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Purification and enzymatic characterization of secretory glycoside hydrolase family 3 (GH3) aryl β-glucosidases screened from *Aspergillus oryzae* genome

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By a global search of the genome database of *Aspergillus oryzae*, we found 23 genes encoding putative β-glucosidases, among which 10 genes with a signal peptide belonging to glycoside hydrolase family 3 (GH3) were overexpressed in *A. oryzae* using the improved *glaA* gene promoter. Consequently, crude enzyme preparations from three strains, each harboring the genes AO090038000223 (*bglA*), AO090103000127 (*bglF*), and AO090003001511 (*bglJ*), showed a substrate preference toward *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc) and thus were purified to homogeneity and enzymatically characterized. All the purified enzymes (BglA, BglF, and BglJ) preferentially hydrolyzed aryl β-glycosides, including *p*NPGlc, rather than cellobiose, and these enzymes were proven to be aryl β-glucosidases. Although the specific activity of BglF toward all the substrates tested was significantly low, BglA and BglJ showed appreciably high activities toward *p*NPGlc and arbutin. The kinetic parameters of BglA and BglJ for *p*NPGlc suggested that both the enzymes had relatively higher hydrolytic activity toward *p*NPGlc among the fungal β-glucosidases reported. The thermal and pH stabilities of BglA were higher than those of BglJ, and BglA was particularly stable in a wide pH range (pH 4.5–10). In contrast, BglJ was the most heat- and alkaline-labile among the three β-glucosidases. Furthermore, BglA was more tolerant to ethanol than BglJ; as a result, it showed much higher hydrolytic activity toward isoflavone glycosides in the presence of ethanol than BglJ. This study suggested that the mining of novel β-glucosidases exhibiting higher activity from microbial genome sequences is of great use for the production of beneficial compounds such as isoflavone aglycones.

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[**Key words:** *Aspergillus oryzae*; β-Glucosidase; Glycoside hydrolase family 3 (GH3); Aryl β-glucoside; Isoflavone aglycone]

β-Glucosidases (EC 3.2.1.21) are biologically and industrially important enzymes (1). They hydrolyze alkyl and aryl β-glycosides and can be classified into three groups on the basis of their substrate specificities (2,3). Cellobiases, a type of β-glucosidases, efficiently hydrolyze cellobiose and cellooligosaccharides. Other β-glucosidases show higher specificities toward aryl β-D-glycosides than cellobiose and are therefore called aryl β-glucosidases. On the other hand, most β-glucosidases are called broad-specificity β-glucosidases because they exhibit broad substrate specificity and can equivalently degrade both the types of substrates. In many biological stages, β-glucosidases play various important roles corresponding to these substrate specificities.

Together with endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91), cellobiases, the first type of β-glucosidases, are also involved in the degradation of cellulosic biomass (4). Although the activities of cellobiohydrolases and endoglucanases are inhibited by the reaction product (cellobiose) when they hydrolyze cellulose, β-glucosidases can overcome such inhibition by the reaction product (5,6). These catalytic functions of β-glucosidases are very important for bioethanol production. On the other hand, numerous secondary metabolites are accumulated in glycosylated forms in fruits and plants (7). These glycosylated compounds include many flavor precursors that are cleaved into sugar and aglycone by the action of aryl β-glucosidases, the second type of β-glucosidases. Recently, the application of β-glucosidases for flavor enhancement in plants has attracted increased attention (8). In addition, some of these aryl β-glucosidases can hydrolyze isoflavone glycosides to isoflavone aglycones (9,10). Isoflavones are known to have estrogenic activity and are abundantly found in soybean in aglycone forms (e.g., daidzein, genistein, glycitein) and glycoside forms (e.g., daidzin, genistin, glycitin) (11), and they possess useful preventive properties against cancer (12–14). As the aglycone forms of isoflavones are absorbed into the human body more effectively and are more biologically active than their glycoside forms (15–19), hydrolysis of isoflavone glycosides to isoflavone aglycones by β-glucosidases has significant importance in the field of biotechnology.

In addition to their hydrolytic activities, β-glucosidases also catalyze glycosidic bond formation (2,20). This transglycosylation activity is an industrially useful property. For example, in chemical syntheses, some moieties of substrates need to be protected during reactions; however, enzyme-catalyzed syntheses have high selectivities, and thus, glycosidic bond formation can be achieved without the protection of these moieties (21). Numerous glycosyltransferases catalyze the transfer of sugars and have been used for the syntheses of flavor precursors, glycosylated oligopeptides, and oligosaccharides (22,23). However, the application of these

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enzymes to industrial processes is limited because of the requirement of expensive nucleotide sugar precursors (21). Compared with these glycosyltransferases, β-glucosidases are more suitable for oligosaccharide synthesis because of their commercial availability and lower cost (24). However, in industrial applications, their utility in glycoside synthesis is limited due to lower transglycosylation activities (6). As a result, there is a need to find more suitable β-glucosidases.

β-Glucosidases are distributed in all types of living organisms, from bacteria, fungi, and plants to animals (21,25). *Aspergillus oryzae* is a filamentous fungus and an important genetic resource because it can produce various useful enzymes (26). The genome sequence of *A. oryzae* has already been determined (27) and has been proven to comprise nearly 20 β-glucosidase genes (28). Among these β-glucosidases, only one β-glucosidase encoded by AO090009000356 has been purified and enzymatically characterized (29,30), and recently, genetically engineered yeast strains displaying two β-glucosidases (encoded by AO090003001511 and AO090001000544) from *A. oryzae* on their cell surfaces have been reported (31). However, other β-glucosidases have not yet been studied.

In the present study, we globally screened novel β-glucosidase genes from the *A. oryzae* genome to identify more suitable enzymes for industrial applications. From the CAZy database (http://www.cazy.org/), we chose 23 genes encoding enzymes belonging to glycoside hydrolase family 3 (GH3) and then attempted to overexpress 10 genes that were presumed to encode putative β-glucosidases with a signal peptide under the control of the improved *glaA* gene promoter (32) with *A. oryzae* as the host. Consequently, three strains, each harboring AO090038000223 (*bglA*), AO090103000127 (*bglF*), and AO090003001511 (*bglJ*), showed higher hydrolytic activities toward aryl β-D-glycosides. Therefore, we purified and enzymatically characterized these enzymes from the overexpression strains. Although there are several studies on the secretory production of BglJ (Bgl1) from *A. oryzae* (31,33,34), they focused on the basic characterization and isoflavone aglycone production by the crude enzyme. In the present study, we describe the enzymatic properties of these three purified enzymes in detail.

MATERIALS AND METHODS

Strains, media, and cultivation conditions An *A. oryzae* mutant deficient in a nitrate reductase gene (*niaD*$^-$), derived from the industrial strain *A. oryzae* AOK11 (Akita Konno Shoten, Akita, Japan) (35), which can produce higher amount of secreted proteins, was used as the host for the overexpression of putative β-glucosidase genes. *A. oryzae* RIB40 (27) was used as the DNA donor. Transformation of *A. oryzae* was performed using the method described by Gomi et al. (36). The *A. oryzae* transformants were grown in the Czapek–Dox (CD) medium consisting of 0.3% $NaNO_3$, 0.15% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, trace amounts of $FeSO_4$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $(NH_4)_6Mo_7O_{24}$, 0.8 M NaCl, and 1% sugar supplemented with appropriate nutrients, which was used as the minimal medium. The culture was grown at 30°C for 5–7 days and purified thrice on CD medium plates. For β-glucosidase production, the *A. oryzae* transformants were cultured in the YPM medium [0.5% Bacto yeast extract (Becton–Dickinson, Sparks, MD, USA), 1% Bacto peptone (Becton–Dickinson), and 1% maltose (Wako Pure Chemical Industries, Ltd., Osaka, Japan)] at 30°C for 48 h after inoculation with approximately 1×10^6 conidia per 100 ml of the medium. Subsequently, the cultures were harvested by filtration with Miracloth (Calbiochem, CA, USA). *Escherichia coli* DH5α (*supE44, hsdR17, recA1, gyrA96, endA1, thi-1, relA1*) was used for plasmid construction and was grown in the Luria–Bertani medium [1% (w/v) Bacto tryptone (Becton–Dickinson), 0.5% (w/v) Bacto yeast extract, and 0.5% (w/v) NaCl] containing 50 μg/ml ampicillin (Wako Pure Chemical Industries, Ltd.) at 37°C.

