16]. Both enzymes showed highest activity toward barley β-glucan, followed by lichenan and laminarin, but degraded neither CMC-Na nor birchwood xylan. The results of sequence alignment and substrate specificity indicate that Bgl16C1 is a classical nonspecific endo-1,3(4)-β-D-glucanase which can catalyze hydrolysis of β-1,3 or β-1,4 glycosidic bonds.

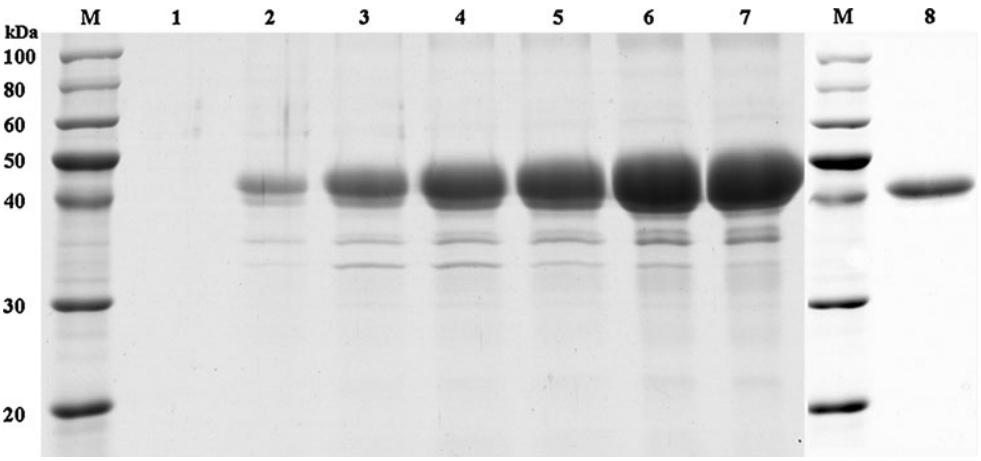

Fig. 2 SDS-PAGE analyses of the expression time course of recombinant Bgl16C1 in *P. pastoris*. *Lanes*: *M* molecular mass markers, *1* culture supernatant before induction, *2–7* culture supernatants after induction for 24, 48, 72, 96, 120, and 144 h, respectively, *8* purified recombinant Bgl16C1

