1 **Title**

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

5 **Running title**

6 Novel β -1,6-glucosidase from *A. oryzae*

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19 **[Key words:** *Aspergillus oryzae*; β -1,6-glucosidase; β -glucosidase; gentiobiose; glycoside
20 hydrolase family 3 (GH3)]

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Abstract

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.

1 **Introduction**

2 β -glucosidases (EC 3.2.1.21) are enzymes that catalyze the hydrolysis of glycosidic bonds
3 to release nonreducing terminal glucosyl residues from β -glycosides and β -linked
4 oligosaccharides (1). They have various substrate specificities; some prefer β -linked di- or
5 oligosaccharides, whereas others preferentially hydrolyze heterogeneous substrates such as aryl
6 β -glucosides. In addition to their hydrolytic activities, some β -glucosidases possess a
7 transglycosylation activity that allows them to produce glycosidic bonds (2, 3). β -glucosidases
8 play various important roles, depending on these substrate specificities, in many biological
9 stages, so they are thought to be biologically and industrially important enzymes (4). However,
10 in industrial fields, their use is limited because of the specific activities and substrate
11 specificities of β -glucosidases, which are insufficient to meet the requirements of these fields
12 (5). Thus, many researchers are still aiming to find more suitable β -glucosidases for applications
13 in this context (5, 6, 7).

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

23 In the present study, among the 10 strains in which putative β -glucosidase genes were
24 overexpressed, we found that a novel strain harboring AO090038000425, which we named
25 *bglH*, preferentially hydrolyzed gentiobiose rather than other oligosaccharides and aryl β -
26 glucosides, thereby demonstrating that BglH is a β -1,6-glucosidase. We describe the

1 purification and enzymatic properties of this novel enzyme. To the best of our knowledge, this
2 is the first study to purify and characterize β -1,6-glucosidase from *Aspergillus* or from other
3 fungi.

4 5 **Materials and Methods**

6
7 **Strains, media, and cultivation conditions** A *niaD*-deficient mutant of *A. oryzae*
8 AOK11 (*niaD*⁻), *A. oryzae* RIB40, and *Escherichia coli* DH5 α were used for overexpressing
9 putative β -glucosidase genes, as the DNA donor, and for plasmid construction/propagation,
10 respectively, as described previously (8). Transformation of *A. oryzae* was performed using the
11 method described by Gomi et al. (11). The *A. oryzae* transformants were grown in Czapek–Dox
12 medium, which was used as the minimal medium, while the other cultivation conditions for *A.*
13 *oryzae* or *E. coli* DH5 α were described previously (8).

14 **Construction of a plasmid for overexpression of the putative β -glucosidase, BglH**
15 The insert DNA containing an open reading frame with an intron for a putative β -glucosidase
16 gene (*bglH*) was amplified by polymerase chain reaction (PCR) using the appropriate forward
17 and reverse primers with the genomic DNA of *A. oryzae* RIB40 as the template. The primers
18 used for *bglH* gene amplification were SN-*bglH*-*Hind*III (5'-
19 CCCAAGCTTGGGCGTCGTCATGTTGTCACC-3') and ASN-*bglH*-*Spe*I (5'-
20 GGACTAGTCCTTGGGTTCGGTGGTACATC-3'). Cleavage sites (underlined) for the
21 restriction enzymes *Hind*III or *Spe*I, which were included in the multicloning site (MCS) of the
22 expression vector plasmid pNGA142 (10), were attached at the 5'-end of each primer. PCR
23 amplification and the insertion of the resulting PCR fragments into the MCS of the expression
24 vector plasmid were performed as described previously (8).

