

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

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Abstract Most reported microbial β -1,3-1,4-glucanases belong to the glycoside hydrolase family 16. Here, we report a new acidic family 7 endo- β -1,3-1,4-glucanase (Bgl7A) from the acidophilic fungus *Bispora* sp. MEY-1. The cDNA of Bgl7A was isolated and over-expressed in *Pichia pastoris*, with a yield of about 1,000 U ml⁻¹ in a 3.7-l fermentor. The purified recombinant Bgl7A had three activity peaks at pH 1.5, 3.5, and 5.0 (maximum), respectively, and a temperature optimum at 60°C. The enzyme was stable at pH 1.0–8.0 and highly resistant to both pepsin and trypsin. Belonging to the group of non-specific endoglucanase, Bgl7A can hydrolyze not only β -glucan and cellulose but also laminarin and oat spelt xylan. The specific activity of Bgl7A against barley β -glucan and lichenan (4,040 and 2,740 U mg⁻¹) was higher than toward carboxymethyl cellulose sodium (395 U mg⁻¹), which was different from other family 7 endo- β -glucanases.

Keywords Endo- β -1,3-1,4-glucanase · Acidophilic fungus · *Bispora* sp. MEY-1 · *Pichia pastoris*

Introduction

β -Glucans are the major component of cereal cell walls and are particularly abundant in the endosperm cell walls of cereals such as barley, rye, rice, and wheat (Buliga et al. 1986). The endosperm cell wall is composed of β -D-glycosyl residues linked through β -1,3 and/or β -1,4 glycosidic bonds. Degradation of β -glucans in nature is catalyzed by β -glucanases, which can be grouped into four main categories according to the type of glycosidic linkage they cleave: β -1,3-1,4-glucanases (lichenases, EC 3.2.1.73), β -1,4-glucanases (cellulases, EC 3.2.1.4), β -1,3-glucanases (laminarinases, EC 3.2.1.39), and β -1,3(4)-glucanases (EC 3.2.1.6; McCarthy et al. 2003; Boyce and Walsh 2007; Yang et al. 2008).

β -1,3-1,4-Glucanases (lichenases) have received significant attention because of their enzymatic functions and importance in industrial applications. For example, in brewing industry, the addition of β -1,3-1,4-glucanases can reduce brewer mash viscosity and turbidity, increase yields of extracts, and produce high-quality brewers malt (Celestino et al. 2006); in animal feed industry, supplementation of this enzyme can increase β -glucan digestibility in feed stuffs, improve feed conversion efficiency, and reduce sanitary problems such as sticky droppings (Mathlouthi et al. 2002; Beckmann et al. 2006).

Most reported microbial β -1,3-1,4-glucanases belong to the glycoside hydrolase (GH) family 16 and are produced by bacteria, especially the genus *Bacillus* (Ekinci et al. 1997; Planas 2000; Teng et al. 2006; Huang et al. 2008).

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And, a number of extracellular β -1,3-1,4-glucanases have been purified from a variety of fungal sources such as *Orpinomyces* sp. PC-2 (Chen et al. 1997), *Cochliobolus carbonum* (Görlach et al. 1998), *Talaromyces emersonii* (Murray et al. 2001), *Aspergillus japonicus* (Grishutin et al. 2006), *Rhizopus microsporus* var. *microsporus* (Celestino et al. 2006), and *Paecilomyces thermophila* (Yang et al. 2008). But, only a few of them such as those from *Orpinomyces* sp. PC-2, *C. carbonum*, and *A. japonicus* have been reported on gene cloning, enzyme characterization, and classification. Some GH family 3, 5, 7, and 12 cellulases (endo- β -1,4-glucanases) are active toward β -1,3-1,4-linked barley β -glucan and lichenan as well as β -1,4-linked carboxymethyl cellulose (CMC; Hasper et al. 2002; Grishutin et al. 2006; Feng et al. 2007). However, little is known about the specificity or mode of action of the fungal β -1,3-1,4-glucanases.

The acidophilic fungus *Bispora* sp. MEY-1 can secrete a variety of GHs with special enzymatic properties. Gene cloning and expression of an acidic β -mannanase (Luo et al. 2009a) and an acidic xylanase (Luo et al. 2009b) from this strain have been reported. In this paper, we describe the molecular cloning, overexpression, and biochemical properties of Bgl7A, a family 7 endo- β -glucanase from *Bispora* sp. MEY-1. The acidic enzyme was stable at pH 1.0–8.0, showed excellent protease resistance, and had high catalytic activity on both β -1,3-1,4-glucan and β -1,4-glucan. These biochemical properties make it ideal for use in brewing and animal feed industrial applications.