Construction of plasmids for the overexpression of putative β-glucosidases, BglA, BglF, and BglJ Each insert DNA that contained the ORF of each putative β-glucosidase gene was amplified by PCR using appropriate forward and reverse primers with the genomic DNA of *A. oryzae* RIB40 as the template. For *bglA* gene amplification, the PCR fragment containing BglA ORF was amplified with primers SN-*bglA*-*Spe*I and ASN-*bglA*-*Spe*I. For *bglF* gene amplification, the PCR fragment containing BglF ORF was amplified with primers SN-*bglF*-*Spe*I and ASN-*bglF*-*Spe*I. For *bglJ* gene amplification, the PCR fragment containing BglJ ORF was amplified with primers SN-*BglJ*-*Hin*dIII and ASN-*BglJ*-*Xba*I. Cleavage sites (underlined) for the restriction enzymes *Spe*I, *Hin*dIII, or *Xba*I, which were included in the multicloning site (MCS) of the expression vector plasmid pNGA142 (32), were attached at the 5′-end of each primer. PCR amplification was performed using *PfuUltra* II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA). The reaction mixture was first incubated at 95°C for 5 min and then subjected to 50 cycles of 95°C for 0.5 min, 56°C for 0.5 min, and 72°C for 1.5 min, followed by incubation at 72°C for 7 min. The resulting PCR fragments were inserted between the improved *glaA* promoter and the *agdA* terminator of the expression vector with the *niaD* gene as the selectable marker. The nucleotide sequences of all the primers used in this study are shown in Supplementary Table S1.

Purification of BglA, BglF, and BglJ The culture filtrates of the *A. oryzae* AOK11 transformants, each harboring the overexpressed construct of the *bglA, bglF,* and *bglJ* genes, grown for 48 h at 30°C in the YPM liquid medium (400, 700, and 700 ml, respectively) were prepared by filtration. After filtration, all the steps of enzyme purification were performed at 4°C. Solid ammonium sulfate was added to the filtrates containing BglA, BglF, and BglJ under stirring to obtain 70%, 50%, and 65% saturation, respectively, for removing contaminant proteins. β-Glucosidase activities in the supernatants were recovered at 80%, 65%, and 75% saturation, respectively. The precipitates formed were collected by centrifugation at 12,000 ×*g* for 30 min and dissolved in 20 mM phosphate buffer (pH 6.8) and dialyzed against the same buffer containing 1.5 M (BglA), 1.05 M (BglF), or 0 M (BglJ) of ammonium sulfate overnight at 4°C.

Following ammonium sulfate precipitation, the dialysate of BglA was added to the Hitrap Butyl FF column (1.6 × 2.5 cm, GE Healthcare, Tokyo, Japan) equilibrated with 20 mM phosphate buffer (pH 6.8) containing 1.5 M ammonium sulfate. The column was washed with the same phosphate buffer, and then, elution was performed with a linear gradient of ammonium sulfate from 1.5 to 0.9 M in the same buffer. All the eluted fractions were checked for β-glucosidase activity using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPGlc; Wako Pure Chemical Industries, Ltd.) as the substrate and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions that exhibited high β-glucosidase activity were collected and concentrated by Centricut W-10 (Kurabo, Osaka, Japan) to 1.5 ml. In the case of BglF, the dialysate was added to the Hitrap Butyl FF column equilibrated with 20 mM phosphate buffer (pH 6.8) containing 1.05 M ammonium sulfate. Elution was performed with a linear gradient of ammonium sulfate from 1.05 to 0.825 M in the same buffer. The fractions with high β-glucosidase activity were collected and dialyzed overnight against 20 mM phosphate buffer (pH 6.8).

Following hydrophobic interaction chromatography, the dialyzed enzyme solution of BglF was added to an anion exchange column (1.6 × 4.0 cm) of TOYOPEARL SuperQ-650M (Tosoh Corp., Tokyo, Japan) equilibrated with 20 mM phosphate buffer (pH 6.8). After the column was washed with the same buffer, proteins were eluted with a linear gradient of NaCl from 50 to 88 mM in the same buffer. All the eluted fractions were used for enzyme activity assay and SDS-PAGE analyses. The active fractions were concentrated by Centricut W-10 to 1.5 ml. In the case of BglJ, following ammonium sulfate precipitation, the dialysate was added to the TOYOPEARL SuperQ-650M column (1.6 × 4.0 cm) equilibrated with 20 mM phosphate buffer (pH 6.8). Elution was performed with a linear gradient of NaCl from 0 to 0.05 M in the same buffer. The fractions with high beta-glucosidase activity were collected and concentrated by Centricut W-10 to 1.5 ml.

The concentrated enzyme solution of BglA obtained after hydrophobic interaction chromatography was added to a gel filtration column (1.6 × 60 cm) of HiLoad 16/60 Superdex-200 prep grade (GE Healthcare). The column was equilibrated and eluted with 150 mM NaCl in 20 mM phosphate buffer (pH 6.8), and all the eluted fractions were used for enzyme activity assay and SDS-PAGE analyses. The active fractions were collected and concentrated by Centricut W-10 to an appropriate concentration of the enzyme preparation and used for enzyme characterization. In the case of BglF and BglJ, the concentrated enzyme solutions were added to the same gel filtration column, and elution and concentration procedures were performed similar to those employed for BglA. All the chromatography procedures were performed using ÄKTAprime plus (GE Healthcare).

SDS-PAGE and glycosylation analysis SDS-PAGE was performed using the method described by Laemmli (37). The stacking and separating gels comprised 4.5% and 10% polyacrylamide, respectively. Proteins were stained with Coomassie Brilliant Blue R-250. The molecular weight standards used were Precision Plus Protein Standards, Unstained (Bio-Rad Laboratories, Hercules, CA, USA). For glycosylation analysis, purified BglA, BglF, and BglJ were electrophoresed on a 10% acrylamide gel following incubation with or without 200 U of Endo Hf (New England Biolabs, Beverly, MA, USA) at 37°C for 5 h, according to the manufacturer's instruction, and then stained with Coomassie Brilliant Blue R-250.

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

β-Glucosidase activity assay Plate assay to evaluate the β-glucosidase activity of the overexpression strains was conducted using a fluorogenic substrate for β-glucosidase, 4-methylumbelliferyl β-D-glucopyranoside (MUG; Wako Pure Chemical Industries, Ltd.). The transformants obtained were cultured for 3 days at 30°C on CD agar plates containing 1% maltose. Then, each plate was overlaid by soft agar containing 0.1% MUG and incubated for 1 h at 37°C, followed by detection of a clear zone around the colony under UV light.

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β-Glucosidase activity was assayed using the method developed by Riou et al. (38) with slight modifications. As a standard assay procedure, several concentrations of the *p*-nitrophenol (Wako Pure Chemical Industries, Ltd.) solution were used to construct a standard curve. β-Glucosidase activity was measured at the optimal temperature of each enzyme with 1 mM *p*NPGlc as the substrate in 0.4 ml of 20 mM citrate buffer (at the optimal pH of each enzyme) and the appropriately diluted enzyme preparation (2 μl). After 20 min of incubation, the reaction was stopped by adding 0.8 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 minute under the assay conditions.

For substrate specificity analyses, we used a 0.3-ml reaction mixture containing 2 mM *p*NPGlc, 100 mM acetate buffer (at the optimal pH of each enzyme), and an aliquot (2 μl) of the enzyme reparation. After 20 min of incubation at the optimal temperature of each enzyme, the reaction was stopped by adding 0.6 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 minute under the assay conditions. Furthermore, the activities toward other chromogenic aryl substrates were determined under the same conditions.

The activities toward natural oligosaccharides were assayed using a 0.3-ml reaction mixture containing 2 mM sophorose (Sigma–Aldrich, St. Louis, MO), laminaribiose (Funakoshi, Ltd., Tokyo, Japan), cellobiose (Wako Pure Chemical Industries, Ltd.), gentiobiose (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), maltose (Wako Pure Chemical Industries, Ltd.), lactose (Wako Pure Chemical Industries, Ltd.), or cellotriose (Sigma–Aldrich), 100 mM acetate buffer (at the optimal pH of each enzyme), and 2 μl of the enzyme preparation. After 20 min of incubation at the optimal temperature of each enzyme, the reaction was stopped by boiling for 2 min, and the amount of released glucose was measured by HPLC. The HPLC system (Model 8020; Tosoh Corp.) consisted of a dual pump (DP-8020) and a column oven (CO-8020) equipped with a refractive index detector (RI-8020) and an Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad). The HPLC conditions were as follows: the column oven temperature was 65°C, the flow rate was 0.6 ml/min, and the mobile phase (isocratic) was 5 mM sulfuric acid. 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 minute under the assay conditions.

The activities toward aryl β-glycosides were assayed using a 0.3-ml reaction mixture containing 2 mM arbutin (Tokyo Chemical Industry Co., Ltd.), salicin (Wako Pure Chemical Industries, Ltd.), or indican (Wako Pure Chemical Industries, Ltd.), 100 mM acetate buffer (at the optimal pH of each enzyme), and 2 μl of the enzyme preparation. After 20 min of incubation at the optimal temperature of each enzyme, the amount of reducing sugar released was immediately measured using the method proposed by Nelson (39) and Somogyi (40). Alkaline copper reagent (0.3 ml) was added to the reaction mixture, boiled for 10 min, and chilled quickly on ice, followed by adding 0.3 ml of arsenic molybdate reagent and mixing well. After incubation at room temperature for 15 min, absorbance was measured at 500 nm. 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 sugar equivalent to 1 μmol of glucose per minute under the assay conditions. The K_m and k_{cat} values for the activity of BglA and BglJ toward *p*NPGlc and the most hydrolyzed substrate were calculated using the double reciprocal plot method of Lineweaver and Burk (41).