25 **Purification of BglH** The culture filtrates of the *A. oryzae* AOK11 transformants that

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

10 Following ammonium sulfate precipitation, the BglH dialysate was added to a
11 TOYOPEARL Super Q-650M column (1.6 $\times$ 4.0 cm, Tosoh Corp., Tokyo, Japan) which had
12 been equilibrated with 20 mM phosphate buffer (pH 6.6). The column was washed with the
13 same phosphate buffer and elution was then performed with a linear gradient of NaCl from 0 to
14 0.2 M in the same buffer. All of the eluted fractions were checked to assess their β -glucosidase
15 activity levels using *p*-nitrophenyl- β -D-glucopyranoside (*p*NPGLc) as the substrate and then
16 analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The
17 fractions with high β -glucosidase activity levels were collected and concentrated using a
18 Centricut W-10 (Kurabo, Osaka, Japan) to obtain an appropriate concentration of the enzyme
19 preparation, and these fractions were then used for enzyme characterization. The
20 chromatography procedure was performed using an ÄKTA prime plus chromatography system
21 (GE Healthcare, Tokyo, Japan).

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

24 **Protein measurement** The protein content of the samples was measured using a BCA
25 Protein Assay Kit- Reducing Agent Compatible (Thermo Fisher Scientific, Yokohama, Japan)

1 according to the manufacturer's instructions, with bovine serum albumin as the standard.

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

4 The β -glucosidase activity was assayed using the method developed by Riou et al. (13),
5 with slight modifications. As a standard assay procedure, several concentrations of *p*-
6 nitrophenol solution were used to construct a standard curve. The β -glucosidase activity was
7 measured at the optimal temperature for BglH (50°C) with 1 mM *p*NPGlc as the substrate in
8 0.1 ml of 20 mM acetate buffer (at the optimal pH for BglH, i.e., pH 4.5) and with the
9 appropriately diluted enzyme preparation (2 μ l). After incubation for 20 min, the reaction was
10 stopped by adding 0.2 ml of 1 M Na₂CO₃, and the release of *p*-nitrophenol was measured at
11 A₄₀₀. The results were calculated using the equation obtained from the standard curve. One unit
12 of β -glucosidase activity was defined as the amount of enzyme that yielded 1 μ mol of *p*-
13 nitrophenol per min under the assay conditions.

14 In the substrate specificity analyses, we used a 0.1-ml reaction mixture containing 2 mM
15 *p*NPGlc, 100 mM acetate buffer (pH 4.5), and an aliquot (2 μ l) of the enzyme preparation. After
16 incubation for 20 min at 50°C, the reaction was stopped by adding 0.2 ml of 1 M Na₂CO₃, and
17 the release of *p*-nitrophenol was monitored at A₄₀₀. One unit of β -glucosidase activity was
18 defined as the amount of enzyme that yielded 1 μ mol of *p*-nitrophenol per min under the assay
19 conditions. Furthermore, the activities against other chromogenic aryl substrates were
20 determined under the same conditions.

21 The activities against natural oligosaccharides were assayed using a 0.1-ml reaction mixture
22 containing 2 mM sophorose, laminaribiose, cellobiose, cellotriose, cellotetraose, cellopentaose,
23 cellohexaose, gentiobiose, lactose, laminarin, arbutin, or salicin, with 100 mM acetate buffer
24 (pH 4.5), and 2 μ l of the enzyme preparation. After incubation for 40 min at 50°C, the reaction
25 was stopped by boiling for 5 min, and the amount of glucose released was determined using

1 glucose oxidase–mutarotase reagent (Glucose CII Test Wako). Several concentrations of the
2 glucose solution were used to construct a standard curve. One unit of β -glucosidase activity was
3 defined as the amount of enzyme that yielded 1 μ mol of glucose per min under the assay
4 conditions. BglH substrates such as *p*NPGlc, lactose, salicin, *p*-nitrophenol, and glucose
5 oxidase–mutarotase reagent were purchased from Wako Pure Chemical Industries Ltd (Osaka,
6 Japan). Other BglH substrates such as sophorose and cellotriose were purchased from Sigma–
7 Aldrich (St Louis, MO, USA). Laminaribiose and cellohexaose were acquired from Funakoshi
8 Ltd (Tokyo, Japan). Cellobiose, cellotetraose, and laminarin were obtained from Nacalai Tesque,
9 Inc. (Kyoto, Japan). Cellopentaose, gentiobiose, and arbutin were provided by Tokyo Chemical
10 Industry Co. Ltd (Tokyo, Japan).

