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NOTE

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Molecular identification of a novel β-1,3-glucanase from alkaliphilic *Nocardiopsis* sp. strain F96

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Abstract Alkaliphilic *Nocardiopsis* sp. strain F96 produced three *β*-1,3-glucanase isozymes of different molecular masses (BglF1, BglF2 and BglF3). The N-terminal amino acid sequences of BglFs indicated that these isozymes were the products of a single gene. The *β*-1,3-glucanase gene (*bglF*) was cloned from the chromosomal DNA of strain F96. The *bglF* gene encoded a polypeptide of 270 amino acids including a signal sequence. The deduced amino acid sequence of mature BglF exhibited the highest homology to those of glycoside hydrolase (GH) family 16 *β*-1,3-glucanases, suggesting that the enzyme belonged to the GH family 16. The mature region of *bglF* gene was functionally expressed in *Escherichia coli*. The optimum pH and temperature of purified recombinant BglF were pH 9.0 and 70°C, respectively. This enzyme efficiently hydrolyzed insoluble *β*-1,3-glucans and showed the highest activity toward a *β*-1,3-1,4-glucan rather than *β*-1,3-glucans. These results suggested that BglF would be a novel *β*-1,3-glucanse. Mutational analysis revealed that Glu123 and Glu128 should be the catalytic residues of BglF.

Keywords Alkaliphile · Actinomycete · *Nocardiopsis* sp. · *β*-1,3-Glucanase · Glycoside hydrolase (GH) family 16 · Gene cloning · Overexpression · Catalytic residue

β-1,3-Glucan, a polymer of *β*-1,3-linked glucose, is the main constitute of botanical and fungal cell walls. *β*-1,3-Glucanase (EC 3.2.1.39) hydrolyzes *β*-1,3 bonds of *β*-1,3-glucan. They are classified as endo-*β*-1,3-glucanase (EC 3.2.1.6 and EC 3.2.1.39) and exo-*β*-1,3-glucanase (EC 3.2.1.58). Decomposition of *β*-1,3-glucan is very important because there are lots of industrial needs for *β*-1,3-glucan hydrolysate and it is an effective solution for the depletion of natural resources. Alkaliphilic *Nocardiopsis* sp. strain F96, a producer of chitinolytic enzymes, was isolated from a soil sample at Fuji city in Shizuoka prefecture, Japan (Endo et al. 2003). In this study, we found that strain F96 secreted *β*-1,3-glucanase isozymes. Cloning and overexpression of the *β*-1,3-glucanase gene in *Escherichia coli* were performed followed by characterization of the recombinant protein. Mutational analysis was also done to reveal the catalytic residues of the enzyme.

Strain F96 was grown aerobically in the neutral medium (1% polypeptone, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.25% pachyman, pH 6.8) at 37°C for 96 h. Pachyman was prepared from commercial fruiting bodies of *Poria cocos*. Culture supernatant was prepared by centrifugation (at 10,000×g and 4°C for 30 min) and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel (Laemmli 1970). After electrophoresis, proteins in the gel were stained with Coomassie brilliant blue (CBB) R-250. The molecular mass markers used were the low-molecular weight calibration kit (Amersham Biosciences, Piscataway, NJ, USA). Zymogram analysis was performed by the method of Nakamura et al. (1993). Samples were electrophoresed on an SDS-12.5% polyacrylamide gel containing 0.1% laminarin (Nakalai Tesque, Kyoto, Japan). Then, the gel was washed four times for 30 min in 100 mM NaH_2PO_4–NaOH buffer (pH 6.0) [the first two washes contained 25% (v/v) isopropyl alcohol] to remove SDS and renature proteins in the gel, and further incubated in the buffer at 37°C for 10 min. The gel was soaked in 0.1% Congo red solution for 15 min at room temperature and washed with 1 M NaCl until excess dye was removed from the gel. To make the active bands clear, the gel was soaked in 0.5% acetic acid. The background turned dark blue, and clear zones were observed in the areas exposed to *β*-1,3-glu-

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canase activity. Three active bands were observed in zymogram analysis, suggesting that strain F96 secreted three β-1,3-glucanase isozymes (named BglF1, BglF2 and BglF3) in the culture supernatant (Fig. 1). Molecular masses of BglF1, BglF2 and BglF3 were 34.1, 32.4 and 30.7 kDa, respectively.

