Journal of Bioscience and Bioengineering
VOL. 121 No. 3, 259–264, 2016

www.elsevier.com/locate/jbiosc

Purification and enzymatic characterization of a novel β-1,6-glucosidase from *Aspergillus oryzae*

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Received 11 June 2015; accepted 27 July 2015
Available online 29 August 2015

In this study, among the 10 genes that encode putative β-glucosidases in the glycoside hydrolase family 3 (GH3) with a signal peptide in the *Aspergillus oryzae* genome, we found a novel gene (AO090038000425) encoding β-1,6-glucosidase with a substrate specificity for gentiobiose. The transformant harboring AO090038000425, which we named *bglH*, was overexpressed under the control of the improved *glaA* gene promoter to form a small clear zone around the colony in a plate assay using 4-methylumbelliferyl β-D-glucopyranoside as the fluorogenic substrate for β-glucosidase. We purified BglH to homogeneity and enzymatically characterize this enzyme. The thermal and pH stabilities of BglH were higher than those of other previously studied *A. oryzae* β-glucosidases, and BglH was stable over a wide temperature range (4°C–60°C). BglH was inhibited by Hg^{2+}, Zn^{2+}, glucono-δ-lactone, glucose, dimethyl sulfoxide, and ethanol, but not by ethylenediaminetetraacetic acid. Interestingly, BglH preferentially hydrolyzed gentiobiose rather than other oligosaccharides and aryl β-glucosides, thereby demonstrating that this enzyme is a β-1,6-glucosidase. To the best of our knowledge, this is the first report of the purification and characterization of β-1,6-glucosidase from *Aspergillus* fungi or from other eukaryotes. This study suggests that it may be possible to find a more suitable β-glucosidase such as BglH for reducing the bitter taste of gentiobiose, and thus for controlling the sweetness of starch hydrolysates in the food industry via genome mining.

[**Key words:** *Aspergillus oryzae*; β-1,6-Glucosidase; β-Glucosidase; Gentiobiose; Glycoside hydrolase family 3]

β-Glucosidases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of glycosidic bonds to release nonreducing terminal glucosyl residues from β-glycosides and β-linked oligosaccharides (1). They have various substrate specificities; some prefer β-linked di- or oligo-saccharides, whereas others preferentially hydrolyze heterogeneous substrates such as aryl β-glucosides. In addition to their hydrolytic activities, some β-glucosidases possess a transglycosylation activity that allows them to produce glycosidic bonds (2,3). β-Glucosidases play various important roles, depending on these substrate specificities, in many biological stages, so they are thought to be biologically and industrially important enzymes (4). However, in industrial fields, their use is limited because of the specific activities and substrate specificities of β-glucosidases, which are insufficient to meet the requirements of these fields (5). Thus, many researchers are still aiming to find more suitable β-glucosidases for applications in this context (5–7).

In a previous study (8), we screened novel β-glucosidase genes from *Aspergillus oryzae*, the genome sequence of which has already been determined (9) to identify more suitable enzymes for industrial applications. From the CAZy database (http://www.cazy.org/), we selected 23 genes encoding enzymes belonging to glycoside hydrolase family 3 (GH3), and we then tried to overexpress 10 genes encoding putative β-glucosidases with a signal peptide under the control of the improved *glaA* gene promoter (10) with *A. oryzae* as the host. As a result, we found that the genes AO090038000223, AO090103000127, and AO090003001511 encoded aryl β-glucosidases with useful hydrolytic activities against isoflavone glycosides, and we designated these genes as *bglA*, *bglF*, and *bglJ*, respectively (8).

In the present study, among the 10 strains in which putative β-glucosidase genes were overexpressed, we found that a novel strain harboring AO090038000425, which we named *bglH*, preferentially hydrolyzed gentiobiose rather than other oligosaccharides and aryl β-glucosides, thereby demonstrating that BglH is a β-1,6-glucosidase. We describe the purification and enzymatic properties of this novel enzyme. To the best of our knowledge, this is the first study to purify and characterize β-1,6-glucosidase from *Aspergillus* or from other fungi.

MATERIALS AND METHODS

Strains, media, and cultivation conditions A *niaD*-deficient mutant of *A. oryzae* AOK11 (*niaD*$^{-}$), *A. oryzae* RIB40, and *Escherichia coli* DH5α were used for overexpressing putative β-glucosidase genes, as the DNA donor, and for plasmid construction/propagation, respectively, as described previously (8). Transformation of *A. oryzae* was performed using the method described by Gomi et al. (11). The *A. oryzae* transformants were grown in Czapek–Dox medium, which was used as the minimal medium, while the other cultivation conditions for *A. oryzae* or *E. coli* DH5α were described previously (8).