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

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

22 **Effects of pH on the activity and stability of BglH** The effect of pH on the activity of
23 purified BglH was determined by measuring the enzyme activity at various pH values for 20
24 min at 50°C, according to the assay method described earlier. The pH stability of purified BglH
25 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

1 UV light (data not shown). Thus, we purified BglH from the overexpression transformant.

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

17 **Effects of temperature and pH on the activity and stability of BglH** We examined
18 the effects of temperature on the activity and stability of BglH. As shown in Fig. 2A, the
19 maximum activity of BglH was obtained at 50°C at pH 4.5. Although BglH was stable below
20 60°C, it completely lost its activity at $\geq 70^\circ\text{C}$ (Fig. 2B).

21 The optimal pH for the activity of purified BglH was 4.5 at 50°C, and more than 80% of its
22 maximum activity was retained between pH 4.0 and 5.0 (Fig. 2C). In addition, BglH was stable
23 over a wide pH range (2.0 and 8.5), and it retained more than 70% of its maximum activity
24 between pH 2.0 and 9.0, whereas it lost its activity at pH > 9.0 (Fig. 2D).

25 **Substrate specificity of BglH** We examined the substrate specificity of BglH and the

1 results are summarized in Fig. 3. First, we investigated the hydrolytic activity of purified BglH
2 against various chromogenic aryl substrates, including *p*NPGlc, and we compared these relative
3 activities with *p*NPGlc (100%) (Fig. 3A). BglH had the highest hydrolytic activity against
4 *p*NPGlc. Two other substrates, *p*-nitrophenyl- β -D-xylopyranoside (*p*NPXyl) (0.511%) and *p*-
5 nitrophenyl- β -D-cellobioside (*p*NPCel) (1.75%), were also hydrolyzed by BglH, but only very
6 weakly, whereas BglH had no hydrolytic activity against *p*-nitrophenyl- β -D-fucopyranoside
7 (*p*NPFuc), *p*-nitrophenyl- β -D-lactopyranoside (*p*NPLac), or *p*-nitrophenyl- β -D-
8 galactopyranoside (*p*NPGal). Next, we investigated the hydrolytic activity of purified BglH
9 against various oligosaccharides and aryl β -glucosides (Fig. 3B). BglH had the highest
10 hydrolytic activity against gentiobiose. BglH had significantly lower hydrolytic activities
11 against laminarin (0.4%), sophorose (10.0%), laminaribiose (3.3%), cellotriose (4.9%),
12 cellotetraose (1.4%), cellohexaose (1.1%), arbutin (5.6%), and salicin (2.8%) than that against
13 gentiobiose (100%). Furthermore, BglH had no hydrolytic activity against cellobiose,
14 cellopentaose, or lactose. Based on these results, we concluded that BglH is a β -1,6-glucosidase
15 because BglH preferentially hydrolyzed gentiobiose rather than other oligosaccharides and aryl
16 β -glucosides.