Assay of the hydrolytic activities of BglA and BglJ toward isoflavone glycosides The hydrolytic activities toward isoflavone glycosides were assayed using a modified method of Kudou et al. (42). Isoflavone glycosides [daidzin, genistin, and glycitin (Nagara Science, Ltd., Gifu, Japan)] were dissolved in 70% (v/v) ethanol at a concentration of 0.1 mg/ml and mixed with 100 mM acetate buffer (at the optimal pH of each enzyme) at a ratio of 1:4. An aliquot (2 μl) of the diluted enzyme solution was added to 0.3 ml of these substrate solutions and incubated at the optimal temperature of each enzyme for 40 min. After incubation, the reaction was stopped by boiling for 2 min, and the amount of released aglycones was measured by the HPLC system (model 8020) equipped with a UV detector (UV-8020) and an YMC-pack ODS-AM column (5 μm, 25 cm × 4.6 mm; YMC, Kyoto, Japan). The HPLC conditions were as follows: the column oven temperature was 35°C, and the concentration of the mobile phase increased with a linear gradient of acetonitrile from 15% to 35% containing 0.1% acetic acid within 50 min at the flow rate of 0.6 ml/min. Absorbance was measured at 260 nm. Several concentrations of the aglycone solutions 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 aglycone per minute under the assay conditions.

For comparison of the hydrolytic activities of BglA and BglJ toward isoflavone glycosides with those toward *p*NPGlc, the hydrolytic activity of each enzyme toward *p*NPGlc was assayed under the same condition as that toward isoflavone glycosides. Similar to the method to determine the hydrolytic activity of each enzyme toward isoflavone glycosides, *p*NPGlc was dissolved in 70% (v/v) ethanol or distilled water at a concentration of 230 μM, which was equivalent to 0.1 mg/ml isoflavone glycosides, and mixed with 100 mM acetate buffer (at the optimal pH of each enzyme) at a ratio of 1:4. Then, 2 μl of the enzyme preparation was added to 0.3 ml of the *p*NPGlc solution and incubated for 40 min. The reaction was stopped by adding 0.6 ml of 1 M Na_2CO_3, and *p*-nitrophenol released was monitored at A_{400}.

Effects of temperature on the activity and stability of BglA, BglF, and BglJ The effect of temperature on the activity of purified BglA, BglF, and BglJ was determined by incubating each enzyme at various temperatures ranging from 10°C to 80°C for 20 min in 200 mM citrate buffer (at the optimal pH of each enzyme) using 1 mM *p*NPGlc as the substrate, according to the assay method described earlier (see the β-Glucosidase activity assay section). The thermal stabilities of the enzymes were examined by incubating the enzymes for 30 min at various temperatures ranging from 10°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 200 mM citrate buffer (at the optimal pH of each enzyme). The maximum activity obtained was defined as 100%.

Effects of pH on the activity and stability of BglA, BglF, and BglJ The effects of the pH on the activity of purified BglA, BglF, and BglJ were determined by measuring the enzyme activity at various pHs for 20 min at the optimal temperature of each enzyme, according to the assay method described earlier (see the β-Glucosidase activity assay section). The pH stability of purified BglA, BglF, and BglJ was examined after incubating each enzyme in various 20 mM buffers at different pHs 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 citrate buffer (at the optimal pH of each enzyme). The buffers used were 20 mM HCl–KCl buffer (pH 1.5–2.0), 20 mM glycine-HCl buffer (pH 2.5–3.5), 20 mM citrate buffer (pH 3.0–6.0), 20 mM phosphate buffer (pH 6.0–8.0), 20 mM TAPS buffer (pH 8.0–9.0), 20 mM glycine buffer (pH 9.0–10.0), and 20 mM CAPS buffer (pH 10.0–11.0). The maximum activity obtained was defined as 100%.

RESULTS

Selection and overexpression of putative β-glucosidase genes from *A. oryzae* genome First, we chose 23 genes

TABLE 1. Summary of the purification of BglA, BglF, and BglJ.

Purification step	Total activity (U)[a]	Total protein (mg)[b]	Specific activity (U/mg)	Purification (fold)
BglA				
Culture filtrate	21.0	401	0.052	1.0
$(NH_4)_2SO_4$ precipitation	1.53	6.42	0.24	4.6
Hitrap Butyl FF	0.90	0.14	6.43	124
Superdex	0.36	0.053	6.79	131
BglF				
Culture filtrate	1.40	613	0.0023	1.0
$(NH_4)_2SO_4$ precipitation	0.82	29.6	0.028	12.2
Hitrap Butyl FF	0.26	1.66	0.16	69.6
TOYOPEARL SuperQ	0.032	0.050	0.64	278
Superdex	0.028	0.048	0.58	254
BglJ				
Culture filtrate	160	1610	0.099	1.0
$(NH_4)_2SO_4$ precipitation	53.2	30.7	1.73	17.5
TOYOPEARL SuperQ	11.0	0.94	11.7	118
Superdex	1.04	0.099	10.5	106

[a] Activity was measured in 20 mM citrate buffer [pH 5.5 (BglA), pH 6.0 (BglF), and pH 4.5 (BglJ)] at 50°C (BglA), 45°C (BglF), and 40°C (BglJ) using 1 mM *p*NPGlc as the substrate.

[b] The protein was assayed using the BCA Protein Assay Kit with BSA as the standard.

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encoding enzymes that belong to GH3 from the CAZy database (http://www.cazy.org/). Then, 10 genes encoding putative β-glucosidases with a signal peptide were screened from the SignalP 4.1 database (http://www.cbs.dtu.dk/services/SignalP/) (Supplementary Table S2) and overexpressed in *A. oryzae*, because these gene products were expected to be secreted into the culture media so that they could be easily purified and analyzed. Subsequently, plasmids for the overexpression of these genes were constructed using the expression vector plasmid pNGA142 and introduced into *A. oryzae* AOK11 to construct the β-glucosidase-overexpressing strains. Through plate assay using MUG as the fluorogenic substrate for β-glucosidase, four transformant strains, each harboring AO090038000223 (*bglA*), AO090009000356 (*bglC*), AO090103000127 (*bglF*), and AO090003001511 (*bglJ*), formed a large clear zone around the colony when detected under UV light (data not shown). As the β-glucosidase encoded by AO090009000356 (BglC) had already been purified and characterized (29,30) and because our preliminary experiment suggested that the other three gene products had higher activity toward *p*NPGlc than cellobiose, the three β-glucosidases BglA, BglF, and BglJ were purified to homogeneity from the overexpression transformants.

Purification of BglA, BglF, and BglJ BglA, BglF, and BglJ, were purified from the culture filtrates of the *A. oryzae* AOK11 transformants that overexpressed the *bglA*, *bglF*, or *bglJ* genes by employing the procedure described earlier (see Materials and methods). The β-glucosidase activities of all the fractions were assayed using *p*NPGlc as the substrate. The purification of BglA, BglF, and BglJ is summarized in Table 1. From the culture filtrates, proteins were concentrated by ammonium sulfate precipitation, and the dialyzed enzyme solutions were used in the subsequent step of purification. After ammonium sulfate precipitation, BglA was purified by hydrophobic interaction and gel filtration