The culture was centrifuged at 10,000×*g* and 4°C for 30 min. Proteins in the supernatant (680 ml) were concentrated by ultra-filtration with a 10,000 Da-cutoff membrane (Sartorius, Goettingen, Germany). The concentrated fluid was dialyzed against 10 mM Tris–HCl buffer (pH 7.5). The dialyzed solution was applied to a DEAE-Toyopearl 650 M column (2.5 by 16.5 cm) (Tosoh, Tokyo, Japan) that had been equilibrated with 10 mM Tris–HCl buffer (pH 7.5). The column was washed with 400 ml of the buffer and then eluted with a linear gradient of 0–1,000 mM NaCl in 2 l of the buffer. Fractions were automatically collected, scanned for their absorbance at 280 nm, and assayed for β-1,3-glucanase activity. The active fractions that eluted around 440 mM NaCl were dialyzed against 20 mM Tris–HCl buffer (pH 7.5). The dialyzed solution was applied to a BioAssist Q column (1.0 by 10 cm) (Tosoh) that had been equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The column was washed with 15 ml of the buffer and then eluted with a linear gradient of 0–1,000 mM NaCl in 1 l of the buffer. β-1,3-Glucanase active fractions that eluted around 500 mM NaCl were pooled and stored at 4°C. β-1,3-Glucanase activity was assayed by measuring the amount of reducing sugars librated from β-1,3-glucan by the 3,5-dinitrosalicylic acid (DNS) method (Sumner et al. 1925). Briefly, 40 µl of the enzyme preparation was added to 160 µl of a 1.5% laminarin suspension in 100 mM NaH_2PO_4–NaOH buffer (pH 6.0). The reaction was carried out at 37°C for 15 min. The enzyme was inactivated by adding 400 µl of the DNS reagent and boiling for 5 min. Absorbance at 545 nm was measured immediately after 2.4 ml of distilled water was added to the mixture. One unit was defined as the amount of enzyme that produced reducing sugars equivalent to 1 µmol of glucose per minute under the conditions described above. In the case of analyzing pH profile in various pHs, Britton–Robinson buffers (Britton and Robinson 1931) were used.

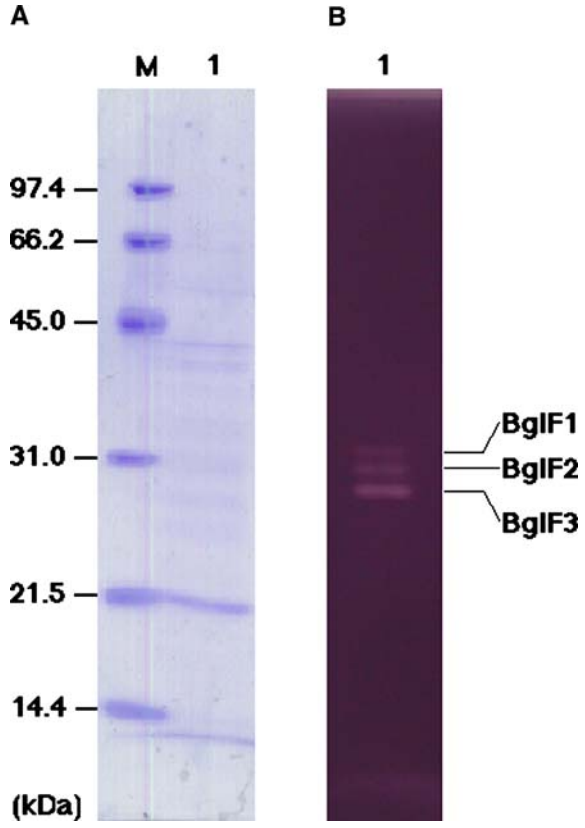

Fig. 1 SDS-PAGE of the culture supernatant of strain F96. **a** Sample was subjected to SDS-12.5% PAGE; the gel was stained with CBB R-250. **b** Sample was electrophoresed on an SDS-12.5% polyacrylamide gel containing 0.1% laminarin; the gel was examined for β-1,3-glucanase activity at 37°C and pH 6.0. *Lane 1* culture supernatant of strain F96; *M* molecular mass markers