Materials and methods

Strains, vectors, media, and chemicals

To induce β -glucanase production, *Bispora* sp. MEY-1 CGMCC 2500 (Luo et al. 2009a) was cultivated at 30°C and pH 2.5 in medium consisting of 5.0 g l⁻¹ (NH₄)₂SO₄, 1.0 g l⁻¹ KH₂PO₄, 0.5 g l⁻¹ MgSO₄·7H₂O, 0.2 g l⁻¹ CaCl₂, 0.01 g l⁻¹ FeSO₄·7H₂O, 30 g l⁻¹ wheat bran, and 30 g l⁻¹ corncob powder. *Escherichia coli* top 10 competent cells (Transgen, Beijing, China) were cultivated at 37°C in Luria–Bertani medium. *Pichia pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) was cultivated at 30°C in yeast extract peptone dextrose medium. The plasmids pEASY-T₃ (Transgen) and pPIC9 (Invitrogen) were used as cloning and expression vectors, respectively.

The substrates lichenan, barley β -glucan, laminarin, oat spelt xylan, carboxymethyl cellulose sodium (CMC-Na), Avicel, glucose, cellobiose and the proteases pepsin (from bovine), and trypsin (from bovine) were purchased from Sigma (San Diego, CA, USA). Cellotriose and cellotetraose were purchased from Seikagaku

(Tokyo, Japan). The DNA purification kit, Genome Walking kit, and LA Taq DNA polymerase were purchased from TaKaRa (Tsu, Japan). Restriction endonucleases and T₄ DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). DNA polymerase *Pfu* was purchased from Tiangen (Beijing, China). All other chemicals were of analytical grade and commercially available.

Cloning of the β -glucanase encoding gene

To clone the β -glucanase encoding gene, a degenerate primer set was designed based on the conserved motifs of family 7 members from subphylum *Ascomycota*. A total of 128 family 7 amino acid sequences were obtained from Pfam (<http://www.sanger.ac.uk/>) and aligned using ClustalX software (<ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/>; Jeanmougin et al. 1998). Based on the alignment, two conserved amino acid sequences, namely GYCDABC and GCGFNPY, were found. The degenerate primers GH7F (5'-GGYACTGYGAYGCNCARTG-3') and GH7R (5'-TAGGGRTTGAANCCRCANCC-3', Y = C/T, R = A/G, N = A/T/C/G) were designed and used to amplify the partial core region of the GH 7 β -glucanase gene, *bgl7A*, from *Bispora* sp. MEY-1. The polymerase chain reaction (PCR) conditions were as follows: 3 min at 95°C, followed by 30 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min. The PCR products with the correct size were purified and ligated into the pEASY-T₃ vector for sequencing. The partial sequence of the core region was analyzed by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). The 5' and 3' flanking regions of the core region were obtained using the Genome Walking kit with thermal asymmetric interlaced (TAIL)-PCR, according to the manufacturer's instructions (Liu and Whittier 1995).

Total RNA extraction and RT-PCR were carried out according to the methods of Luo et al. (2009a), using specific primers Bgl7AF (5'-ATGGCAGTGTTCACCTGACCGTGGCTATTATAAC-3') and Bgl7AR (5'-CTAATCCAACCGCCAGAGCCAGAGCC-3'), with an annealing temperature of 62°C.

Sequence analysis

Sequence assembly and nucleotide sequence were analyzed using the Vector NTI advance 10.0 software (Invitrogen). The amino acid sequences were aligned using the BLASTp program (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the ClustalW program. The signal peptide was predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). Homology modeling was performed using SWISS-MODEL (<http://swissmodel.expasy.org/SWISS-MODEL.html>; Arnold et al. 2006).

Recombinant expression of *bgl7A* in *P. pastoris*

To construct the *bgl7A* vector for expression in *P. pastoris*, a gene fragment containing *bgl7A* without the signal peptide coding sequence was amplified by PCR using primers P*bgl*F (5'-ACCGAATTCCAGCAGATTGGTCGCATACCAGAAGTTCATCC-3', *Eco*RI site underlined) and P*bgl*R (5'-ACTGCGGCCGCCTAATTCCAACCGCCAGAGCCAGAGCC-3', *Not*I site underlined). The DNA polymerase *Pfu* and an annealing temperature of 65°C were used for the PCR amplification. The PCR product was digested with *Eco*RI and *Not*I and cloned into pPIC9 in-frame fusion with the α -factor signal peptide. Recombinant expression and high-cell-density fermentation were performed following the method as previously described (Luo et al. 2009a).