17 **Effects of metal ions, inhibitors, and other reagents on the enzyme activity of BglH**

18 We determined the effects of metal ions, inhibitors, and other reagents on the activity of purified
19 BglH, and the results are summarized in Table 2. Cu^{2+} (5 mM) and EDTA (10 mM) had almost
20 no effect on the enzyme activity. Interestingly, Ca^{2+} , Fe^{3+} , Mg^{2+} , and Mn^{2+} at a concentration of
21 5 mM enhanced the enzyme activity by 10–30% from that measured without their addition.
22 BglH was inhibited by 5 mM Hg^{2+} and Zn^{2+} with residual activities of 14% and 86%,
23 respectively. With 10 mM glucono- δ -lactone, a specific inhibitor of many glucosidases (15),
24 BglH was completely inactivated. We found that 10 mM and 50 mM glucose, which severely
25 inhibits most β -glucosidases (5, 13, 16), also inhibited the enzyme activity of BglH by 67% and

96%, respectively. Furthermore, BglH was unstable in the presence of DMSO and ethanol, with residual activities of 49% and 62%, respectively, at a concentration of 10% (v/v), while it was inactivated by higher concentrations (25% (v/v)) of DMSO and ethanol.

Comparison of the characteristics of BglH with those of β -1,6-glucosidase from *Flavobacterium* sp. M64

Based on the results of the substrate specificity tests for BglH (Fig. 3), we showed that this enzyme is a β -1,6-glucosidase. Previously, no fungal GH3 β -1,6-glucosidase had been reported to be purified to homogeneity with the ability to preferentially hydrolyze gentiobiose. Thus, this is the first report of the purification and characterization of a β -1,6-glucosidase from filamentous fungi. Furthermore, only the enzyme from *Flavobacterium* sp. M64 has been reportedly purified to homogeneity and it is the only previously characterized β -1,6-glucosidase among all types of living organisms, ranging from microbes, to animals, to plants (17). Therefore, we compared the characteristics of BglH with those of the *Flavobacterium* sp. M64 enzyme (Table 3). BglH is an extracellular enzyme, but the *Flavobacterium* enzyme is produced intracellularly. The molecular mass of BglH was calculated as 89 kDa based on the DNA sequence in the genome database, whereas that of the *Flavobacterium* enzyme was determined to be ~59 kDa by gel filtration and SDS-PAGE (17). The specific activities of BglH and the *Flavobacterium* enzyme during gentiobiose hydrolysis were 3.0 and 65.4 U/mg, respectively, and thus the *Flavobacterium* enzyme had ~22 times greater activity against gentiobiose than BglH. BglH and the *Flavobacterium* enzyme were stable at temperatures up to 60°C and 40°C, respectively, and were stable at pH ranges of 2–8.5 and 5–7, respectively. According to these results, BglH has greater temperature and pH stability than the *Flavobacterium* enzyme. Glucono- δ -lactone, which is a specific inhibitor of many glucosidases (15), inhibited the enzyme activities of both BglH and the *Flavobacterium* enzyme. Glucose and Hg²⁺ also inhibited both enzyme activities. Cu²⁺ and Fe³⁺ caused 80 to 100% inhibition of the activity of the *Flavobacterium* enzyme at a concentration of 2 mM (17), but

these two ions had almost no effect on the enzyme activity of BglH or they enhanced this activity (Table 2). In addition, the transglycosylation activity of BglH was not detected against the substrates gentiobiose, *p*NPGLc, and glucose (data not shown), whereas the *Flavobacterium* enzyme catalyzed transglycosylation of gentiobiose to gentiotriose (17).

Kinetic constants of BglH The steady-state kinetic parameters of BglH measured for gentiobiose and *p*NPGLc, both of which were preferentially hydrolyzed by this enzyme, are summarized in Table 4. The K_m , k_{cat} , and k_{cat}/K_m values for the activity of BglH against gentiobiose were 34.9 mM, 13.3 s⁻¹, and 0.38 s⁻¹ mM⁻¹, respectively. In addition, these values for the activity of BglH against *p*NPGLc were 110 mM, 51.6 s⁻¹, and 0.47 s⁻¹ mM⁻¹, respectively. It has been reported that the k_{cat} values for β -glucosidase activities against natural substrates are relatively low (normally with a maximum of 300 s⁻¹ or lower) (1). In the present study, the k_{cat} values for the activity of BglH against gentiobiose and *p*NPGLc were also relatively low.