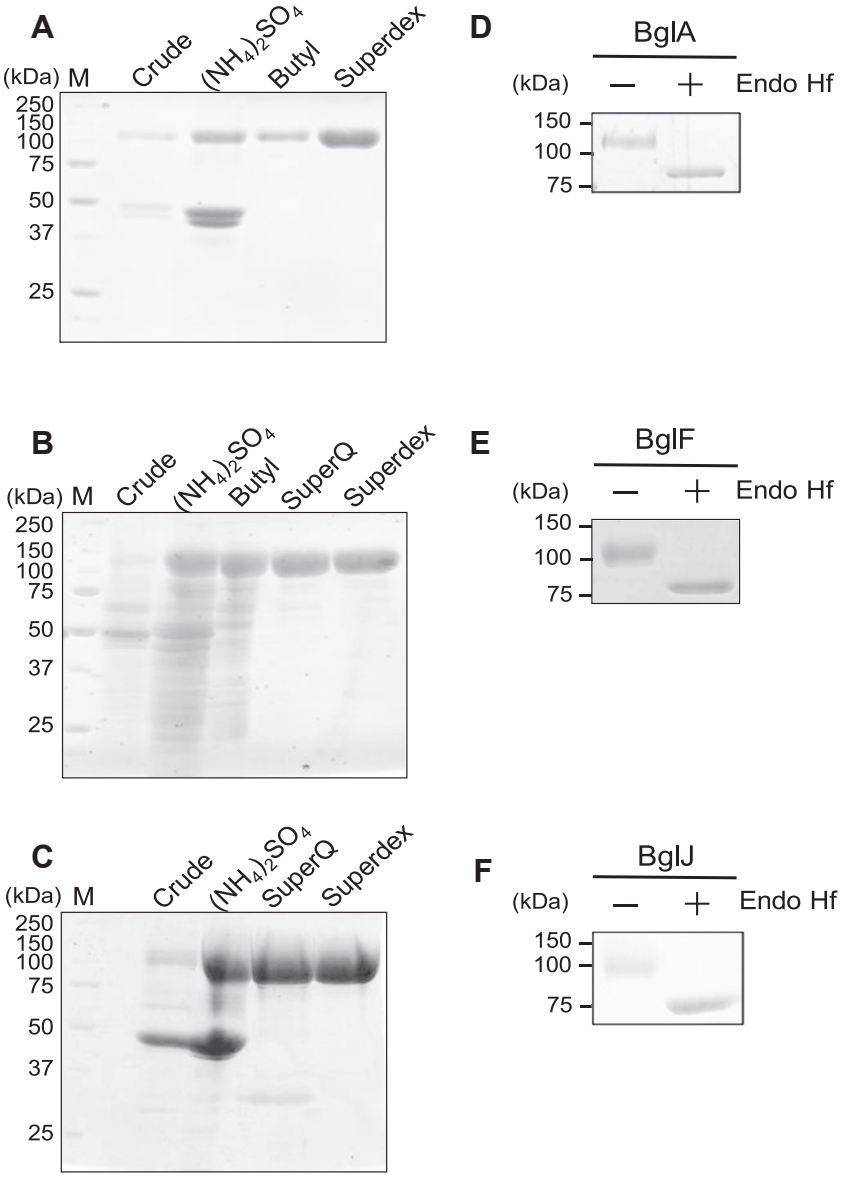

FIG. 1. SDS-PAGE of each purification step of BglA (A), BglF (B), and BglJ (C), and Endo Hf-treated (+) or not (−) BglA (D), BglF (E), and BglJ (F). (A–C) M, molecular weight standard proteins (250, 150, 100, 75, 50, 37, 25 kDa); Crude, crude enzyme sample; $(NH_4)_2SO_4$, enzyme sample after ammonium sulfate precipitation; Butyl, enzyme sample after hydrophobic interaction column (Hitrap Butyl FF) chromatography; SuperQ, enzyme sample after anion exchange column (Toyopearl SuperQ-650M) chromatography; Superdex, enzyme sample after gel filtration column (Superdex 200 prep grade) chromatography. (D–F) Purified BglA, BglF, and BglJ were electrophoresed on a 10% acrylamide gel after incubation with (+) or without (−) 200 U of Endo Hf at 37°C for 5 h and stained with Coomassie Brilliant Blue R-250.

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chromatographies; BglF was purified by hydrophobic interaction, anion exchange, and gel filtration chromatographies; and BglJ was purified by anion exchange and gel filtration chromatographies. The specific activities of purified BglA, BglF, and BglJ were 6.79, 0.58, and 10.5 U/mg, which were approximately 130-, 250-, and 100-fold higher than those of the crude enzyme preparations, respectively. The purified samples of BglA, BglF, and BglJ migrated as single protein bands on SDS-PAGE (Fig. 1A–C), and MUG-containing plate assay revealed that all the protein bands of purified BglA, BglF, and BglJ on PAGE showed β-glucosidase activities (data not shown). The apparent molecular masses of purified BglA, BglF, and BglJ obtained by SDS- PAGE analysis were approximately 130, 120, and 100 kDa, respectively, which were much higher than the values (87.7, 85.9, and 91.9 kDa, respectively) deduced from the amino acid sequences of mature proteins. As differences in the molecular masses were presumed to be due to glycosylation of proteins, purified BglA, BglF, and BglJ were treated with Endo Hf. After treatment with Endo Hf, the protein bands shifted to approximately 88 kDa (BglA; Fig. 1D), 86 kDa (BglF; Fig. 1E), and 84 kDa (BglJ; Fig. 1F), corresponding to the molecular mass estimated from each amino acid sequence; this suggested that proteins were *N*-glycosylated.

Effect of temperature and pH on the activity and stability of BglA, BglF, and BglJ The effect of temperature on the activity and stability of BglA, BglF, and BglJ was examined. As shown in Fig. 2, the maximum activity of BglA, BglF, and BglJ was obtained at 50°C at pH 5.5, 45 C at pH 6.0, and 40°C at pH 4.5, respectively. Although BglA was stable below 50°C, it lost its activity sharply over 50°C. BglF was stable below 40°C and retained over 70% of its maximum activity up to 50°C. Among the three β-glucosidases, BglJ was the most thermolabile enzyme, which was stable below 30°C but lost its activity at 45°C.

The optimal pH for the activity of purified BglA was 5.5 at 50°C, and over 80% of its maximum activity was retained between pH 5.0 and 6.5 (Fig. 3A). In addition, BglA was stable in a wide pH range between 4.5 and 10.0 and retained over 70% of its maximum activity between pH 4.5 and 11.0 but lost its activity at pH 3.0 (Fig. 3B). The optimal pH for the activity of BglF was 6.0 at 45°C, and over 70% of its maximum activity was retained between pH 5.0 and 6.5 (Fig. 3C). Although BglF was stable between pH 6 and 9, it lost its activity sharply at pH higher than 9.5 and gradually at pH below 5.5 (Fig. 3D). In contrast, the optimal pH for the activity of purified BglJ was 4.5 at 40°C (Fig. 3E). Although BglJ was highly stable between pH 4.0 and 7.0, it was significantly unstable even at weak alkaline pH and lost its activity at pH 8.0 (Fig. 3F).

Substrate specificities of BglA, BglF, and BglJ The substrate specificities of BglA, BglF, and BglJ, were examined, and the results are summarized in Table 2. First, we investigated the hydrolytic activities of the purified β-glucosidases toward various oligosaccharides, including cellobiose, and compared these activities with their activities toward *p*NPGlc. BglA, BglF, and BglJ displayed the highest hydrolytic activity toward *p*NPGlc, with specific activities of 319, 0.6, and 168 U/mg, respectively. These three β-glucosidases also hydrolyzed laminaribiose, cellobiose, and gentiobiose, but their specific activities were significantly low when compared with their activities toward *p*NPGlc. In particular, the hydrolytic activities of BglA, BglF, and BglJ toward cellobiose

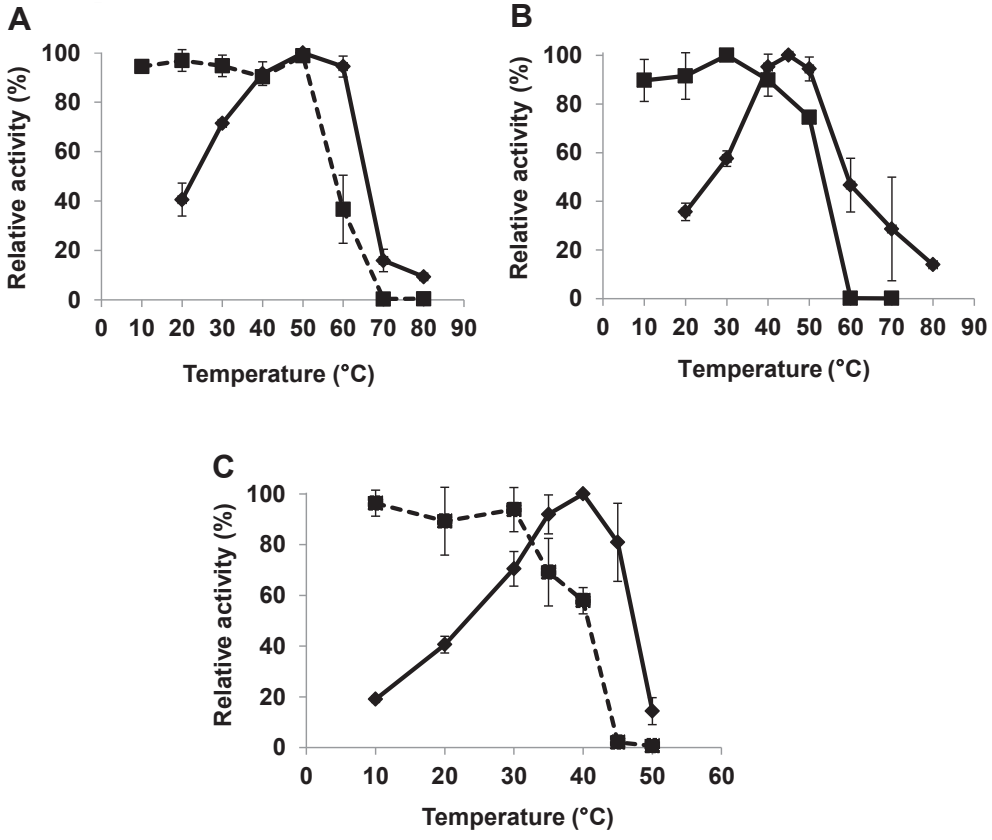

FIG. 2. Effect of temperature on the activity and stability of purified BglA (A), BglF (B), and BglJ (C). For the determination of the optimal temperature, β-glucosidase activities (thin lines) were assayed in 200 mM citrate buffer (at the optimal pH of each enzyme) at various temperatures using 1 mM *p*NPGlc as the substrate. For thermal stability assay, the remaining activities (broken lines) were measured after incubating the enzyme for 30 min at various temperatures in 20 mM phosphate buffer (pH 6.8). The maximum activity obtained was defined as 100%. Error bars are given as mean ± SE of three independent experiments.