Purification of recombinant Bgl7A

To purify recombinant Bgl7A, the induced culture (200 ml) was centrifuged at 12,000 \times g for 10 min at 4°C to remove cell debris. The culture supernatant was then concentrated and desalted by ultrafiltration using vivaflow 50 ultrafiltration membranes with a molecular weight cut-off of 5 kDa (Vivascience, Hannover, Germany). The protein was then precipitated by acetone. The resulting precipitate was dissolved in 5 ml of 20 mM Tris-HCl (pH 7.5) and loaded onto a HiTrap Q Sepharose XL 5 ml FPLC column (Amersham Pharmacia Biotech, Uppsala, Sweden) that was equilibrated with 20 mM Tris-HCl (pH 7.5). Proteins were eluted using a linear gradient of NaCl (0–1.0 M) in the same buffer. Fractions having enzyme activity were pooled and concentrated to 2.0 ml by ultrafiltration at 4,000 \times g for 20 min at 4°C using an Amicon Ultra Centrifugal Filter Device PL-10 (Millipore, Billerica, MA, USA).

Protein expression and purification profiles were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was carried out as described by Laemmli (1970). The protein concentration was determined using a Bradford assay (1976) with bovine serine albumin as a standard. To identify the purified protein, the in-gel tryptic digest of the protein after SDS-PAGE was analyzed by the peptide fingerprinting method using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/MS).

Deglycosylation analysis of Bgl7A

The purified recombinant Bgl7A (10 μ g) was treated with 250 U of endo- β -*N*-acetylglucosaminidase H (Endo H) for 2 h at 37°C according to the supplier's instructions (New England Biolabs) and then analyzed by SDS-PAGE.

Glucanase activity assay

The enzymatic activity of β -1,3-1,4-glucanase was determined by measuring the amount of reducing sugar released from lichenan with the method of 3,5-dinitrosalicylic acid (DNS; Miller 1959; Huang et al. 2008). Citric acid-Na₂HPO₄ buffer (0.1 M, pH 5.0) containing 1.0% (w/v) lichenan was used. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of reducing sugar per minute from 1.0% lichenan at 60°C for 10 min. Each reaction and its control were run in triplicate.

Biochemical characterization of Bgl7A

The pH optimum of the purified recombinant Bgl7A was determined at 60°C and pH 1.0–6.0 for 10 min. The pH stability of Bgl7A was determined by measuring the residual activity under standard conditions (pH 5.0, 60°C and 10 min) after pre-incubation at 37°C and pH 1.0–9.0 for 1 h. The buffers used were 0.1 M KCl-HCl for pH 0.5–2.2, 0.1 M citric acid-Na₂HPO₄ for pH 2.2–8.0, and 0.1 M Tris-HCl for pH 8.0–9.0. The optimal temperature was determined at 25–80°C in 0.1 M citric acid-Na₂HPO₄ (pH 5.0) for 10 min. Thermal stability of the purified recombinant enzyme was determined by assessing the residual enzyme activity under standard conditions after incubation of the enzyme at either 60°C or 70°C for various duration.

The effect of chemicals (Na⁺, K⁺, Ca²⁺, Li⁺, Co²⁺, Cr³⁺, Ni²⁺, Cu²⁺, Mg²⁺, Fe³⁺, Mn²⁺, Zn²⁺, Pb²⁺, Ag⁺, Hg²⁺, SDS, EDTA, and β -mercaptoethanol) on the activity of the purified recombinant Bgl7A and its resistance to proteolysis were determined as described before (Luo et al. 2009a).

To investigate the specificity of Bgl7A for glycosidic bonds, multiple substrates of lichenan, barley β -glucan, CMC-Na, Avicel, laminarin, and oat spelt xylan were selected (Table 1). Enzyme activity was determined after incubation in 0.1 M citric acid-Na₂HPO₄ buffer (pH 5.0) containing 1.0% of each substrate at pH 5.0 and 60°C for 10 min. The amount of reducing sugars produced was estimated using the DNS method as described above.

The kinetic parameters, K_m and V_{max} , were determined in 0.1 M citric acid-Na₂HPO₄ (pH 5.0) containing 0.5–10.0 mg ml⁻¹ lichenan, barley β -glucan, or CMC-Na after incubation with purified recombinant Bgl7A at pH 5.0 and 60°C for 5 min. The data were plotted according to the Lineweaver-Burk method. Each datum was an average of three independent experiments and every experiment included three samples.

Analysis of hydrolysis products by HPAEC

For studying the hydrolysis products of β -glucan under the action of purified enzymes, 50 U purified recombinant

Table 1 Substrate specificity and kinetic parameters of purified recombinant Bgl7A

Substrate	Main linkage (monomer)	Specific activity (U mg ⁻¹)	K _m (mg ml ⁻¹)	V _{max} (U min ⁻¹ mg ⁻¹)
Barley β-glucan	1,3-1,4-β-(glucose)	4,040	9.16	6,737
Lichenan	1,3-1,4-β-(glucose)	2,740	12.01	3,019
CMC-Na	1,4-β-(glucose)	395	287	3,460
Laminarin	1,3-β-(glucose)	4.8	–	–
Oat spelt xylan	1,4-β-(xylose)	4.6	–	–
Avicel	1,4-β-(glucose)	0	–	–

enzyme and 5 mg lichenan or barley β-glucans in 1 ml citric acid–Na₂HPO₄ buffer (pH 5.0) was incubated at 60°C for 6 h. After hydrolysis, the enzyme was removed from the reaction using the Nanosep Centrifugal 3 K Device (Pall, East Hills, NY, USA). The products were analyzed by high-performance anion-exchange chromatography (HPAEC) with a model 2500 system from Dionex (USA; Yang et al. 2007). Glucose, cellobiose, cellotriose, and cellotetraose were used as standards.