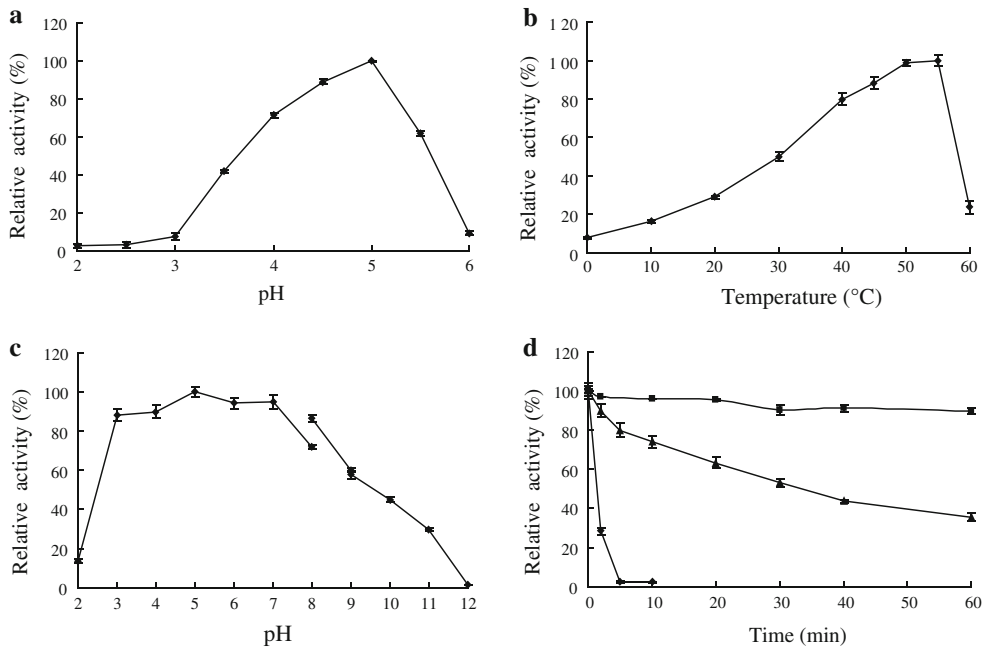

Fig. 3 Characterization of purified recombinant Bgl16C1. **a** Effect of pH on Bgl16C1 activity assayed in 100 mM McIlvaine buffer at 50°C for 10 min. **b** Effect of temperature on enzyme activity measured in 100 mM McIlvaine buffer (pH 5.0). **c** Effect of pH on the stability of Bgl16C1 activity. After incubating purified recombinant Bgl16C1 at 37°C for 1 h in buffers with pH ranging between pH 2.0 and 12.0, residual activity was measured in 100 mM McIlvaine buffer (pH 5.0) at 50°C. **d** Thermostability of purified recombinant Bgl16C1. The enzyme was preincubated at 50°C (*filled squares*), 55°C (*filled triangles*), and 60°C (*filled diamonds*) in 100 mM McIlvaine buffer (pH 5.0), and aliquots were removed at specific time points for measurement of residual activity at 50°C. Each value in the panels represents the mean of triplicates ± standard deviation

Although Bgl16C1 shared high identities with the endo-1,3(4)-β-glucanases PsBgl16A and PtLic16A from *Paecilomyces* sp. J18 and *Paecilomyces* sp. FLH30, respectively [15, 16], it exhibited obviously distinct biochemical properties. PsBgl16A and PtLic16A exhibited their highest activity at neutral pH (7.0) and 70°C, whereas Bgl16C1 was optimally active at acidic pH 5.0 and 55°C and lost all activity under alkaline conditions. Attractively, Bgl16C1 was stable at pH ranging from pH 3.0 to 7.0 and kept 30% activity after treatment at pH 11.0 for 1 h. Bgl16C1 had lower optimum temperature (55°C) than PsBgl16A and PtLic16A (both at 70°C), but its optimal temperature for activity was higher than that of recombinant LicA (45°C) from *Orpinomyces* sp. PC-2 [9] and β-1,3-1,4-glucanase (50°C) from *Bacillus subtilis* 168 [13]. Furthermore, Bgl16C1 still had approximately 50–80% activity at 30–40°C, and so would exhibit good catalytic activity in the gut of animals [38]. In addition, the activity of Bgl16C1 was sharply enhanced by β-ME, which was similar to the β-1,3-1,4-glucanase from *B. licheniformis* [37] and

Table 2 Effects of metal ions and chemical reagents on Bgl16C1 activity

Chemical	Relative activity (%)[a]		Chemical	Relative activity (%)[a]	
	5 mM	10 mM		5 mM	10 mM
None	100.0 ± 1.2	100.0 ± 3.3	Mg^{2+}	109.3 ± 0.5	105.0 ± 0.2
Na^{+}	96.6 ± 0.6	109.0 ± 1.1	Fe^{3+}	117.7 ± 1.0	130.6 ± 1.6
K^{+}	88.7 ± 1.0	103.2 ± 0.4	Mn^{2+}	115.8 ± 2.3	95.6 ± 5.7
Ca^{2+}	97.3 ± 2.0	108.6 ± 2.6	Pb^{2+}	95.4 ± 0.2	91.3 ± 4.2
Li^{+}	95.6 ± 1.1	103.6 ± 2.3	Ag^{+}	26.9 ± 1.6	12.4 ± 2.1
Co^{2+}	108.1 ± 0.7	114.0 ± 3.8	β-mercaptoethanol	131.6 ± 1.5	157.2 ± 3.2
Cu^{2+}	102.9 ± 0.5	85.5 ± 1.4	EDTA	114.8 ± 1.7	123.9 ± 0.5
Ni^{2+}	99.0 ± 0.4	103.1 ± 2.9	SDS	9.5 ± 1.5	3.2 ± 0.8

[a] Values represent means ± SD ($n = 3$) relative to untreated control samples

PsBg16A from *Paecilomyces* sp. FLH30 [16]. Fe^{3+} ion moderately activated the activity of Bgl16C1, too.