Construction of a plasmid for overexpression of the putative β-glucosidase, BglH The insert DNA containing an open reading frame with an intron for a

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http://dx.doi.org/10.1016/j.jbiosc.2015.07.011

putative β-glucosidase gene (*bglH*) was amplified by polymerase chain reaction (PCR) using the appropriate forward and reverse primers with the genomic DNA of *A. oryzae* RIB40 as the template. The primers used for *bglH* gene amplification were SN-*bglH*-*Hin*dIII (5′-CCCAAGCTTGGGCGTCGTCATGTTGTCACC-3′) and ASN-*bglH*-*Spe*I (5′-GGACTAGTCCTTGGGTTCCGGTGGTACATC-3′). Cleavage sites (underlined) for the restriction enzymes *Hin*dIII or *Spe*I, which were included in the multicloning site (MCS) of the expression vector plasmid pNGA142 (10), were attached at the 5′-end of each primer. PCR amplification and the insertion of the resulting PCR fragments into the MCS of the expression vector plasmid were performed as described previously (8).

Purification of BglH The culture filtrates of the *A. oryzae* AOK11 transformants that harbored the overexpressed construct of the *bglH* gene, which had been grown for 48 h at 30°C in YPM liquid medium (500 ml) [0.5% Bacto yeast extract (Bectone–Dickinson, Sparks, MD, USA), 1% Bacto peptone (Bectone–Dickinson), and 1% maltose (Wako Pure Chemical Industries Ltd., Osaka, Japan)], were prepared by filtration. After filtration, all of the enzyme purification steps were performed at 4°C. Solid ammonium sulfate was added to the filtrates containing BglH under stirring to obtain 65% saturation for contaminant protein removal. The β-glucosidase activities in the supernatants were recovered at 80% saturation. The precipitate formed was collected by centrifugation at 12,000 ×*g* for 20 min and dissolved in 20 mM phosphate buffer (pH 6.6) and then dialyzed overnight against the same buffer at 4°C.

Following ammonium sulfate precipitation, the BglH dialysate was added to a Toyopearl Super Q-650M column (1.6 × 4.0 cm, Tosoh Corp., Tokyo, Japan) which had been equilibrated with 20 mM phosphate buffer (pH 6.6). The column was washed with the same phosphate buffer and elution was then performed with a linear gradient of NaCl from 0 to 0.2 M in the same buffer. All of the eluted fractions were checked to assess their β-glucosidase activity levels using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) as the substrate and then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions with high β-glucosidase activity levels were collected and concentrated using a Centricut W-10 (Kurabo, Osaka, Japan) to obtain an appropriate concentration of the enzyme preparation, and these fractions were then used for enzyme characterization. The chromatography procedure was performed using an ÄKTA prime plus chromatography system (GE Healthcare, Tokyo, Japan).

SDS-PAGE and glycosylation analysis SDS-PAGE and glycosylation analysis were performed using the method described by Laemmli (12) as previously described (8).

Protein measurement The protein content of the samples was measured using a BCA Protein Assay Kit-Reducing Agent Compatible (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions, with bovine serum albumin as the standard.

β-Glucosidase activity assay The plate assay employed to evaluate the β-glucosidase activity levels of the overexpression strains was conducted as previously described (8).

The β-glucosidase activity was assayed using the method developed by Riou et al. (13), with slight modifications. As a standard assay procedure, several concentrations of *p*-nitrophenol solution were used to construct a standard curve. The β-glucosidase activity was measured at the optimal temperature for BglH (50°C) with 1 mM *p*NPGlc as the substrate in 0.1 ml of 20 mM acetate buffer (at the optimal pH for BglH, i.e., pH 4.5) and with the appropriately diluted enzyme preparation (2 μl). After incubation for 20 min, the reaction was stopped by adding 0.2 ml of 1 M Na_2CO_3, and the release of *p*-nitrophenol was measured at A_{400}. The results were calculated using the equation obtained from the standard curve. One unit of β-glucosidase activity was defined as the amount of enzyme that yielded 1 μmol of *p*-nitrophenol per min under the assay conditions.

In the substrate specificity analyses, we used a 0.1-ml reaction mixture containing 2 mM *p*NPGlc, 100 mM acetate buffer (pH 4.5), and an aliquot (2 μl) of the enzyme preparation. After incubation for 20 min at 50°C, the reaction was stopped by adding 0.2 ml of 1 M Na_2CO_3, and the release of *p*-nitrophenol was monitored at A_{400}. One unit of β-glucosidase activity was defined as the amount of enzyme that yielded 1 μmol of *p*-nitrophenol per min under the assay conditions. Furthermore, the activities against other chromogenic aryl substrates were determined under the same conditions.