Nucleotide sequence accession numbers

The nucleotide sequence for the *Bispora* sp. MEY-1 β-glucanase gene *bgl7A* was deposited in the GenBank database under accession number FJ695140.

Results

Cloning of the β-glucanase gene *bgl7A* and sequence analysis

Using degenerate PCR and TAIL-PCR techniques, a 2,787-bp fragment containing a β-glucanase coding gene (1,341 bp) was cloned from the acidophilic *Bispora* sp. MEY-1. The full-length cDNA sequence (1,281 bp) was obtained via RT-PCR using the specific primers Bgl7AF and Bgl7AR. The chromosomal gene was interrupted by one intron of 60 bp and coded for a peptide of 427 amino acids with a theoretical molecular mass of 45.3 kDa. SignalP analysis indicated the presence of an N-terminal signal peptide at residues 1–19. Five potential *N*-glycosylation sites (Asn-Xaa-Thr/Ser-Zaa, where Zaa is not Pro) were found.

The deduced amino acid sequence of Bgl7A exhibited the highest identity of 59.5% with a putative family 7 endoglucanase EG-1 from *Aspergillus terreus* and of 54.5% with an endo-1,4-β-glucanase from *Aspergillus oryzae* (Kitamoto et al. 1996). The alignment with other endo-β-glucanases is shown in Fig. 1. No carbohydrate-binding domain was identified based on sequence alignment of Bgl7A. The putative catalytic residues in Bgl7A, Glu215,

Asp217, and Glu220 are highly conserved among GH family 7 members (Kleywegt et al. 1997).

The three-dimensional structure of *Bispora* sp. MEY-1 β-glucanase was predicted using the SWISS-MODEL server with the automatically selected *Trichoderma reesei* endoglucanase I (Protein data bank code, 1EG1) as the closest template. The theoretical results show that Bgl7A consists of two large anti-parallel β-sheets that pack face to face to form a β-sandwich and four short helical segments (Fig. 2). The extensive loops are stabilized by seven disulphide bridges although eight disulphide bridges stabilize the loops in *T. reesei* endoglucanase I. The active-site residues, EMDLWE, and two carbohydrate-binding tryptophan residues, W366 and W375, in Bgl7A are conserved in family 7 members (EMDL(I)W(L)E) and are similar to that of family 16 endo-1,3-1,4-β-glucanases (EIDIE). Moreover, a conserved histidine residue exists in the active site of Bgl7A (His231), which is identical to His212 in *T. reesei* endoglucanase I. This conserved residue is replaced by tyrosine in GH family 16 endo-1,3-1,4-β-glucanases. As a result, the wider and kinked solvent-accessible surface of *T. reesei* endoglucanase I becomes narrow and straight in GH 16 endo-1,3-1,4-β-glucanases. Such variation may possibly tailor the enzyme for its specific substrate and function.

Expression and purification of Bgl7A in *P. pastoris*

Recombinant Bgl7A was successfully expressed in *P. pastoris* GS115 and secreted into the medium. The highest β-1,3-1,4-glucanase activity was 93 U ml⁻¹ in a shaker flask culture after induction with methanol for 72 h. SDS-PAGE revealed that recombinant Bgl7A was the major protein in the culture supernatant. A faint band (~60–80 kDa) was visible, which was much higher than the predicted molecular weight, perhaps due to over-glycosylation (Fig. 3). A piece of the faint smear was digested with trypsin, and the resulting peptides were analyzed by MALDI-TOF/MS. The amino acid sequences obtained from the mass peaks were compared with the amino acid sequence of Bgl7A. The peptides QYLNID GTEEEVSPR, VYLLDPSGR, DYEILK, VYLLDPSG RDYEILK, and PFTVVTQFLTSDGTSTGTLSQLR com-

Fig. 1 Amino acid sequence alignment of Bgl7A from *Bispora* sp. MEY-1 with the known endo- β -glucanase from *Aspergillus oryzae* (D83732), endo-glucanase I from *Trichoderma reesei* (P07981), and lichenase from *Bacillus macerans* (P23904) using the ClustalW program. Identical and similar amino acids are indicated by solid black and gray boxes, respectively. The active-site residues are indicated by black frame and catalytic glutamate residues are indicated by asterisks