Many methods have been developed to increase the catalytic efficiency of enzymes and improve their properties and expression levels. Construction of fusion proteins has been reported to enhance the hydrolysis activity and thermostability of a β-1,3-1,4-glucanase [34]. Several heterologous expression systems including *E. coli* [30], *P. pastoris* [35], *Saccharomyces cerevisia* [18], and *Aspergillus oryzae* [28] have been used to produce glucanases. Of them, *P. pastoris* has a secretion system for eukaryotic proteins which can perform appropriate post-translational modifications to facilitate correct folding of heterologous into correct functional higher-order structures [8, 11]. Moreover, *P. pastoris* has a strong, inducible promoter and can be easily manipulated as *E. coli* for high-cell-density fermentation under inexpensive growth conditions, thus representing an ideal heterologous expression system [26]. So far, many glucanases have been successfully expressed in *P. pastoris* with high expression levels [17, 23, 35]. In this study, the total secreted proteins of *P. pastoris* reached a rather high level of 2.61 g l^{-1}, and recombinant Bgl16C1 constituted approximately 95% of them. This value is higher than the expression level of β-1,3-1,4-glucanase (250 mg l^{-1}) from *B. licheniformis* after codon optimization [37], Bgl7A (370 mg l^{-1}) from *Bispora* sp. MEY-1 [25], and Bgl (0.8 g l^{-1}) from *Paenibacillus* sp. F-40 [39], but lower than that of a truncated r-Bgl (3.0 g l^{-1}) from *F. succinogenes* [17], PsBgl16A (8.4 g l^{-1}) from *Paecilomyces* sp. FLH30 [16], and PtLic16A (9.1 g l^{-1}) from *P. thermophila* [15]. However, Bgl16C1 had high specific activity toward barley β-glucan (12,622 U mg^{-1}), being slightly higher than PtLic16A from *P. thermophila* (11,938 U mg^{-1}), but significantly higher than PsBgl16A from *Paecilomyces* sp. FLH30 (8,649 U mg^{-1}), r-Bgl from *F. succinogenes* (5,180 U mg^{-1}), Bgl7A from *Bispora* sp. MEY-1 (4,040 U mg^{-1}), Bgl from *Paenibacillus* sp. F-40 (3,076 U mg^{-1}), β-1,3-1,4-glucanase from *B. subtilis* MA139 (728.79 U mg^{-1}) [30], and EglA from *Aspergillus niger* (63.83 U mg^{-1}) [31]. Considering its optimal activity at acidic and mesothermal conditions, high expression level, and specific activity as well as simple processing steps, Bgl16C1 would represent an ideal candidate for use in the animal feed industry.

Acknowledgments This research was supported by the National Science and Technology Support Program (2011BADB02), the China Modern Agriculture Research System (CARS-42), and the Key Program of Transgenic Plant Breeding (2009ZX08003-020B).