As a result, 0.19 mg of BglF1 was purified to attain homogeneity, although BglF2 and BglF3 did not separate from each other. The specific activity of purified BglF1 was 3.3 U/mg of protein. The optimum pH and temperature of purified BglF1 were pH 8–9 and 70°C, respectively. The partially purified BglFs were separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Sequi-Blot PVDF Membrane; Bio-Rad, Hercules, CA, USA), and subsequently stained by CBB G-250. Protein bands corresponding to BglFs were cut off, and then subjected to a PPSQ-10 Protein Sequencer (Shimadzu, Kyoto, Japan). The N-terminal amino acid sequences of BglFs were found to overlap each other (Fig. 2), indicating that these isozymes were the products of a single gene. The discrepancy between molecular masses and N-terminal amino acid sequences of BglFs would suggest the possibility of C-terminal processing, as well as N-terminal processing.

To generate a probe for the β-1,3-glucanase gene (*bglF*), PCR was performed on the chromosomal DNA of strain F96 using an iCycler Thermalcycler (Bio-Rad). The chromosomal DNA of strain F96 was prepared as described previously (Endo et al. 2003). The primers used were as follows: 5′-TGG (A/T)(G/C)(G/C) GAC GA(G/A) TTC GAC GG-3′ (sense primer: corresponding to the N-terminal sequence of BglFs) and 5′-GTA (G/C)AC (G/C)CG (G/C)AC GTA GTC (G/C)AC-3′ (antisense primer: corresponding to the conserved sequence of several β-1,3-glucanases that had high homology with the N-terminal sequence of BglFs). The amplified DNA fragment of about 700 bp was

Fig. 2 N-terminal amino acid sequences of BglFs. Homologous residues observed were shadowed

cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA), labeled with digoxigenin using a DIG DNA Labeling Kit (Roche, Basel, Switzerland). The chromosomal DNA of strain F96 was digested by several restriction enzymes and applied onto electrophoresis with a 0.7% agarose gel. The DNA fragments on the gel were transferred onto Hybond N+ membrane (Amersham Biosciences), and Southern analysis was performed according to the standard protocol (Sambrook et al. 1989) using the DIG-labeled probe. The temperature for hybridization was 60°C. Chromosomal DNA digested with *Bam*HI showed a single hybridization band at 4.4 kb. DNA fragments of about 4.4 kb were isolated by preparative agarose gel electrophoresis, ligated into the *Bam*HI site of pUC119, and then introduced into *E. coli* JM109. Transformants were cultured on Luria-Bertani (LB) broth containing 50 μg/ml ampicillin, and screened for *bglF* gene by colony hybridization (Sambrook et al. 1989). Several positive clones were obtained and found to contain recombinant plasmids with an identical 4.4-kb genomic insert (named pBglF). The nucleotide sequence of the 4.4-kb insert was determined using a DSQ-2000L DNA Sequencer (Shimadzu) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). If necessary, 10% (v/v) of dimethyl sulfoxide (DMSO) was added to the sequence reaction mixtures. Sequence data were analyzed with programs FASTA (http://fasta.bioch.virginia.edu/) and GENETYX-MAC ver.11.1.2 (Genetyx, Tokyo, Japan). An open reading frame of 810 bp, encoding a 270-amino acid precursor enzyme, was found. The DNA sequence of *bglF* gene has been deposited to DDBJ/EMBL/GenBank with the accession number of AB244275. Typical promoter and Shine-Dalgarno sequences were observed upstream from the possible ATG start codon. The N-terminal sequences of native BglFs (see Fig. 2) agreed with the deduced amino acid sequence between the positions 26 and 45, suggesting that the polypeptide region between Met1 and Thr25 should be a signal sequence. The deduced amino acid sequence of mature BglF was homologous with those of other microbial glycoside hydrolase (GH) family 16 (http://www.cazy.org/CAZY/index.html) β-1,3-glucanases, such as BglII (74.7%) and BglIIa (72.0%) from *Oerskovia xanthineolytica* [reclassified as *Cellulomonas cellulans* (Stackebrandt et al. 1980) and then *Cellulosimicrobium cellulans* (Schumann et al. 2001)] LL G109 (Ventom and Asenjo 1991; Parrado et al. 1996; Ferrer et al. 1996a, b), BglM (48.5%) from *Bacillus circulans* IAM1165 (Asano et al. 2002), LamR (44.7%) from *Rhodothermus marinus* ITI278 (Krah et al. 1998), BglH (38.9%) from *B. circulans* IAM1165 (Yamamoto et al. 1993), and GlcA (37.8%) from *B. circulans* WL-12 (Yahata et al. 1990). The consensus motif specific in GH family 16 β-1,3-glucanases (Juncosa et al. 1994), E-[LIV]-D-[LIVF]-x(0,1)-E-x(2)-[GQ]-[KRNF]-x-[PSTA] (in single-letter codes; x denotes a certain amino acid residue) was found in BglF as well as other GH family 16 β-1,3-glucanases (Fig. 3). These results suggested that BglF also belonged to the GH family 16.