<i>Bispora</i> sp.	1	MA---VFTLTVAILITLIRFAS SQQ IGR-IPEVHPRLTTQTCT-KHGCTTQQTS VLDALS
<i>A. oryzae</i>	1	M---IWTL-APFVALPLVIAQQVGT-TADAHPRLLTTYKCTSQNGCTRQNTS VLDAAI
<i>T. reesei</i>	1	MAPSVTLPLTTALIAIARLVAAQQPGTSTPEVHPKLTTYKCTKSGGCVAGDTS VLDWNY
<i>B. macerans</i>	1	MK-----KKSCTLVVTTAFSLIFSVALAGS VFWPELS
<i>Bispora</i> sp.	56	HPIDDIHTNESETSSGGVNTTICPTVEVCAENCALEGVN-YASHGVYINGDS VTLRQYL
<i>A. oryzae</i>	55	HFIIHKKGTCTSCTNSNG-IDTALCPDKQTCADNCVVDGITDYASYGVQTKNDITLQOYL
<i>T. reesei</i>	61	RWMHDANYN-SCTVNGG-VNNTLCPDEATCGKNGFIEGVD-YAASGVTTSGSSLTMNQYM
<i>B. macerans</i>	35	YFNRSTWEKADGYSNGGVFNCTWRAN-----NVN-TNDGKCLKG-LTSSAYN
<i>Bispora</i> sp.	115	NID-GTEEEVSPRVYLLDPSGRDYEILKLLNQEISFTVDVSNLPCGMNGALYLLSMDASG
<i>A. oryzae</i>	114	QTG-NATKSLSPRVYLLAEDGENYSMLKLLNQEFITFDVDASTLYCGMNGALYLSMEASG
<i>T. reesei</i>	118	PSSSGGYSSVSPRYLLDSDGE-YVMLKLNQQLSFDVDLSALPCGNGSLYLSQMDENG
<i>B. macerans</i>	81	KFDCAEYRSTN-----TYGYGLYEVSMLKPAKNTGIVSSFYTYG
<i>Bispora</i> sp.	174	GRSQLNPAGATYGTGYCDAQCNAPAWINGVANLKGKGCCEMDLWEANSATCLTPHAC
<i>A. oryzae</i>	173	GKSSLNQAGAKYGTGYCDAQCYTTPWINGEGNTEVGSCCQEMDIWEANARATGLTPHC
<i>T. reesei</i>	177	GANQVNTAGANYGSGYCDAQCPVQTVRNGLTNTSHQGFCCNEMDITLEGNSRAALTPHSC
<i>B. macerans</i>	120	-----PAHG GTQW EIDI-EFL KDT K QFY
<i>Bispora</i> sp.	234	NTTGFYECSGAACGSNGVCDKDGCGFNPYGLGD SFYGPSVTDITDTKFTVVTQFLTS
<i>A. oryzae</i>	233	NTTGLYECSGSGCGDSGVCDKAGCGFNPYGLGAK YYG-YGLVVTNFTVVTQFLTN
<i>T. reesei</i>	237	TAI-----CDAGCGFNPYGSYKSYG-PGDTVDTKTFTITQFNID
<i>B. macerans</i>	136	YTN-----VGGHEVI LGLFASKG-FHTYFDWQPGYIKWYVDGL
<i>Bispora</i> sp.	294	DGTSTGILSIRRLYLQNGKVIQNAKVDFDSTLDSITPAYCEATAATFEAEGG PQMGK
<i>A. oryzae</i>	291	DITTSGLSIRRLYLQNGQVIQNAAVTSGGKTVDSITKDFCSGEGSAFNRLGGLLEEMGL
<i>T. reesei</i>	281	NGS SGNLVSITRKYQNGVDIPAAQPG-GDTISCSASVYG-----GLATMGK
<i>B. macerans</i>	178	KHTTANISTPG-----

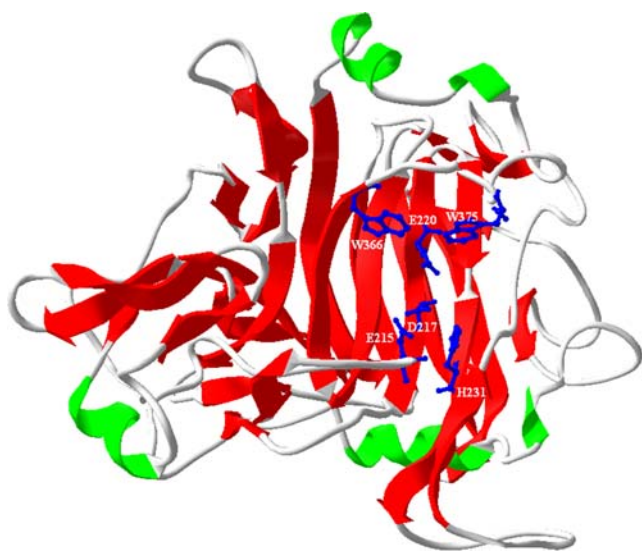


Fig. 2 The predicted structure of *Bispora* sp. MEY-1 Bgl7A. The active sites and the substrate binding sites are indicated

pletely corresponded to the amino acid sequence of Bgl7A, and no peptides from other proteins were detected, confirming the purity of the band and identity of Bgl7A.