Discussion

β -Glucosidases are involved in the chemical defense against herbivores and pathogens (18), lignification (19), the biological activation of phytohormones (20) in higher plants, the degradation of cellulosic biomass (5), and other bioconversion processes (2, 3), so they are considered to be biologically and industrially important enzymes (4). *A. oryzae* has been used for a long time in Japanese fermentation industries (21) and it is an industrially significant filamentous fungus. This fungus can produce various useful enzymes, including β -glucosidases (22, 23). In the present study, we found a novel β -glucosidase encoded by AO090038000425 (*bglH*). We purified BglH from the overexpression transformant, which formed a small clear-zone around the colony in a plate assay using MUG, and we characterized it enzymatically.

Our analysis of the substrate specificity for various oligosaccharides and aryl β -

1 glucosides, including chromogenic aryl substrates such as *p*NPGLc, demonstrated that BglH is a
2 β -1,6-glucosidase because this enzyme barely hydrolyzed natural oligosaccharides with β -1,4-,
3 β -1,3- and β -1,2-glucosidic linkages as well as aryl β -glucosides, except *p*NPGLc, when
4 compared with its activity with gentiobiose (Fig. 3). Previously, there were no reports of the
5 purification of fungal GH3 family β -1,6-glucosidase to homogeneity with the ability to
6 preferentially hydrolyze gentiobiose. Thus, this is the first report of the purification and
7 characterization of a β -1,6-glucosidase from the genus *Aspergillus* or from other fungi.
8 Furthermore, only the *Flavobacterium* sp. M64 enzyme has been reportedly purified and
9 characterized as a β -1,6-glucosidase among all types of living organisms, ranging from
10 microbes, to animals, to plants (17).

11 BglH is an extracellular β -glucosidase with a relatively high molecular mass of 89 kDa that
12 belongs to the GH3 family. In contrast, the *Flavobacterium* enzyme is intracellular with a lower
13 molecular mass of 59 kDa, thereby suggesting that this enzyme belongs to the GH1 family
14 because the molecular weights of GH1 enzymes are normally lower than those of GH3 enzymes
15 (5, 24). However, it is unclear whether this enzyme definitively belongs to the GH1 family
16 because its amino acid sequence has not yet been determined. The temperature stabilities of
17 BglH and the *Flavobacterium* enzyme reached up to 60°C and 40°C, respectively, and they
18 were stable at pH ranges of 2–8.5 and 5–7, respectively, thereby indicating that BglH is more
19 stable than the *Flavobacterium* enzyme. Moreover, BglH not only exhibited better thermal
20 stability (up to 60°C) than the *Flavobacterium* enzyme but it is also more stable than other *A.*
21 *oryzae* β -glucosidases (7, 8, 13, 25). This feature also makes BglH a good candidate enzyme
22 for industrial applications, although its enzymatic stability and activity should be improved
23 using the family shuffling method because the specific activity of BglH against gentiobiose was
24 lower than that of the *Flavobacterium* enzyme.

25 Compared with other sugars, gentiobiose possesses a more bitter taste (26). When glucose

1 is produced via the acid hydrolysis of starch in the food industry, gentiobiose remains in the
2 mother liquor and makes it bitter, and this is the most serious problem that affects the use of
3 this hydrolysate in the food industry (27). Thus, β -1,6-glucosidases such as BglH may solve
4 this problem because these β -glucosidases preferentially hydrolyze gentiobiose rather than
5 other sugar components, so a sweet taste will still remain in the residual hydrolysate.
6 Furthermore, the transglycosylation activity of BglH was not detectable against the substrates
7 gentiobiose, *p*NPGlc, and glucose (as mentioned above), so BglH may be more suitable for use
8 in the food industry to degrade gentiobiose, thereby reducing the bitter taste of the starch
9 hydrolysate better than the *Flavobacterium* enzyme.