To obtain a large amount of BglF, overexpression of the *bglF* gene in *E. coli* was performed. The mature region of *bglF* gene was amplified by PCR using the following primers: 5′-TCC CCC ACC ACC GCC TCA GCA CAT ATG ACG GAG T-3′ (sense primer) and 5′-ACG GGG CCC CAC CTC GAT CTC TCC CGG ATC CGG GTG TT-3′ (antisense primer). The *Nde*I and *Bam*HI sites had been created in the sense and antisense primers, respectively. The PCR products were digested with *Nde*I and *Bam*HI, and ligated into pET-21b(+) (Novagen, Madison, WI, USA) to construct the expression plasmid pET-BglF. *E. coli* BL21(DE3) containing pET-BglF was cultured at 37°C on LB broth containing ampicillin. When the optical density at 660 nm of the culture reached 0.6, 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce *bglF* gene expression. Transformant cells were further cultured at 37°C for 3 h and harvested by centrifugation at 5,000×g and 4°C for 10 min. Cells were suspended in 10 mM Tris–HCl buffer (pH 7.5) and sonicated to

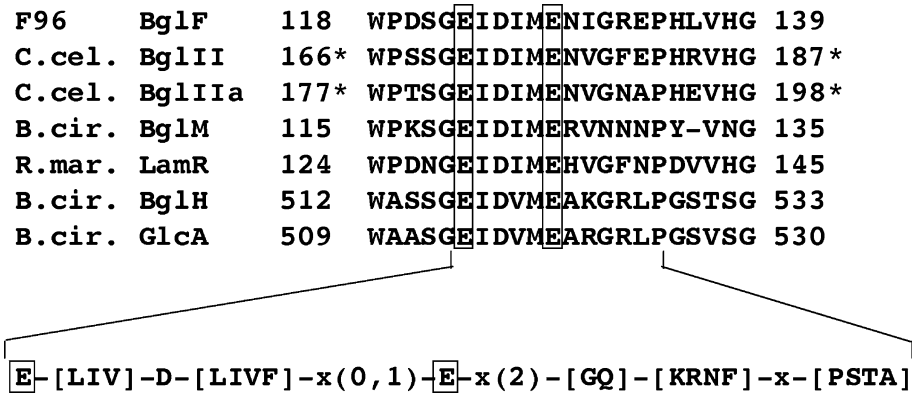

Fig. 3 Partial amino acid sequence alignment of GH family 16 β-1,3-glucanases. The consensus motif of GH family 16?β-1,3-glucanases was also shown below the alignment. The deduced catalytic residues were *boxed*. F96 BglF, BglF from *Nocardiopsis* sp. F96; C.cel. BglII, BglII from *C. cellulans* LL G109; C.cel. BglIIa, BglIIa from *C. cellulans* LL G109; B.cir. BglM, BglM from *B. circulans* IAM1165; R.mar. LamR, LamR from *R. marinus* ITI278; B.cir. BglH, BglH from *B. circulans* IAM1165; B.cir. GlcA, GlcA from *B. circulans* WL-12. *Residue numbers of precursor enzymes

prepare the cell extract. SDS-PAGE of the cell extract revealed that the *bglF* gene was functionally overexpressed in *E. coli* (data not shown).

Recombinant BglF was purified as described above and 20.3 mg of purified enzyme was obtained from 800 ml culture. The specific activity of recombinant BglF was 3.8 U/mg, which was comparable to that of native BglF1 (3.3 U/mg). The optimum pH and temperature of recombinant BglF were around pH 9.0 (at 37°C) and 70°C (at pH 6.0), respectively. The pH and temperature profiles of recombinant BglF were almost the same as those of purified native BglF1 (data not shown). Furthermore, BglF was almost stable after the heat