The transformant that showed the highest β -1,3-1,4-glucanase activity in the shaker flask culture was subjected to high-cell-density fermentation. Before the induction phase, no β -1,3-1,4-glucanase activity was detected in the culture supernatant. After methanol induction, the β -1,3-1,4-glucanase activity in the supernatant increased substantially, reaching a maximum of about 1,000 U ml⁻¹ after 120-h induction. The maximal concentration of secreted Bgl7A was 370 mg l⁻¹.

Purification and deglycosylation of Bgl7A

The recombinant Bgl7A was purified by ion exchange chromatography. The specific activity of the purified Bgl7A was 2,740 U mg⁻¹ after 1.3-fold purification, with a final activity yield of 32.3%. The purified enzyme yielded a faint smear band with a molecular mass of 60–80 kDa by SDS-PAGE (Fig. 3). After Endo H treatment, purified Bgl7A migrated as a single band of ~48 kDa on SDS-PAGE,

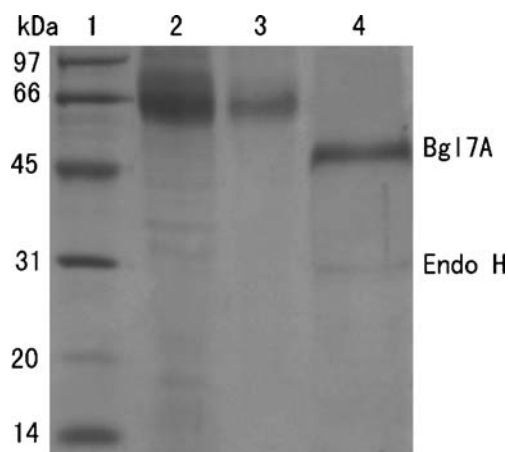


Fig. 3 SDS-PAGE analysis of purified recombinant Bgl7A before and after deglycosylation with Endo H. Lanes 1 standard protein molecular weight markers (sizes indicated on left); 2 culture supernatant from the induced transformant; 3 purified recombinant Bgl7A; 4 purified recombinant Bgl7A after deglycosylation with Endo H

which was a little higher than the calculated molecular weight of the protein (45.3 kDa; Fig. 3).

Biochemical properties of purified recombinant Bgl7A

Purified recombinant Bgl7A exhibited optimal activity around pH 5.0 at 60°C, and the activity curve had three peaks pH 1.5 (61%), 3.5 (91%), and 5.0 (100%), respectively (Fig. 4a). Following incubation at 37°C for 1 h without substrate, the enzyme retained greater than 85% activity at pH 1.0–8.0 (Fig. 4b). Enzyme activity was optimal at 60°C (Fig. 4c) and remained stable at 60°C for at least 60 min (Fig. 4d). After incubation at 70°C for 5 and 60 min, the enzyme retained more than 48% and 30% of the initial activity, respectively.

We tested the effects of various cations and compounds (5 and 10 mM) on Bgl7A activity. The enzymatic activity was enhanced or not affected by the presence of Na⁺, K⁺, Li⁺, Ca²⁺, or β-mercaptoethanol. Ag⁺, Hg²⁺, SDS, and EDTA strongly inhibited the enzyme activity of Bgl7A. Partial inhibition (< 30%) was observed in the presence of certain metal ions (5 or 10 mM), including Mg²⁺, Co²⁺, Cr³⁺, Cu²⁺, Ni²⁺, Zn²⁺, Pb²⁺, Fe³⁺, and Mn²⁺.

Bgl7A was strongly resistant to pepsin and trypsin digestion. After treatment with pepsin and trypsin at 37°C for 1 h, the enzyme retained 99.6% and 107.3% activity, respectively.

Substrate specificity and kinetic parameters of Bgl7A was examined by measuring the release of reducing sugars from several substrates with various linkages (Table 1). The highest activity was detected for barley β-glucan (100%), followed by lichenan (72.0%) and CMC-Na (9.8%). Laminarin and oat spelt xylan were also degraded by the

enzyme, but Avicel was not. Kinetic parameters were determined for lichenan, barley β-glucan, and CMC-Na. These results confirmed that Bgl7A is a β-1,3-1,4-glucanase (lichenase).