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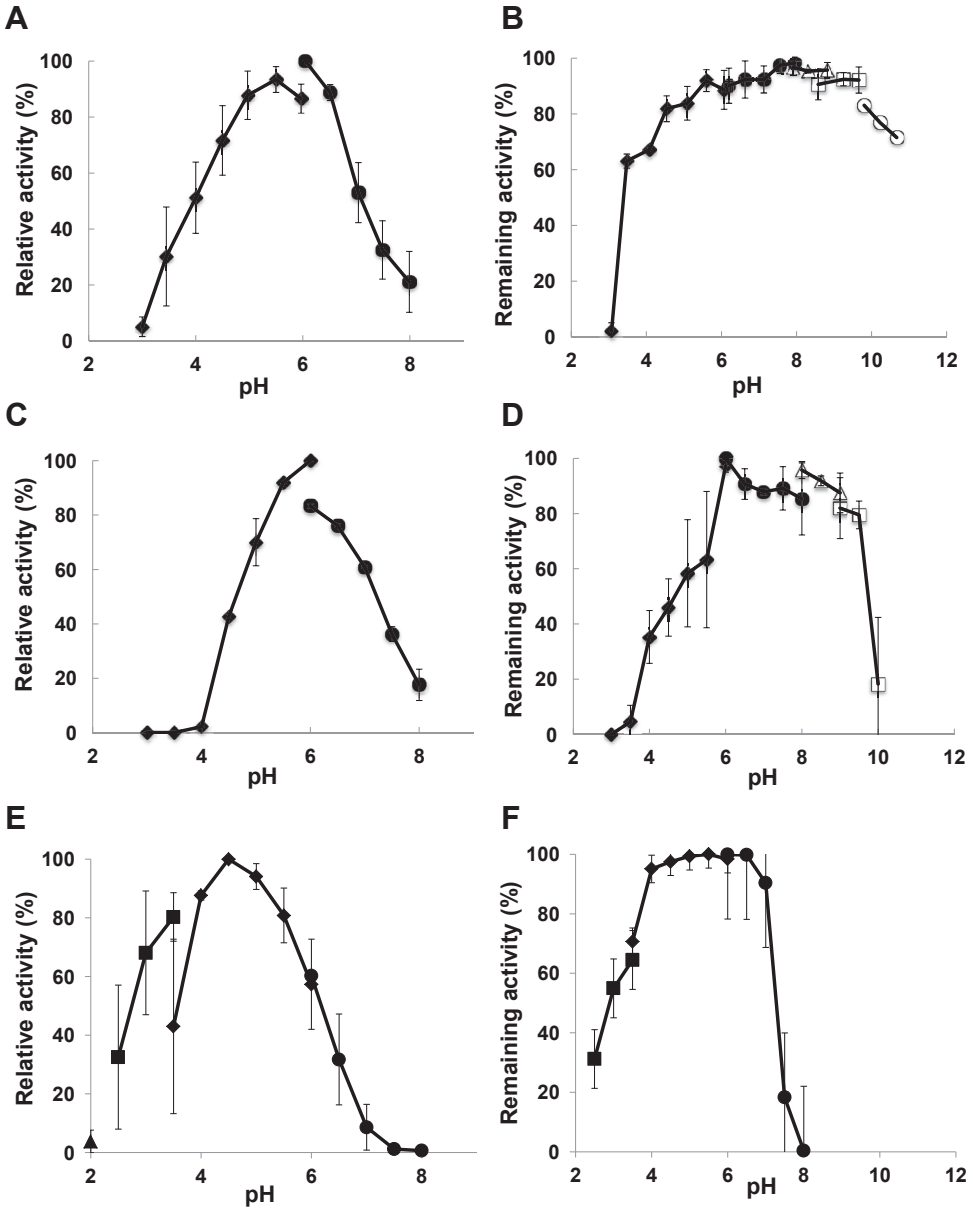

FIG. 3. Effect of pH on the activity and stability of purified BglA (A, B), BglF (C, D), and BglJ (E, F). (A, C, E) For the determination of the optimal pH, β-glucosidase activities were assayed in different buffers at the optimal temperature of each enzyme using 1 mM *p*NPGlc as the substrate. The buffers used were 20 mM HCl–KCl buffer (closed triangles), 20 mM glycine-HCl buffer (closed squares), 20 mM citrate buffer (closed diamonds), and 20 mM phosphate buffer (closed circles). (B, D, F) For pH stability assay, the remaining activities were measured after incubating each enzyme for 30 min at 30°C in the following buffers: 20 mM glycine-HCl buffer (closed squares), 20 mM citrate buffer (closed diamonds), 20 mM phosphate buffer (closed circles), 20 mM TAPS buffer (open triangles), 20 mM glycine buffer (open squares), and 20 mM CAPS buffer (open circles) indicated by. The maximum activity obtained was defined as 100%. Error bars are given as mean ± SE of three independent experiments.

were only approximately 6%, 1%, and 12% of those noted toward *p*NPGlc, respectively. Although BglA and BglF could not hydrolyze sophorose, maltose, lactose, and cellotriose, BglJ showed little hydrolytic activity toward maltose. From these results, we assumed that BglA, BglF, and BglJ were aryl β-glucosidases. As the specific activities of BglF were significantly lower than those of BglA and BglJ toward the various substrates tested (Table 2), further analyses to determine the substrate specificities of BglA and BglJ toward various aryl β-glycosides were performed. Interestingly, BglA could effectively hydrolyze arbutin, with a specific activity of 327 U/mg, when compared with its activity toward *p*NPGlc. In addition, BglA showed little hydrolytic activity toward *p*-nitrophenyl-β-D-xylopyranoside (*p*NPXyl) and indican and could not hydrolyze *p*-nitrophenyl-β-D-galactopyranoside (*p*NPGal), *p*-nitrophenyl-β-D-cellobioside (*p*NPCel), and salicin. Conversely, BglJ exhibited hydrolytic activities toward salicin as well as *p*NPXyl, arbutin, and indican. In particular, BglJ showed considerably higher activity toward arbutin, resulting in a specific activity of 439 U/mg, which was 1.3-fold higher than that of BglA. However, BglJ showed no hydrolytic activities toward *p*NPGal and *p*NPCel. Thus, BglA and BglJ preferentially hydrolyzed aryl β-glycosides rather than oligosaccharides, and both the enzymes were proven to be aryl β-glucosidases.

Kinetic constants of BglA and BglJ The steady-state kinetic parameters of BglA and BglJ measured for *p*NPGlc and arbutin, both of which were preferentially hydrolyzed by these enzymes, are summarized in Table 3. The K_m, k_{cat}, and k_{cat}/K_m values for the

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TABLE 2. Substrate specificities of BglA, BglF, and BglJ.

Substrate	Linkage of glycosyl group	BglA		BglF		BglJ	
		Specific act. (U/mg)	Relative act. (%)	Specific act. (U/mg)	Relative act. (%)	Specific act. (U/mg)	Relative act. (%)
Oligosaccharides							
Sophorose	β-(1,2)-Glucose	N.D.	N.D.	N.D.	N.D.	–	–
Laminaribiose	β-(1,3)-Glucose	22.8 ± 2.36	109	0.010 ± 0.0	169	21.7 ± 9.75	12.9
Cellobiose	β-(1,4)-Glucose	21.0 ± 1.44	100	0.0059 ± 0.0004	100	20.2 ± 12.4	100
Gentiobiose	β-(1,6)-Glucose	49.3 ± 2.68	235	0.0046 ± 0.0013	78.0	1.07 ± 0.07	5.3
Lactose	β-(1,4)-Galactose	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Cellotriose	β-(1,4)-Glucose	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Aryl β-glycosides							
*p*NPGlc	β-Glucose	319 ± 15.4	100	0.60 ± 0.002	100	168 ± 5.80	100
*p*NPGal	β-Galactose	N.D.	N.D.	–	–	N.D.	N.D.
*p*NPXyl	β-Xylose	2.70 ± 0.35	0.8	–	–	10.6 ± 1.02	6.3
*p*NPCel	β-Cellobiose	N.D.	N.D.	–	–	N.D.	N.D.
Arbutin	β-Glucose	327 ± 2.35	103	–	–	439 ± 3.29	261
Salicin	β-Glucose	N.D.	N.D.	–	–	28.8 ± 3.76	17.1
Indican	β-Glucose	42.2 ± 0.42	13.2	–	–	78.7 ± 6.85	46.8

