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Characterization of Three Extracellular β-Glucosidases Produced by a Fungal Isolate *Aspergillus* sp. YDJ14 and Their Hydrolyzing Activity for a Flavone Glycoside

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A cellulolytic fungus, YDJ14, was isolated from compost and identified as an *Aspergillus* sp. strain. Three extracellular β-glucosidases, BGL-A1, BGL-A2, and BGL-A3, were separated using ultrafiltration, ammonium sulfate fractionation, and High-Q chromatography. The molecular masses of the three enzymes were estimated to be 100, 45, and 40 kDa, respectively, by SDS-PAGE. The optimum pH and temperature of BGL-A3 were 5.0 and 50°C, respectively, whereas the optimum pH and temperature of BGL-A1 and BGL-A2 were identical (4.0 and 60°C, respectively). The half-life of BGL-A3 at 70°C (2.8 min) was shorter than that of BGL-A1 and BGL-A2 (12.1 and 8.8 min, respectively). All three enzymes preferred *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) and hardly hydrolyzed cellobiose, suggesting that these enzymes were aryl β-glucosidases. The K_m of BGL-A3 (1.26 mM) for *p*NPG was much higher than that of BGL-A1 and BGL-A2 (0.25 and 0.27 mM, respectively). These results suggested that BGL-A1 and BGL-A2 were similar in their enzymatic properties, whereas BGL-A3 differed from the two enzymes. When tilianin (a flavone glycoside of acacetin) was reacted with the three enzymes, the inhibitory activity for monoamine oxidase, a target in the treatment of neurological disorders, was similar to that shown by acacetin. We conclude that these enzymes may be useful in the hydrolysis of flavone glycosides to improve their inhibitory activities.

Keywords: *Aspergillus* sp. YDJ14, extracellular β-glucosidases, aryl β-glucosidases, flavone glycoside hydrolysis

Introduction

β-Glucosidases (E.C. 3.2.1.21) catalyze the hydrolysis of β-1,4-glycosidic linkages to release nonreducing terminal glucosyl residues from disaccharides, oligosaccharides, and alkyl or aryl β-glucosides [1, 2]. According to their substrate specificity, enzymes are divided into three groups: (i) aryl β-glucosidases, which hydrolyze only aryl β-glucosidic linkages; (ii) cellobiases, which hydrolyze cellobiose; and (iii) broad-substrate specificity β-glucosidases, which hydrolyze a wide range of substrates with different bonds [3, 4]. Most of the β-glucosidases are broad specificity β-glucosidases [2].

Fungal β-glucosidases are used in bioflavorings and in the development production of novel carbohydrate foods, animal feeds, bioethanol, and pharmaceuticals [4–6]. They are also employed in the hydrolysis of isoflavone glycosides [7]. Recently, fungal β-glucosidase from several *Aspergillus* species, including *A. fumigatus* [8], *A. saccharolyticus* [9], *A. niger* [10], *A. ochraceus* [11], *A. oryzae* [12], *A. terreus* [13], and *Aspergillus* sp. [15], have been characterized. The production of active β-glucosidases from microbial compost communities has also been studied [16].

Many researchers have studied the hydrolysis of flavonoid

glycosides by β-glucosidases, with the majority of studies focusing on isoflavone glycosides from soybean [17–19], and flavanone glycosides from citrus extracts [20]. However, little information is available on flavone glycosides.

Previous research demonstrated that acacetin, a flavonoid, was a potent inhibitor of monoamine oxidases (MAOs), catalyzing the oxidative deamination of monoamine neurotransmitters and serving as a target for the treatment for neurological disorders [21]. The same study showed that the inhibitory activity of tilianin, a glycoside of acacetin, was lower than that of acacetin.

In this report, three β-glucosidases were separated from *Aspergillus* sp. YDJ14, a cellulolytic fungal strain isolated from compost, and their biochemical properties were characterized. The potential applications of these β-glucosidases in the hydrolysis of flavone glycosides and in improving the inhibitory activity of a pharmaceutical enzyme were also investigated.

Materials and Methods

Chemicals and Enzymes

Glucose, cellobiose, xylobiose, *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-β-D-cellobioside (*p*NPC), *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), 4-methylumbelliferyl-β-D-glucopyranoside (MUG), carboxymethyl-cellulose (CMC), barley β-glucan, birchwood xylan, dinitrosalicylic acid (DNS), trypan blue, and other chemicals were purchased from Sigma-Aldrich (USA). Acacetin and tilianin (acacetin 7-glucoside) were isolated from *Agastache rugosa*, and MAO inhibition was assayed using recombinant human MAO-A, as described previously [21].