10 Interestingly, according to the phylogenetic analysis of 23 putative β -glucosidases from *A.*
11 *oryzae*, BglH shares the highest homology (62% identity) with BglA (8) (Fig. S1). Furthermore,
12 the signature motifs encompassing an active site of GH3, which were found using the PROSITE
13 database (<http://prosite.expasy.org/>), are highly conserved in their deduced amino acid
14 sequences. However, BglH was categorized as a β -1,6-glucosidase and BglA as an aryl β -
15 glucosidase. This suggests that the substrate specificities of β -glucosidases cannot be predicted
16 based only on the shared homology of their amino acid sequences. Therefore, a comparative
17 study of the three-dimensional structures of BglH and BglA is needed to clarify the causes of
18 the difference in their substrate specificities.

19 In conclusion, the present study suggests that it may be possible to obtain a more suitable
20 β -glucosidase, such as BglH, for reducing the bitter taste of gentiobiose by mining microbial
21 genomes, thereby facilitating the control of sweetness in starch hydrolysate in the food industry.

22 23 Acknowledgments

24
25 This study was financially supported by the A-STEP Program of Japan Science and

Technology Agency and in part by the Ministry of Economy, Trade, and Industry, Japan.

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Figure legends

FIG. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses of purified BglH.

(A) SDS-PAGE of purified BglH. Lane 1, molecular weight standard proteins (250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa); lane 2, purified BglH. (B) SDS-PAGE of BglH treated with the recombinant protein Endo Hf. Lane 1, molecular weight standard proteins (250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa); lane 2, purified BglH; lane 3, Endo Hf-treated BglH. The *closed arrowhead* (150 kDa) and *open arrowhead* (89 kDa) indicate glycosylated BglH and deglycosylated BglH, respectively. The asterisk indicates the band of Endo Hf.

FIG. 2. Effects of temperature and pH on the activity and stability of purified BglH.

(A) The effect of temperature on the activity of BglH was assayed in 100 mM acetate buffer (pH 4.5) at various temperatures using 1 mM *p*-nitrophenyl- β -D-glucopyranoside (*p*NPGlc) as the substrate. (B) The remaining activities were measured after incubating BglH for 30 min at various temperatures in 20 mM phosphate buffer (pH 6.8). The maximum activity obtained was defined as 100%. (C) The effect of pH on the activity of BglH was assayed in McIlvaine buffers (pH 3–8) at 50°C using 1 mM *p*NPGlc as the substrate. (D) The remaining activities were measured after incubating BglH for 30 min at 30°C in the following buffers; 20 mM HCl-KCl buffer (pH 1.4 to 2.5; *closed circles*), 20 mM glycine-HCl buffer (pH 2.2 to 2.9; *open squares*), 20 mM citrate buffer (pH 2.7 to 5.7; *closed triangles*), 20 mM phosphate buffer (pH 6.1 to 8.1; *open circles*), 20 mM *N*-Tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid buffer (pH 7.8 to 9.0; *closed squares*), 20 mM glycine-NaOH buffer (pH 9.1 to 10; *open triangles*), and 20 mM *N*-cyclohexyl-3-aminopropanesulfonic acid buffer (pH 9.9 to 11; *closed diamonds*). The maximum activity obtained was defined as 100%. Error bars represent the means $\pm$ SE based on three independent experiments.

1 **FIG. 3.** Substrate specificity of BglH.

2 All substrates were tested at a concentration of 2 mM in 100 mM acetate buffer (pH 4.5).

3 Each activity was determined by measuring the amount of released *p*-nitrophenol (A) or

4 glucose (B) after incubation with BglH for 20 min at 50°C as described in the Materials and

5 Methods section. The relative activity for *p*-nitrophenyl- β -D-glucopyranoside (*p*NPGlc) (A)

6 or gentiobiose (B) was defined as 100%. Error bars represent means $\pm$ SE of three

7 independent experiments.

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TABLE 1. Purification of BglH.