The activities against natural oligosaccharides were assayed using a 0.1-ml reaction mixture containing 2 mM sophorose, laminaribiose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, gentiobiose, lactose, laminarin, arbutin, or salicin, with 100 mM acetate buffer (pH 4.5), and 2 μl of the enzyme preparation. After incubation for 40 min at 50°C, the reaction was stopped by boiling for 5 min, and the amount of glucose released was determined using glucose oxidase–mutarotase reagent (Glucose CII Test Wako). Several concentrations of the glucose solution were used to construct a standard curve. One unit of β-glucosidase activity was defined as the amount of enzyme that yielded 1 μmol of glucose per min under the assay conditions. BglH substrates such as *p*NPGlc, lactose, salicin, *p*-nitrophenol, and glucose oxidase–mutarotase reagent were purchased from Wako Pure Chemical Industries Ltd. Other BglH substrates such as sophorose and cellotriose were purchased from Sigma–Aldrich (St Louis, MO, USA). Laminaribiose and cellohexaose were acquired from Funakoshi Ltd. (Tokyo, Japan). Cellobiose, cellotetraose, and laminarin were obtained from Nacalai Tesque, Inc. (Kyoto, Japan). Cellopentaose, gentiobiose, and arbutin were provided by Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

The Michaelis constant (K_m) and the catalytic constant (k_{cat}) values for the activity of BglH against *p*NPGlc and gentiobiose were calculated using the double reciprocal plot method of Lineweaver and Burk (14).

Effects of temperature on the activity and stability of BglH The effect of temperature on the activity of purified BglH was determined by incubating the enzyme at various temperatures ranging from 20°C to 80°C for 20 min in 100 mM acetate buffer (pH 4.5) using 1 mM *p*NPGlc as the substrate, according to the assay method described earlier. The thermal stability of BglH was examined by incubating the enzyme for 30 min at various temperatures ranging from 4°C to 80°C in 20 mM phosphate buffer (pH 6.8) without any substrate and assaying the residual activity using 1 mM *p*NPGlc as the substrate in 100 mM acetate buffer (pH 4.5). The maximum activity obtained was defined as 100%.

Effects of pH on the activity and stability of BglH The effect of pH on the activity of purified BglH was determined by measuring the enzyme activity at various pH values for 20 min at 50°C, according to the assay method described earlier. The pH stability of purified BglH was examined after incubating the enzyme in various 20 mM buffers at different pH values for 30 min at 30°C without any substrate and assaying the residual activities using 1 mM *p*NPGlc as the substrate in 200 mM acetate buffer (pH 4.5). The buffers used were 20 mM HCl–KCl buffer (pH 1.5–2.5), 20 mM glycine-HCl buffer (pH 2.2–2.9), 20 mM citrate buffer (pH 2.7–6.0), 20 mM phosphate buffer (pH 6.0–8.0), 20 mM *N*-Tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid (TAPS) buffer (pH 7.8–9.0), 20 mM glycine-NaOH buffer (pH 9.0–10.0), and 20 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) buffer (pH 10.0–11.0). The maximum activity obtained was defined as 100%.

Effects of metal ions, inhibitors, and other reagents on the enzyme activity of BglH The effects of metal ions, inhibitors, and other reagents on the activity of purified BglH were determined by measuring the enzyme activity for 20 min at 50°C with 5 mM metal ions (Ca^{2+}, Fe^{3+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, or Zn^{2+}), inhibitors (10 mM glucono-δ-lactone, or 10 or 50 mM glucose), or other reagents (10 mM ethylenediaminetetraacetic acid (EDTA), 10% or 25% (v/v) dimethyl sulfoxide (DMSO), or 10% or 25% (v/v) ethanol), according to the assay method described earlier.

RESULTS

Selection of a novel β-glucosidase (BglH) gene from *A. oryzae* genome As reported in our previous study (8), 10 genes that encoded putative β-glucosidases belonging to the GH3 family were screened (Table S1) from the *A. oryzae* genome and overexpressed in *A. oryzae* AOK11. As a result, in addition to three transformant strains that each harbored the gene, i.e., AO090038000223 (*bglA*), AO090103000127 (*bglF*), and AO090003001511 (*bglJ*) (8), another transformant strain that harbored the gene, AO090038000425 (*bglH*), formed a small clear-zone around its colonies as shown in the plate assay using 4-methylumbelliferyl β-D-glucopyranoside (MUG) as the fluorogenic substrate for β-glucosidase when detected under UV light (data not shown). Thus, we purified BglH from the overexpression transformant.

Purification of BglH BglH was purified from the culture filtrates of the *A. oryzae* AOK11 transformants that overexpressed the *bglH* gene using the method described above. The purification of BglH is summarized in Table 1. From the culture filtrate, proteins were concentrated by ammonium sulfate precipitation, and the dialyzed enzyme solutions obtained after precipitation were used in the next step. After ammonium sulfate precipitation, BglH was purified by anion exchange chromatography. The specific activity of purified BglH was 0.37 U/mg, which was 686-fold higher than that of the crude enzyme preparation. The purified BglH was detected as a single band by SDS-PAGE (Fig. 1A), and the MUG-containing plate assay showed that the protein band of purified BglH in PAGE exhibited β-glucosidase activity (data not shown). The apparent molecular mass of this purified BglH was approximately 150 kDa, which was much higher than the value (89.2 kDa) deduced from the amino acid sequence of the mature protein. After treatment with the recombinant protein Endo Hf, the protein band moved to approximately 89 kDa (Fig. 1B), which was almost the same value as the molecular mass estimated from