References

1. Boyce A, Walsh G (2007) Production, purification and application-relevant characterisation of an endo-1,3(4)-β-glucanase from *Rhizomucor miehei*. Appl Microbiol Biotechnol 76:835–841
2. Bai Y, Wang J, Zhang Z, Shi P, Luo H, Huang H, Luo C, Yao B (2010) A novel family 9 β-1,3(4)-glucanase from thermoacidophilic *Alicyclobacillus* sp. A4 with potential applications in the brewing industry. Appl Microbiol Biotechnol 87:251–259
3. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
4. Buliga GS, Brant DA, Fincher GB (1986) The sequence statistics and solution conformation of a barley (1 → 3,1 → 4)-β-D-glucan. Carbohydr Res 157:139–156
5. Cai H, Shi P, Bai Y, Huang H, Yuan T, Yang P, Luo H, Meng K, Yao B (2011) A novel thermoacidophilic family 10 xylanase from *Penicillium pinophilum* C1. Process Biochem 46:2341–2346
6. Cai H, Shi P, Luo H, Bai Y, Huang H, Yang P, Yao B (2011) Acidic β-mannanase from *Penicillium pinophilum* C1: cloning, characterization and assessment of its potential for animal feed application. J Biosci Bioeng. doi:10.1016/j.jbiosc.2011.08.018
7. Celestino KR, Cunha RB, Felix CR (2006) Characterization of a β-glucanase produced by *Rhizopus microsporus* var. *microsporus*, and its potential for application in the brewing industry. BMC Biochem 7:23
8. Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66
9. Chen H, Li XL, Ljungdahl LG (1997) Sequencing of a 1,3–1,4-β-D-glucanase (lichenase) from the anaerobic fungus *Orpinomyces* strain PC-2: properties of the enzyme expressed in *Escherichia coli* and evidence that the gene has a bacterial origin. J Bacteriol 179:6028–6034