Isolation and Identification of the Fungal Strain

A compost sample was obtained from the Compost Factory [22], and cellulolytic fungi were isolated on PDA (potato dextrose agar) plates containing 0.4% CMC (PDA/CMC) or xylan (PDA/xylan), as described previously [15]. Based on the halo size around the colony, a strain was selected and named YDJ14. The isolated strain was identified by 18S rRNA sequencing by Solgent (Korea). Sequence similarity was searched, and a phylogenetic tree was constructed using the BLASTN program [23, 24] on the NCBI web site. The nucleotide sequence of the YDJ14 isolate was deposited in GenBank under the accession number MG976613.

Enzyme Production and Isolation

The isolate was cultured at 30°C, with shaking at 150 rpm in 200 ml of potato dextrose broth, as described previously [15], with some modifications (*i.e.*, 21 days of culture to analyze the extracellular β-glucosidase activity in the culture supernatant). The proteins were separated by ultrafiltration, ammonium sulfate fractionation (40–60%), and High-Q column chromatography, as described previously [15]. The concentrations of protein were analyzed by the Bradford method [26]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 11.5 % polyacrylamide gels [27].

Activity Staining

Activity staining for β-glucosidase activity in active High-Q chromatography fractions was performed in a gel after SDS-PAGE and renaturation, as previously described, except for the use of MUG instead of MUC [15, 28].

Enzyme Assay

The activity of β-glucosidase was assayed in a reaction mixture of 2 mM *p*NPG in 50 mM sodium citrate buffer (pH 5.0) as substrate, with the reaction at 50°C stopped after 30 min [15]. One unit of β-glucosidase activity was defined as the amount of enzyme that generated 1 μmol of *p*-nitrophenol in 1 min. Substrate specificity was analyzed using 0.2 mM *p*NPG, *p*NPC, *p*NPX, cellobiose, and xylobiose as small substrates, and 0.5% CMC, β-glucan, and xylan as polysaccharides. The substrates *p*NPG, *p*NPC and *p*NPX were analyzed by measuring the amounts of *p*-nitrophenol released. The others were analyzed by measuring the amounts of reducing sugar released using the DNS method [29]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per minute under the conditions as above.

Biochemical Characterization of the Purified Enzyme

The optimum pH for enzyme activity was analyzed using 50 mM universal buffer (from pH 3.0 to 8.0), and the optimum temperature was determined from 30°C to 80°C in 50 mM of sodium citrate buffer (pH 5.0). Thermostability was analyzed by preincubation of the enzyme without substrate for up to 1 h at 60°C or 70°C. The effects of various cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+}, and Ba^{2+}) at 2.0 mM, and 10% isopropanol, 1% Triton X-100, and 1% SDS on enzyme activity were determined. The K_m and V_{max} values for the enzymes were determined using Lineweaver-Burk plots at five concentrations of *p*NPG (0.1–4.5 mM) at 50°C.

Binding Analysis with Insoluble Avicel

The binding properties of the enzymes to carbohydrate were analyzed as described previously, with slight modifications [30]. Briefly, the crude extract was mixed with 1.5% Avicel in 0.3 ml of 50 mM sodium citrate buffer, pH 5.0, by shaking for 40 min on ice. The mixtures were then centrifuged. The amount of unbound enzyme was determined by measuring the cellulase activity in the supernatant.

Hydrolysis of a Flavone Glycoside and Analysis of MAO-A Inhibitory Activity

Tilianin (100 μM), a flavone glycoside, was reacted for 6 h at 50°C with approximately 0.1 U of each β-glucosidase in 50 μl of

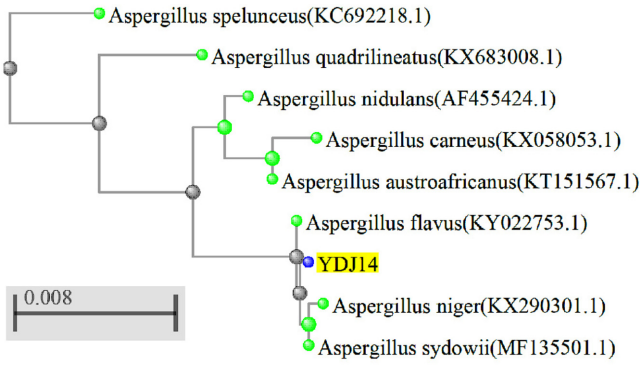

Fig. 1. Phylogenetic tree of the strain YDJ14.