All the substrates were tested at a concentration of 2 mM (BglA and BglJ) in 100 mM acetate buffer (at the optimal pH of each enzyme) or 5 mM (BglF) in 20 mM citrate buffer (pH 6.0). The specific activities were determined by measuring the amount of released glucose (oligosaccharides, arbutin, salicin, and indican) or *p*-nitrophenol (*p*-nitrophenyl glycosides) after incubating them with the enzymes for 20 min at the optimal temperature of each enzyme. The relative activity toward cellobiose (for oligosaccharides) and *p*NPGlc (for aryl β-glycosides) was defined as 100%. The values are expressed means ± SE of three different experiments. N.D., no activity was detected; –; not determined.

activity of BglA and BglJ toward *p*NPGlc were 0.75 and 0.48 mM, 651 and 373 s^{-1}, 868 and 777 s^{-1} mM^{-1}, respectively. Conversely, the K_m, k_{cat}, and k_{cat}/K_m values for the activity of BglA and BglJ toward arbutin were 2.48 and 0.82 mM, 759 and 810 s^{-1}, 361 and 987 s^{-1} mM^{-1}, respectively. It has been reported that the k_{cat} values for β-glucosidase activities toward natural substrates are relatively low (normally, a maximum of 300 s^{-1} or lower) (25). In the present study, the k_{cat} values for the activity of both these enzymes toward arbutin and *p*NPGlc were significantly high. In addition, transglycosylation activities of BglA and BglJ were scarcely detected toward *p*NPGlc, cellobiose, and gentiobiose as substrates (data not shown).

Hydrolytic activity of purified BglA and BglJ toward isoflavone glycosides Purified BglA and BglJ were aryl β-glucosidases because both the enzymes displayed strong specificity toward aryl β-glycosides. It was reported that BglJ on the yeast surface exhibited high hydrolytic activity toward soybean isoflavones (31,34); hence, apart from purified BglJ, we investigated whether BglA also possessed this activity or not. Both BglA and BglJ were incubated with isoflavone glycosides (daidzin, glycitin, and genistin), and the results are summarized in Table 4. As isoflavone glycosides are difficult to dissolve in buffers or water, they were dissolved in 70% (v/v) ethanol at a concentration of 0.1 mg/ml (equivalent to 230 μM), followed by mixing with 100 mM acetate buffer by following the method developed by Kudou et al. (42). Similarly, the hydrolytic reaction toward *p*NPGlc was also tested by employing the same procedure: *p*NPGlc was dissolved in 70% ethanol or water at a concentration of 230 μM. The specific activities of BglA and BglJ were determined by measuring the amount of released aglycons (daidzein, glycitein, and genistein) or *p*-nitrophenol after incubating the substrates with the enzymes. Consequently, as shown in Table 4, BglA showed much higher hydrolytic activities toward all the isoflavone glycosides tested than BglJ. Interestingly, when compared with hydrolytic activity toward *p*NPGlc in the absence of ethanol (ethanol –), BglJ showed a 86% decrease in hydrolytic activity in the presence of ethanol [14% (v/v)], while BglA retained about 60% activity even in the presence of ethanol. Indeed, the ethanol tolerance of BglA was much higher than that of BglJ (Fig. 4). Thus, BglA was more tolerant to ethanol than BglJ, and we assumed that the ethanol tolerance of both the enzymes resulted in the difference in the hydrolytic activities toward isoflavone glycosides.

TABLE 3. Steady-state kinetic constants of BglA and BglJ.

Enzyme	Substrate	V_{max} (U mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
BglA	*p*NPGlc	456 ± 10	0.75 ± 0.06	651 ± 14	868
	Arbutin	531 ± 8	2.48 ± 0.20	759 ± 12	316
BglJ	*p*NPGlc	264 ± 21	0.48 ± 0.003	373 ± 30	777
	Arbutin	573 ± 36	0.82 ± 0.008	810 ± 51	987

DISCUSSION

β-Glucosidase plays an important role in the degradation of cellulosic biomass, isoflavone aglycones production, and other bioconversion processes; thus, numerous studies have been conducted to characterize β-glucosidases (10,43,44). *A. oryzae* is an industrially important filamentous fungus that has been used for a long time in Japanese fermentation industries (26), and this fungus can produce various useful enzymes, including β-glucosidases (45,46). In the present study, we screened and attempted to overexpress putative β-glucosidase genes from the *A. oryzae* genome, for which sequencing analysis had been completed (27). Besides the three β-glucosidases [AO090009000356 (BglC), AO090003001511 (BglJ), and AO090001000544 (BglG)] previously reported, we found seven additional genes encoding putative β-glucosidases with a signal peptide in the genome, all of which belonged to GH3 according to the CAZy database. The signature motifs encompassing an active site of GH3, found using the PROSITE database (http://prosite.expasy.org/) (consensus pattern is [LIVM](2)-[KR]-x-[EQKRD]-x(4)-G-[LIVMFTC]-[LIVT]-[LIVMF]-[ST]-D-x(2)-[SGADNIT] and D is the active site residue; PROSITE accession no. PDOC00621), were highly conserved in their deduced amino acid sequences (data not shown).

Among these 10 genes, the transformants overexpressing four genes, *bglC*, *bglJ*, AO090038000223 (*bglA*), and AO090103000127 (*bglF*), clearly showed β-glucosidase activities in plate assay using MUG as the fluorogenic substrate (data not shown). The enzymatic characterization of purified BglC has been previously reported (29,30), and BglC has been suggested to be a broad-specificity β-glucosidase. Alternatively, the other three β-glucosidases appeared to exhibit high specificity toward aryl β-glycosides; hence, we purified and enzymatically characterized these enzymes. Analysis of the substrate specificities toward various oligosaccharides and *p*NPGlc revealed that all these β-glucosidases could be categorized into aryl β-glucosidases because natural oligosaccharides with β-

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TABLE 4. Hydrolytic activities of BglA and BglJ toward isoflavone glycosides.

Substrate	Ethanol	BglA		BglJ	
		Specific act. (U/mg)	Relative act. (%)	Specific act. (U/mg)	Relative act. (%)
Daidzin	+	8.31 ± 1.99	49.2	1.09 ± 0.02	7.6
Glycitin	+	7.93 ± 1.66	46.9	0.67 ± 0.02	4.6
Genistin	+	10.5 ± 2.02	62.1	1.18 ± 0.13	8.2
*p*NPGlc	+	10.4 ± 0.87	61.5	1.97 ± 0.01	13.7
*p*NPGlc	−	16.9 ± 2.06	100	14.4 ± 0.76	100

The reaction mixture contained 240 μl of 100 mM acetate buffer (at the optimal pH of each enzyme), 60 μl of daidzin, glycitin, or genistin solutions dissolved in 70% (v/v) ethanol at a concentration of 0.1 mg/ml (about 230 μM), and an appropriate dilution of the enzyme preparation. The hydrolytic reaction toward *p*NPGlc was also tested with 240 μl of 100 mM acetate buffer (at the optimal pH of each enzyme) plus 60 μl of 230 μM *p*NPGlc, which was dissolved in 70% ethanol (+) or water (−). Specific activity was determined by measuring the amount of released aglycones (daidzein, glycitein, and genistein) or *p*-nitrophenol after incubating the substrates with enzymes for 40 min at the optimal temperature of each enzyme. The relative activity toward *p*NPGlc (ethanol −) was defined as 100%. The values are expressed means ± SE of three different experiments.

1,4-, β-1,3-, and β-1,6-glucosidic linkages were appreciably poor substrates when compared with *p*NPGlc (Table 2). The substrate specificities and kinetic parameters of both BglA and BglJ were subsequently examined, although BglF was not subjected to further enzymatic characterization due to its very low specific activity. Among the substrates tested, both BglA and BglJ preferentially hydrolyzed aryl β-glycosides, particularly *p*NPGlc and arbutin, rather than oligosaccharides. To date, only two fungal GH3 β-glucosidases that exhibit a marked preference toward hydrolysis of aryl β-glycosides have been reported to be purified to homogeneity (8,47). Decker et al. (8) purified four β-glucosidases from the culture broth of *Aspergillus tubingensis* and found that β-glycosidase II exhibited high substrate specificity toward aryl β-glucosides. Based on the partial amino acid sequences of β-glucosidase II, this enzyme was noted to be most likely to be the one encoded by Asptu1_0124418 by a BLAST search of the AspGD database (http://www.aspergillusgenome.org/). The amino acid sequences of BglA, BglF, and BglJ were highly homologous to the sequence of β-glucosidase II (Asptu1_0124418-P) (46%, 51%, and 53% amino acid identities, respectively); thus, these enzymes might also exhibit similar substrate specificities. However, the amino acid sequence of

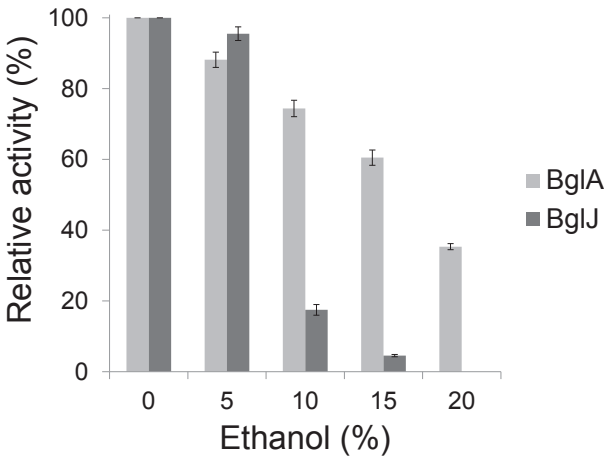

FIG. 4. Effect of ethanol on the activity of BglA and BglJ. To examine the ethanol tolerance of BglA and BglJ, the remaining activities were measured after incubating each enzyme at various concentrations of ethanol for 20 min at their optimal temperatures, according to the assay method described in Materials and methods. The maximum activity obtained was defined as 100%. Error bars are given as mean ± SE of three independent experiments.