Analysis of hydrolysis products

The hydrolysis products of barley β-glucan and lichenan by recombinant Bgl7A were analyzed by HPAEC. Bgl7A hydrolyzed barley β-glucan and lichenan to produce glucose and cellobiose as the predominant products. The composition of the hydrolysis products from barley β-glucan was 6.8 μmol ml⁻¹ glucose and 9.9 μmol ml⁻¹ cellobiose, and the product composition from lichenan was 9.1 μmol ml⁻¹ glucose, 16.3 μmol ml⁻¹ cellobiose, and 0.45 μmol ml⁻¹ cellotetraose. The results indicated that Bgl7A could effectively hydrolyze β-1,4 bonds and some β-1,3 linkages in β-glucan.

Discussion

In this study, a GH family 7 endo-β-glucanase gene from *Bispora* sp. MEY-1 was cloned and successfully expressed in *P. pastoris*. This is the first report of a β-glucanase from the genus *Bispora*. Most of the known GH 7 members listed in the CAZy database are classified as cellulases (<http://www.cazy.org>). However, the enzymatic activity of Bgl7A is much higher on barley β-glucan and lichenan, both of which consist of mixed β-1,4- and β-1,3-glucosidic linkages, than against CMC. Such substrate specificity resembles that of bacterial lichenases that belong to GH family 16. Furthermore, Bgl7A could hydrolyze not only β-glucan and cellulose but also laminarin and oat spelt xylan, thus belonging to the group of non-specific endoglucanase. Similar substrate preferences have also been found in *T. reesei* endoglucanase I (Claeysens et al. 1990) and *Humicola insolens* endoglucanase I (Schou et al. 1993). Both endoglucanases have a β-sandwich structure formed by two large anti-parallel β-sheets assembling face-to-face, very similar to that of *Bacillus* β-glucanases but distinct from those β-sandwich folds specific for family 11 xylanases (Kleywegt et al. 1997). Therefore the “non-specific” characteristic might ascribe to the special structure of these enzymes. From another point of view, the substrates xylan, barley β-glucan, lichenan, and cellulose have similar structure and are probably able to fit the same active center of the enzyme (Hrmova and Fincher 2001). Both of these presumptions have no experimental evidence, and thus, Bgl7A might be a good candidate to study the mechanism underlying.

The deduced amino acid sequence of Bgl7A showed significant similarity to that of endo-1,4-β-glucanase CelB