0.1 M sodium phosphate buffer (pH 6.0) [15]. After boiling for 5 min and centrifugation, the supernatant was added to 450 µl of MAO-A assay mixture in 0.1 M sodium phosphate, pH 7.4, making 10 µM of tilianin, and the absorbance was measured at 316 nm, as described previously [21]. At the same time, the absorbances of a control containing MAO-A only, an inhibitory control containing MAO-A and 10 µM of acacetin or tilianin, and incubated inhibitory controls (MAO-A and each flavone, and β-glucosidase but not reacted) were measured.

Results

Isolation of Fungal Strains

When the sample was grown on PDA/CMC or PDA/xylan, four isolates showed both CMC- and xylan-degrading activities. The isolate showing the largest halo on the plate was selected and named YDJ14. The 18S rRNA sequence of the YDJ14 isolate showed 99% similarity with many other *Aspergillus* sp. strains, such as *A. flavus* (KY022753.1), *A. niger* (KX290301.1), *A. sydowii* (MF135501.1), and *A. nidulans* (AF455424.1), and showed 98% similarity with *A. carneus* (KX058053.1) and *A. austroafricanus* (KT151567.1) in the NCBI server. A phylogenetic tree was constructed, as illustrated in Fig. 1. The isolate was named *Aspergillus* sp. YDJ14.

Production and Separation of β-Glucosidases

The *Aspergillus* sp. YDJ14 isolate produced the maximum amount of β-glucosidase at 21 days after inoculation. When the culture supernatant was concentrated to approximately 20-fold, the yield was 85.3% (Table 1). High-Q chromatography of the ammonium sulfate fractionate showed major (fractions from 36 to 40) and minor (fractions from 46 to 48) activity peaks (Fig. 2A). In SDS-PAGE analysis, the fractions in the major peak exhibited two kinds of protein bands at each side of the maximum peak (fraction 38); a protein band from fraction 37 (100 kDa) and a protein band from fraction 40 (45 kDa). The protein band from fraction 47 in the minor peak was 40 kDa (left of Fig. 2B). The three enzymes were named BGL-A1, BGL-A2, and BGL-A3. Zymogram analysis of the fractions following activity staining using MUG after SDS-PAGE and renaturation revealed an active band that matched that of BGL-A3, whereas BGL-A1 and BGL-A2 did not produce active bands (right of Fig. 2B). The molecular mass of this band corresponded to 40 kDa. When the MUG-hydrolyzing activity was measured in a liquid-type assay, the intensity of BGL-A1 was greater than that of BGL-A2 and BGL-A3, which was contrary to expectations, based on the results of activity staining on the gel (Fig. 2C).

Characterization of the β-Glucosidases

The optimum pH values of BGL-A1 and BGL-A2 were identical (pH 4.0). The optimum pH of BGL-A3 was pH 5.0. The optimum temperatures of BGL-A1 and BGL-A2 were also identical (60°C), whereas that of BGL-A3 was 50°C. In the thermostability experiments, BGL-A3 was almost inactivated after 30 min at 70°C, whereas BGL-A1 and BGL-A2 remained stable, with more than 70% of residual

Table 1. Separation of three extracellular β-glucosidases from *Aspergillus* sp. YDJ14.

Procedure	Fraction number	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	-	326	253.3	15.29	16.6	1	100
Ultrafiltration	-	16	216.0	3.65	59.2	3.57	85.3
$(NH_4)_2SO_4$ fractionate (40–60%)	-	1	63.9	1.50	42.6	2.57	25.2
High-Q chromatography	37 (A1)	1	5.87	0.019	308.9	18.6	2.32
	40 (A2)	1	1.62	0.017	95.3	5.74	0.64
	47 (A3)	1	1.63	0.0084	194.0	11.7	0.64