β-glucosidase (Cel3a; accession no. AAL69548) from *Talaromyces emersonii* (47) showed higher homology to that of BglC, a broad-specificity β-glucosidase (69% amino acid identity), when compared with those of BglA and BglJ (47% and 41% amino acid identities, respectively). These results suggested that the substrate specificities of β-glucosidases could not be predicted only based on the homology of amino acid sequences. There had been no reports on the crystal structures of fungal β-glucosidases until the most recent studies on those of *Aspergillus aculeatus* BGL1 and *Trichoderma reesei* Cel3A (48,49). Hence, a comparative study on the three-dimensional structures of BglC and BglA or BglJ could reveal the difference in their substrate specificities.

The substrate specificities and kinetic parameters of BglA and BglJ were slightly different from each other. For example, BglA showed a higher substrate preference toward aryl β-glycosides than BglJ. In addition, the K_m value for the activity of BglA (2.48 mM) toward arbutin was relatively higher than that for the activity of BglJ (0.82 mM). The V_{max} values for the activity of BglA toward *p*NPGlc were comparable with those for the activity of *T. emersonii* β-glucosidase (47), which was approximately 2-fold higher than those for the activity of BglJ. However, the K_m values for the activities of both BglA and BglJ toward *p*NPGlc were 4–6-fold higher than those for the activity of *T. emersonii* β-glucosidase; thus, their k_{cat} values were lower than the latter to a similar extent. Nevertheless, according to the BRENDA database (http://www.brenda-enzymes.org/), the kinetic parameters of BglA and BglJ for *p*NPGlc suggested that these enzymes had relatively higher hydrolytic activity toward *p*NPGlc among the β-glucosidases reported.

The thermal and pH stabilities of BglA were relatively higher than those of BglF and BglJ, and BglA was particularly stable in a wide pH range (pH 4.5–10). In contrast, BglJ was the most heat- and alkaline-labile among the three β-glucosidases, because it was stable only below 30°C and up to pH 7.0. In previous studies, the thermal and pH stabilities of BglJ produced by *Saccharomyces cerevisiae* and *A. oryzae* were determined (33,34). When compared with purified BglJ examined in the present study, BglJ secreted by the yeast was similarly unstable at weak alkaline pH but was more stable over 40°C (34), whereas BglJ produced by *A. oryzae* was more stable at pH 8.0 (33). Although the reason for this difference in enzyme stability between the data obtained in the present study and those previously reported is still unclear, it could be concluded that BglJ is more unstable than BglA and BglF.

Interestingly, we found that the hydrolytic activities of BglA toward isoflavone glycosides, such as daidzin, glycitin, and genistin, were significantly higher (7–12-fold) than those of BglJ under ethanol-containing assay conditions (Table 4), in which isoflavone glycosides were dissolved in ethanol due to their insolubility in water. Although the specific activities of BglA and BglJ toward *p*NPGlc (230 μM) in the absence of ethanol were almost identical, BglJ showed a remarkable reduction (approximately 10%) in the activity toward *p*NPGlc in the presence of ethanol [14% (v/v)], while BglA showed moderately decreased activity under the same condition. Thus, BglA seemed to be more tolerant to ethanol when compared with BglJ. The ethanol tolerance of β-glucosidases is an important characteristic for industrial applications, particularly for wine making, because β-glucosidases that can efficiently hydrolyze aryl β-glycosides are used for liberating aromatic compounds from glucosidic precursors present in wine grapes (50). Moreover, BglA exhibits better thermal stability than BglJ, which also makes BglA a good candidate enzyme for industrial applications, although its enzymatic stability and activity should be much improved.

In conclusion, the present study suggested that the mining of novel β-glucosidases showing higher activity from microbial genome sequences is of great use for the production of beneficial compounds such as isoflavone aglycones. Moreover, certain

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potentially useful enzymes for industrial applications are expected to be found among other uncharacterized β-glucosidase genes in the *A. oryzae* genome. In addition to β-glucosidases, useful β-1,4-endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91) as well as hemicellulases such as xylanase and β-xylosidase could be screened from the *A. oryzae* genome. Currently, construction of transformants that overexpress these candidate genes and characterization of the gene products are in progress.