10. Cheng YM, Hong TY, Liu CC, Meng M (2009) Cloning and functional characterization of a complex endo-β-1,3-glucanase from *Paenibacillus* sp. Appl Microbiol Biotechnol 81:1051–1061
11. Daly R, Hearn MT (2005) Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J Mol Recognit 18:119–138
12. Dashtban M, Schraft H, Qin W (2009) Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. Int J Biol Sci 5:578–595
13. Furtado GP, Ribeiro LF, Santos CR, Tonoli CC, de Souza AR, Oliveira RR, Murakami MT, Ward RJ (2011) Biochemical and structural characterization of a β-1,3–1,4-glucanase from *Bacillus subtilis* 168. Process Biochem 46:1202–1206
14. Grishutin SG, Gusakov AV, Dzedzyulya EI, Sinitsyn AP (2006) A lichenase-like family 12 endo-(1 → 4)-β-glucanase from *Aspergillus japonicus*: study of the substrate specificity and mode of action on β-glucans in comparison with other glycoside hydrolases. Carbohydr Res 341:218–229
15. Hua C, Yan Q, Jiang Z, Li Y, Katrolia P (2010) High-level expression of a specific β-1,3–1,4-glucanase from the thermophilic fungus *Paecilomyces thermophila* in *Pichia pastoris*. Appl Microbiol Biotechnol 88:509–518
16. Hua C, Yi H, Jiao L (2011) Cloning and expression of the endo-1,3(4)-β-glucanase gene from *Paecilomyces* sp. FLH30 and characterization of the recombinant enzyme. Biosci Biotechnol Biochem 75:1807–1812
17. Huang H, Yang P, Luo H, Tang H, Shao N, Yuan T, Wang Y, Bai Y, Yao B (2008) High-level expression of a truncated 1,3–1,4-β-d-glucanase from *Fibrobacter succinogenes* in *Pichia pastoris* by optimization of codons and fermentation. Appl Microbiol Biotechnol 78:95–103
18. Huang XM, Yang Q, Liu ZH, Fan JX, Chen XL, Song JZ, Wang Y (2010) Cloning and heterologous expression of a novel endoglucanase gene *egVIII* from *Trichoderma viride* in *Saccharomyces cerevisiae*. Appl Biochem Biotechnol 162:103–115
19. Jeya M, Joo AR, Lee KM, Sim WI, Oh DK, Kim YS, Kim IW, Lee JK (2010) Characterization of endo-β-1,4-glucanase from a novel strain of *Penicillium pinophilum* KMJ601. Appl Microbiol Biotechnol 85:1005–1014
20. Karboune S, ĽHocine L, Anthoni J, Geraert PA, Kermasha S (2009) Properties of selected hemicellulases of a multi-enzymatic system from *Penicillium funiculosum*. Biosci Biotechnol Biochem 73:1286–1292
21. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
22. Lee KM, Jeya M, Joo AR, Singh R, Kim IW, Lee JK (2010) Purification and characterization of a thermostable endo-β-1,4-glucanase from a novel strain of *Penicillium purpurogenum*. Enzyme Microb Technol 46:206–211
23. Li J, Tang C, Shi H, Wu M (2011) Cloning and optimized expression of a neutral endoglucanase gene (*ncel5A*) from *Volvariella volvacea* WX32 in *Pichia pastoris*. J Biosci Bioeng 111:537–540
24. Lineweaver H, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658–666
25. Luo H, Yang J, Yang P, Li J, Huang H, Shi P, Bai Y, Wang Y, Fan Y, Yao B (2010) Gene cloning and expression of a new acidic family 7 endo-β-1,3–1,4-glucanase from the acidophilic fungus *Bispora* sp. MEY-1. Appl Microbiol Biotechnol 85:1015–1023
26. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249–270
27. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
28. Ooi T, Sato H, Matsumoto K, Taguchi S (2009) A unique post-translational processing of an exo-β-1,3-glucanase of *Penicillium* sp. KH10 expressed in *Aspergillus oryzae*. Protein Expr Purif 67:126–131
29. Planas A (2000) Bacterial 1,3–1,4-β-glucanases: structure, function and protein engineering. Biochim Biophys Acta 1543:361–382
30. Qiao J, Dong B, Li Y, Zhang B, Cao Y (2009) Cloning of a β-1,3–1,4-glucanase gene from *Bacillus subtilis* MA139 and its functional expression in *Escherichia coli*. Appl Biochem Biotechnol 152:334–342
31. Quay DHX, Bakar FDA, Rabu A, Said M, Illias RM, Mahadi NM, Hassan O, Murad AMA (2011) Over expression, purification and characterization of the *Aspergillus niger* endoglucanase, *EglA*, in *Pichia pastoris*. Afr J Biotechnol 10:2101–2111
32. Rubini MR, Dillon AJ, Kyaw CM, Faria FP, Pocas-Fonseca MJ, Silva-Pereira I (2010) Cloning, characterization and heterologous expression of the first *Penicillium echinulatum* cellulase gene. J Appl Microbiol 108:1187–1198
33. Shi P, Yao G, Yang P, Li N, Luo H, Bai Y, Wang Y, Yao B (2010) Cloning, characterization, and antifungal activity of an endo-1,3-β-D-glucanase from *Streptomyces* sp. S27. Appl Microbiol Biotechnol 85:1483–1490
34. Sun J, Wang H, Lv W, Ma C, Lou Z, Dai Y (2011) Construction and characterization of a fusion β-1,3–1,4-glucanase to improve hydrolytic activity and thermostability. Biotechnol Lett 33:2193–2199
35. Shumiao Z, Huang J, Zhang C, Deng L, Hu N, Liang Y (2010) High-level expression of an *Aspergillus niger* endo-β-1,4-glucanase in *Pichia pastoris* through gene codon optimization and synthesis. J Microbiol Biotechnol 20:467–473
36. Teather RM, Erfle JD (1990) DNA sequence of a *Fibrobacter succinogenes* mixed-linkage β-glucanase (1,3–1,4-β-d-glucan 4-glucanohydrolase) gene. J Bacteriol 172:3837–3841
37. Teng D, Fan Y, Yang YL, Tian ZG, Luo J, Wang JH (2007) Codon optimization of *Bacillus licheniformis* β-1,3–1,4-glucanase gene and its expression in *Pichia pastoris*. Appl Microbiol Biotechnol 74:1074–1083
38. Walsh GA, Murphy RA, Killeen GF, Headon DR, Power RF (1995) Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. J Anim Sci 73:1074–1076
39. Yang P, Shi P, Wang Y, Bai Y, Meng K, Luo H, Yuan T, Yao B (2007) Cloning and overexpression of a *Paenibacillus* β-glucanase in *Pichia pastoris*: purification and characterization of the recombinant enzyme. J Microbiol Biotechnol 17:58–66