Purification step	Total activity (U) ^a	Total protein (mg) ^b	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	2.62	4870	5.39×10^{-4}	1	100
(NH ₄) ₂ SO ₄ precipitation	2.17	125	0.017	32.2	83
Ion exchange chromatography	1.12	3.0	0.37	686	43

^aActivity was measured in 20 mM acetate buffer (pH 4.5) at 50°C using 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGLc) as the substrate.

^bThe protein content was assayed using a BCA Protein Assay Kit (Pierce) with bovine serum albumin as the standard.

1 **TABLE 2.** Effects of metal ions, inhibitors, and other reagents on the activity of BglH.

Reagent	Concentration	Relative activity (%)
No addition		100
CaCl ₂	5 mM	109 ± 5
FeCl ₃	5 mM	132 ± 6
HgCl ₂	5 mM	13.5 ± 6.7
MgCl ₂	5 mM	116 ± 8
MnCl ₂	5 mM	116 ± 8
CuSO ₄	5 mM	100 ± 12
ZnSO ₄	5 mM	86.1 ± 9.1
Glucono-δ-lactone	10 mM	N.D.
Glucose	10 mM	33.1 ± 0.3
	50 mM	N.D.
Ethylenediaminetetraacetic acid (EDTA)	10 mM	101 ± 12
Dimethyl sulfoxide (DMSO)	10% (v/v)	49.1 ± 1.6
	25% (v/v)	N.D.
Ethanol	10% (v/v)	62.0 ± 8.6
	25% (v/v)	N.D.

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3 The effects of metal ions, inhibitors, and other reagents on the activity of purified BglH were
4 determined by incubating the enzyme with 5 mM of each metal ion, 10 mM glucono-δ-
5 lactone, 10 or 50 mM glucose, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10 or
6 25% (v/v) dimethyl sulfoxide (DMSO) or ethanol in 100 mM acetate buffer (pH 4.5) using 1
7 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGLc) as the substrate, according to the assay
8 method described in the Methods. The relative activity with no additive was defined as 100%.

- 1 The values represent the means $\pm$ SE based on three different experiments. N.D., no activity
- 2 detected.

1 **TABLE 3.** Comparison of the properties of BglH and β -1,6-glucosidase from *Flavobacterium*
2 sp. M64.

	<i>A. oryzae</i> (BglH)	<i>Flavobacterium</i> sp. M64
Localization	extracellular	intracellular
Molecular mass	89.2 kDa	59 kDa
Specific activity (U/mg) (gentiobiose)	3.0	65.4
Optimal temperature (°C)	50	40
Heat stability	$\leq 60^{\circ}\text{C}$	$\leq 40^{\circ}\text{C}$
Optimal pH	pH 4.5	pH 5.8
pH stability	pH 2–8.5	pH 5–7
Inhibitor	Glucono- δ -lactone, Hg^{2+} , Zn^{2+} , Dimethyl sulfoxide, Ethanol, Glucose	Glucono- δ -lactone, Ag^{2+} , Cu^{2+} , Fe^{3+} , Hg^{2+} , <i>N</i> - ethylmaleimide, <i>p</i> - chloromercuribenzoate, Glucose, Trishydroxyaminomethane
Transglycosylation activity	not detected	gentiobiose to gentiotriose
Reference	This study	17

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2 **TABLE 4.** Steady-state kinetic constants of BglH.

Substrate	K_m (mM)	k_{cat} (S ⁻¹)	k_{cat}/K_m (S ⁻¹ · mM ⁻¹)
Gentiobiose	34.9 ± 1.4	13.3 ± 0.3	0.38
<i>p</i> NPGlc ^a	110 ± 3	51.6 ± 1.4	0.47

3 ^a*p*NPGlc; *p*-nitrophenyl-β-D-glucopyranoside

4

5

FIG. 1

A

B

FIG. 2

B

D

FIG. 3

A

B