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References

1. **Siegel, D., Ira, M., Mara, D., Ben-Ami, B., Shouming, H., Stephen, G. W., and Oded, S.:** Cloning, expression, characterization, and nucleophile identification of family 3, *Aspergillus niger* β-glucosidase, J. Biol. Chem., **275**, 4973–4980 (2000).
2. **Bhatia, Y., Mishra, S., and Bisaria, V. S.:** Microbial β-glucosidases: cloning, properties, and applications, Crit. Rev. Biotechnol., **22**, 375–407 (2002).
3. **Rojas, A., Arola, L., and Romeu, A.:** β-Glucosidase families revealed by computer analysis of protein sequences, Biochem. Mol. Biol. Int., **35**, 1223–1231 (1995).
4. **Chen, H., Hayn, M., and Esterbauer, H.:** Purification and characterization of two extracellular β-glucosidases from *Trichoderma reesei*, Biochim. Biophys. Acta, **1121**, 54–60 (1992).
5. **Saha, B. C., Freer, S. N., and Bothast, R. J.:** Production, purification, and characterization of a highly glucose-tolerant novel β-glucosidase from *Candida peltata*, Appl. Environ. Microbiol., **62**, 3165–3170 (1996).
6. **Uchiyama, T., Miyazaki, K., and Yaoi, K.:** Characterization of a novel β-glucosidase from a compost microbial metagenome with strong transglycosylation activity, J. Biol. Chem., **288**, 18325–18334 (2013).
7. **Winterhalter, P. and Skouroumounis, G. K.:** Glycoconjugated aroma compounds: occurrence, role and biotechnological transformation, Adv. Biochem. Eng. Biotechnol., **55**, 73–105 (1997).
8. **Decker, C. H., Visser, J., and Schreier, P.:** β-Glucosidase multiplicity from *Aspergillus tubingensis* CBS 643.92: purification and characterization of four β-glucosidases and their differentiation with respect to substrate specificity, glucose inhibition and acid tolerance, Appl. Microbiol. Biotechnol., **55**, 157–163 (2001).
9. **Kuo, L. C. and Lee, K. T.:** Cloning, expression, and characterization of two β-glucosidases from isoflavone glycoside-hydrolyzing *Bacillus subtilis* natto, J. Agric. Food Chem., **56**, 119–125 (2008).
10. **Yang, L., Ning, Z. S., Shi, C. Z., Chang, Z. Y., and Huan, L. Y.:** Purification and characterization of an isoflavone-conjugates-hydrolyzing β-glucosidase from endophytic bacterium, J. Agric. Food Chem., **52**, 1940–1944 (2004).
11. **Messina, M. J.:** Legumes and soybeans: overview of their nutritional profiles and health effects, Am. J. Clin. Nutr., **70**(suppl.), 439S–450S (1999).
12. **Cappelletti, V., Fioravanti, L., Miodini, P., and Di Fronzo, G.:** Genistein blocks breast cancer cells in the G_2M phase of the cell cycle, J. Cell. Biochem., **14**, 594–600 (2000).
13. **Miura, T., Yuan, L., Sun, B., Fujii, H., Yoshida, M., Wakame, K., and Kosuna, K.:** Isoflavone aglycon produced by culture of soybean extracts with basidiomycetes and its anti-angiogenic activity, Biosci. Biotechnol. Biochem., **66**, 2626–2631 (2002).
14. **Ravindranath, M. H., Muthugounder, S., Presser, N., and Viswanathan, S.:** Anticancer therapeutic potential of soy isoflavone, genistein, Adv. Exp. Med. Biol., **546**, 121–165 (2004).
15. **Brown, J. P.:** Hydrolysis of glycosides and esters, pp. 109–144, in: Rowland, I. R. (Ed.), Role of the gut flora in toxicity and cancer. Academic Press, San Diego (1998).
16. **Hendrich, S.:** Bioavailability of isoflavones, J. Chromatogr. B Anal. Technol. Biomed. Life Sci., **777**, 203–210 (2002).
17. **Kawakami, Y., Tsurugasaki, W., Nakamura, S., and Osada, K.:** Comparison of regulative functions between dietary soy isoflavones aglycone and glucoside on lipid metabolism in rats fed cholesterol, J. Nutr. Biochem., **16**, 205–212 (2005).
18. **Setchell, K. D., Brown, N. M., Zimmer-Nechemias, L., Brashear, W. T., Wolfe, B. E., Kirschner, A. S., and Heubi, J. E.:** Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability, Am. J. Clin. Nutr., **76**, 447–453 (2002).
19. **Xu, X., Harris, K. S., Wang, H. J., Murphy, P. A., and Hendrich, S.:** Bioavailability of soybean isoflavones depends upon gut microflora in women, J. Nutr., **125**, 2307–2315 (1995).
20. **Hancock, S. M., Corbett, K., Fordham-Skelton, A. P., Gatehouse, J. A., and Davis, B. G.:** Developing promiscuous glycosidases for glycoside synthesis. Residues W433 and E432 in *Sulfolobus solfataricus* β-glycosidase are important glucoside- and galactoside-specificity determinants, Chembiochem, **6**, 866–875 (2005).
21. **Wang, Y., Li, J., and Xu, Y.:** Characterization of novel β-glucosidases with transglycosylation properties from *Trichosporon asahii*, J. Agric. Food Chem., **59**, 11219–11227 (2011).
22. **Ducret, A., Trani, M., and Lortie, R.:** Screening of various glycosidases for the synthesis of octyl glucoside, Biotechnol. Bioeng., **77**, 752–757 (2002).
23. **Kono, H., Kawano, S., Tajima, K., Erata, T., and Takai, M.:** Structural analyses of new tri- and tetrasaccharides produced from disaccharides by transglycosylation of purified *Trichoderma viride* β-glucosidase, Glycoconj. J., **16**, 415–423 (1999).
24. **Hancock, S. M., Vaughan, M. D., and Withers, S. G.:** Engineering of glycosidases and glycosyltransferases, Curr. Opin. Chem. Biol., **10**, 509–519 (2006).
25. **Ketudat Cairns, J. R. and Esen, A.:** β-Glucosidases, Cell. Mol. Life Sci., **67**, 3389–3405 (2010).
26. **Machida, M., Yamada, O., and Gomi, K.:** Genomics of *Aspergillus oryzae*: learning from the history of koji mold and exploration of its future, DNA Res., **15**, 173–183 (2008).
27. **Machida, M., Asai, K., Sano, M., Tanaka, T., Kumagai, T., Terai, G., Kusumoto, K., Arima, T., Akita, O., Kashiwagi, Y., and other 54 authors:** Genome sequencing and analysis of *Aspergillus oryzae*, Nature, **438**, 1157–1161 (2005).
28. **Kobayashi, T., Abe, K., Asai, K., Gomi, K., Juvvadi, P. R., Kato, M., Kitamoto, K., Takeuchi, M., and Machida, M.:** Genomics of *Aspergillus oryzae*, Biosci. Biotechnol. Biochem., **71**, 646–670 (2007).
29. **He, H., Qin, Y., Chen, G., Li, N., and Liang, Z.:** Two-step purification of a novel β-glucosidase with high transglycosylation activity and another hypothetical β-glucosidase in *Aspergillus oryzae* HML366 and enzymatic characterization, Appl. Biochem. Biotechnol., **169**, 870–884 (2013).
30. **Langston, J., Sheehy, N., and Xu, F.:** Substrate specificity of *Aspergillus oryzae* family 3 beta- glucosidase, Biochim. Biophys. Acta, **1764**, 972–978 (2006).
31. **Kaya, M., Ito, J., Kotaka, A., Matsumura, K., Bando, H., Sahara, H., Ogino, C., Shibasaki, S., Kuroda, K., Ueda, M., Kondo, A., and Hata, Y.:** Isoflavone aglycones production from isoflavone glycosides by display of β-glucosidase from *Aspergillus oryzae* on yeast cell surface, Appl. Microbiol. Biotechnol., **79**, 51–60 (2008).
32. **Minetoki, T., Tsuboi, H., Koda, A., and Ozeki, K.:** Development of high expression system with the improved promoter using the *cis*-acting element in *Aspergillus* species, J. Biol. Macromol., **3**, 89–96 (2003).
33. **Horii, K., Adachi, T., Matsuda, T., Tanaka, T., Sahara, H., Shibasaki, S., Ogino, C., Hata, Y., Ueda, M., and Kondo, A.:** Improvement of isoflavone aglycones production using β-glucosidase secretory produced in recombinant *Aspergillus oryzae*, J. Mol. Catal. B: Enzym., **59**, 297–301 (2009).
34. **Ito, J., Sahara, H., Kaya, M., Hata, Y., Shibasaki, S., Kawata, K., Ishida, S., Ogino, C., Fukuda, H., and Kondo, A.:** Characterization of yeast cell surface displayed *Aspergillus oryzae* β-glucosidase 1 high hydrolytic activity for soybean isoflavone, J. Mol. Catal. B: Enzym., **55**, 69–75 (2008).
35. **Sato, H., Toyoshima, Y., Shintani, T., and Gomi, K.:** Identification of potential cell wall component that allows Taka-amylase A adsorption in submerged cultures of *Aspergillus oryzae*, Appl. Microbiol. Biotechnol., **92**, 961–969 (2011).
36. **Gomi, K., Iimura, Y., and Hara, S.:** Integrative transformation of *Aspergillus oryzae* with a plasmid containing the *Aspergillus nidulans argB* gene, Agric. Biol. Chem., **51**, 2549–2555 (1987).
37. **Laemmli, U. K.:** Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, **227**, 680–685 (1970).
38. **Riou, C., Salmon, J. M., Vallier, M. J., Gunata, Z., and Barre, P.:** Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*, Appl. Environ. Microbiol., **64**, 3607–3614 (1998).
39. **Nelson, N.:** A photometric adaptation of the Somogyi method for the determination of glucose, J. Biol. Chem., **153**, 375–380 (1944).
40. **Somogyi, M.:** Notes on sugar determination, J. Biol. Chem., **195**, 19–23 (1952).
41. **Lineweaver, H. and Burk, D.:** The determination of enzyme dissociation constants, J. Am. Chem. Soc., **56**, 658–666 (1934).
42. **Kudou, S., Fleury, Y., Welti, D., Magnolato, D., Uchida, T., Kitamura, K., and Okubo, K.:** Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill), Agric. Biol. Chem., **55**, 2227–2233 (1991).
43. **Bhatia, Y., Mishra, S., and Bisaria, V. S.:** Purification and characterization of recombinant *Escherichia coli*-expressed *Pichia etchellsii* β-glucosidase II with high hydrolytic activity on sophorose, Appl. Microbiol. Biotechnol., **66**, 527–535 (2005).
44. **Odoux, E., Chauwin, A., and Brillouet, J. M.:** Purification and characterization of vanilla bean (*Vanilla planifolia* Andrews) β-D-glucosidase, J. Agric. Food Chem., **57**, 3168–3173 (2003).

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45. **Kitamoto, N., Go, M., Shibayama, T., Kimura, T., Kito, Y., Ohmiya, K., and Tsukagoshi, N.:** Molecular cloning, purification and characterization of two endo-1,4-β-glucanases from *Aspergillus oryzae* KBN616, Appl. Microbiol. Biotechnol., **46**, 538–544 (1996).
46. **Matsumura, K., Obata, H., Hata, Y., Kawato, A., Abe, Y., and Akita, O.:** Isolation and characterization of a novel gene encoding α-L-arabinofuranosidase from *Aspergillus oryzae*, J. Biosci. Bioeng., **98**, 77–84 (2004).
47. **Murray, P., Aro, N., Collins, C., Grassick, A., Penttilä, M., Saloheimo, M., and Tuohy, M.:** Expression in *Trichoderma reesei* and characterization of a thermostable family 3 β-glucosidase from the moderately thermophilic fungus *Talaromyces emersonii*, Protein Expr. Purif., **38**, 248–257 (2004).
48. **Karkehabadi, S., Helmich, K. E., Kaper, T., Hansson, H., Mikkelsen, N. E., Gudmundsson, M., Piens, K., Fujdala, M., Banerjee, G., Scott-Craig, J. S., and other 3 authors:** Biochemical characterization and crystal structures of a fungal family 3 β-glucosidase, Cel3A from *Hypocrea jecorina*, J. Biol. Chem., **289**, 31624–31637 (2014).
49. **Suzuki, K., Sumitani, J., Nam, Y., Nishimaki, T., Tani, S., Wakagi, T., Kawaguchi, T., and Fushinobu, S.:** Crystal structures of glycoside hydrolase family 3 β-glucosidase 1 from *Aspergillus aculeatus*, Biochem. J., **452**, 211–221 (2013).
50. **Eyzaguirre, J., Hidalgo, M., and Leschot, A.:** β-Glucosidases from filamentous fungi: properties, structure, and applications, pp. 645–686, in: Yarema, K. J. (Ed.), Handbook of carbohydrate engineering. CRC Press, Boca Raton, FL (2005).

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