treatment at 60°C for 10 min. Table 1 summarizes the substrate specificity of recombinant BglF. The activity was measured by the DNS method with 1.5% suspension of glucans, e.g., laminarin, pachyman, curdlan (Wako, Osaka, Japan), lichenan (Sigma), CM-cellulose (Wako), and avicel (Merck, Darmstadt, Germany). Recombinant BglF efficiently hydrolyzed insoluble β-1,3-glucans, pachyman and curdlan, as well as fairly soluble laminarin. The enzyme showed the highest activity toward lichenan, a β-1,3-1,4-glucan, rather than β-1,3-glucans. From these results, it was suggested that BglF would be a novel β-1,3-glucanase that had remarkable characteristics.

As described above, BglF had the consensus motif found in GH family 16 β-1,3-glucanases. Two Glu residues, conserved in the consensus motif, have been clarified to be the catalytic residues in LamR from *R. marinus* ITI278 (Krah et al. 1998). The two Glu residues correspond to Glu123 and Glu128 in BglF. In order to elucidate the catalytic residues of BglF, Glu123 and Glu128 were targeted for mutational analysis. Site-directed mutagenesis was performed using QuikChange Kit (Stratagene, LaJolla, CA, USA) to construct mutants E123Q (Glu123 was substituted by Gln) and E128Q. The oligonucleotides used for the mutagenesis were as follows: 5′-GTG GCC GGA CTC GGG CCA GAT CGA CAT CAT GGA G-3′ and its complementary sequence for E123Q, and 5′-GAG ATC GAC ATC ATG CAG AAC ATC GGC CGC GAG CCC-3′ and its complementary sequence for E128Q. The cell extracts of *E. coli* BL21(DE3) producing E123Q and E128Q were applied to SDS-PAGE followed by zymogram analysis. Both mutants completely abolished the β-1,3-glucanase activity (data not shown), confirming that Glu123 and Glu128 should be the catalytic residues of BglF.

Structural study would be required to understand the catalytic mechanism of BglF. X-ray crystallographic analysis of BglF is now underway (Fibriansah et al. 2006).

Table 1 Hydrolyzing activity of BglF toward various glucans

Glucan	Main linkage(s)	Activity[a] (%)
Laminarin	β-1,3; β -1,6	100
Pachyman	β-1,3	69.3
Curdlan	β-1,3	159
Lichenan	β-1,3; β-1,4	851
CM-cellulose	β-1,4	ND
Avicel	β-1,4	ND

[a]Activity is shown as the relative activity to that toward laminarin
ND not detected

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