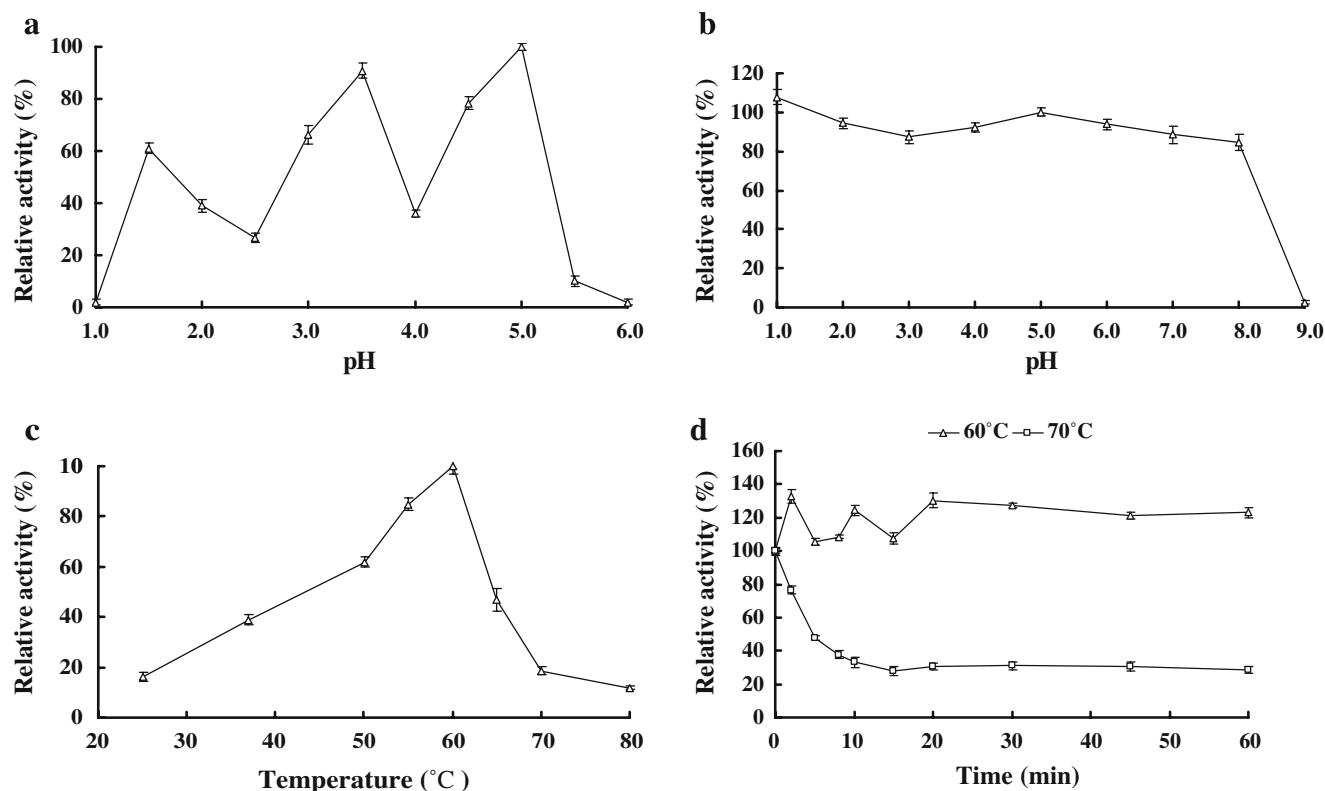


Fig. 4 Characterization of purified recombinant Bgl7A. **a** Effect of pH on β -1,3-1,4-gluconase activity. Activity was assayed at 60°C in buffers ranging from pH 1.0 to 6.0. **b** Effect of pH on the stability of β -1,3-1,4-gluconase activity. After incubation at 37°C for 1 h in buffers ranging from pH 1.0 to 9.0, the purified β -1,3-1,4-gluconase activity of recombinant Bgl7A was assayed in 0.1 M citric acid–

Na_2HPO_4 (pH 5.0) at 60°C. **c** Effect of temperature on β -1,3-1,4-gluconase activity measured in 0.1 M citric acid– Na_2HPO_4 (pH 5.0). **d** Thermostability of Bgl7A. The enzyme was pre-incubated at 60°C or 70°C in 0.1 M citric acid– Na_2HPO_4 (pH 5.0), and aliquots were removed at specific time points to measure residual activity at 60°C and pH 5.0. Each value represents the mean \pm SD of triplicates

from *A. oryzae* (54.5% identity) and endoglucanase I from *T. reesei* (49.4% identity), both of which belong to GH family 7. But, its properties are superior to those of CelB and endoglucanase I. For example, Bgl7A is highly thermostable—remaining active after incubation at 60°C, but CelB and endoglucanase I are not stable above 50°C. Moreover, Bgl7A has excellent pH stability at acidic pH (1.0–3.0), whereas CelB and endoglucanase I have not (Kitamoto et al. 1996; Nakazawa et al. 2008). Furthermore, *A. oryzae* CelB has never been tested for β -1,3-1,4-gluconase activity (Kitamoto et al. 1996), and nobody knows whether the enzyme might have such an activity, but Bgl7A can hydrolyze both β -1,3-1,4-glucon and cellulose. Comparing with bacterial lichenases, Bgl7A is highly active and stable at acidic pH, whereas bacterial lichenases have pH optima around neutrality (6.0–7.5). About substrate specificity, bacterial lichenases have only barley β -glucon activity, but Bgl7A can hydrolyze both β -1,3-1,4-glucon and cellulose (Planas 2000). These characteristics make Bgl7A more suitable for applications in animal feed, food, and beer wort because these industries require the combined activities of various gluconases.

bgl7A was cloned from an acidophilic fungus and encodes a protein that exhibits peak activities at pH 1.5, 3.5, and 5.0, with the highest activity at pH 5.0. Several fungal enzymes have been reported to have multiple pH optima. For example, the endo-1,3(4)- β -gluconase from *Rhizomucor miehei* (Boyce and Walsh 2007) has two peaks of enzyme activity; the multi-optimal pH characteristic of enzymes is also found among the phytases from *Aspergillus* sp. (Oh et al. 2004) and *A. japonicus* (Promdonkoy et al. 2008). It was reported that this property was probably due to the effect of salts present in various buffers used in the experiment (Promdonkoy et al. 2008). Comparing with *T. reesei* endoglucanase I, which has 32 acidic residues and three acidic amino acid residues at the active site, Bgl7A has 44 acidic residues and four (E215, D217, E220, and E224) locate at the active site. We presume that different pH might affect these acidic residues, shift the active sites from E215 and E220 to E215 and D217, E215 and E224, or anything else, thus resulting in multiple activity peaks. The biochemical reasons for the occurrence of multiple activity peaks related with the salts in tested buffer or acid residues in the catalytic domain needs more experimental work for verification.

Recombinant Bgl7A can hydrolyze not only barley β -glucan and cellulose but also laminarin and oat spelt xylan. This characteristic makes Bgl7A more suitable than conventional glucanases for hydrolysis of glucans in animal feed, food, and beer wort because these industries require the combined activities of different types of glucanases. Furthermore, acidophilic stability and high resistance to proteolysis very likely allow Bgl7A to function under the acidic conditions and high degree of proteolytic degradation in the digestive tract of animals. These properties indicate that Bgl7A may be a good candidate for improving nutrient utilization in the animal feed industry.

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