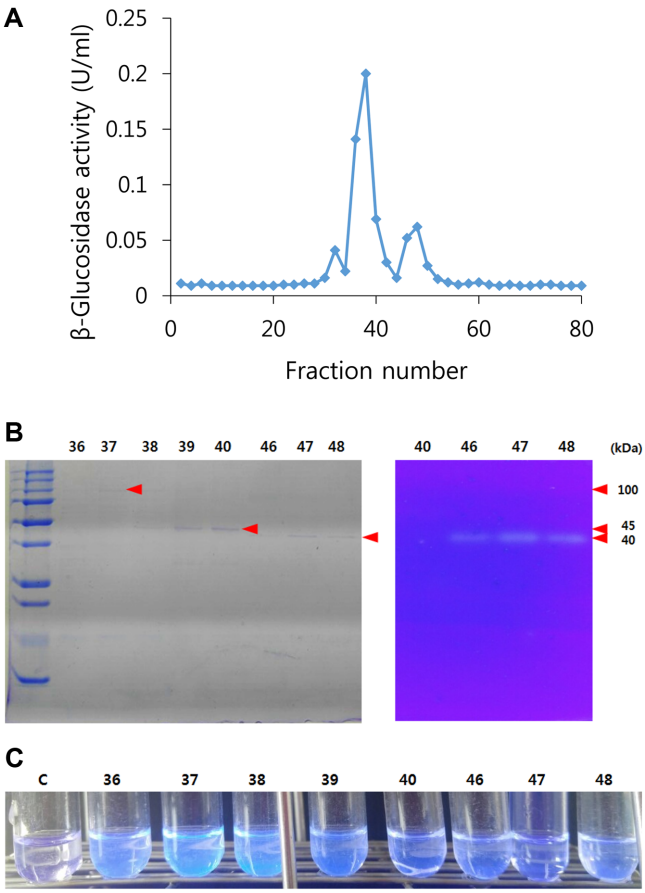

Fig. 2. Analysis of fractions eluted by High-Q chromatography. (**A**) Activity profile. (**B**) SDS-PAGE of active High-Q chromatography fractions (left) and a zymogram obtained after SDS-PAGE/renaturation of the fractions (right). (**C**) Semi-quantitative MUG-hydrolyzing activity assay of the fractions monitored photographically after a reaction with 1 mM MUG for 10 min at 50°C. The numbers on the tops of the figures represent the fractions obtained by High-Q chromatography.

activities. The half-life of BGL-A3 at 70°C (2.8 min) was shorter than that of BGL-A1 and BGL-A2 (12.1 and 8.8 min, respectively). The activities of BGL-A1, BGL-A2, and BGL-A3 were not affected significantly by the divalent ions at 2.0 mM and 10% isopropanol, 1% Triton X-100, and 1% SDS, but BGL-A3 was inhibited by 15.2% and 26.8% in the presence of Ba^{2+} and SDS, respectively (data not shown).

Table 2. Substrate specificities of the three extracellular β-glucosidases isolated from *Aspergillus* sp. YDJ14

	Relative activity (%)*		
	BGL-A1	BGL-A2	BGL-A3
*p*NPG	100	100	100
*p*NPC	0.08 ± 0.01	nd	nd
*p*NPX	nd	nd	nd
Cellobiose	0.4 ± 0.01	1.28 ± 0.17	0.49 ± 0.34
β-Glucan	0.88 ± 0.14	1.01 ± 0.36	0.47 ± 0.70
CMC	nd	nd	nd
Xylan	nd	0.14 ± 0.091	nd
Xylobiose	nd	nd	nd

*Relative activities were calculated on the basis of the specific activities in U/mg protein. Enzyme activities were analyzed using 0.2 mM *p*NPG, *p*NPC, *p*NPX, xylobiose, and cellobiose owing to the high blank value. For polysaccharides, 0.5% CMC, β-glucan, and xylan were used. nd, not detectable.

Among the substrates tested, *p*NPG was the most efficiently hydrolyzed by BGL-A1, BGL-A2, and BGL-A3 (Table 2). The hydrolyzing activities of the three enzymes for *p*NPC, *p*NPX, xylobiose, and cellobiose were negligible or not detectable. The hydrolyzing activities for polysaccharides, such as β-glucan, CMC, and xylan were also negligible or not detectable.

Lineweaver-Burk plots for BGL-A1, BGL-A2, and BGL-A3 were linear (Fig. 3). The K_m values of BGL-A1 and BGL-A2 were similar (0.25 and 0.27 mM, respectively). In contrast, the K_m value of BGL-A3 (1.26 mM) was approximately five times higher than that BGLA1and BGL-A2 (Table 3). The V_{max} of BGL-A1 (131.0 U/mg) was similar to that of BGL-A2 (101.1 U/mg) but lower than that of BGL-A3 (213.5 U/mg). The catalytic efficiency of BGL-A1 (524.0 U/mg·mM) expressed by V_{max}/K_m was higher than that of BGL-A2 (374.4 U/mg·mM) and BGL-A3 (169.4 U/mg·mM).

Binding Properties to Avicel

To investigate the abilities of the three enzymes to bind to carbohydrate, the residual enzyme activities of the supernatants were measured after binding to Avicel. None

Table 3. Kinetic parameters of the three extracellular β-glucosidases from *Aspergillus* sp. YDJ14.

	K_m (mM)	V_{max} (U/mg protein)	V_{max}/K_m (U mg protein^{-1} mM^{-1})
BGL-A1	0.25 ± 0.030	131.0 ± 8.5	524.0
BGL-A2	0.27 ± 0.067	101.1 ± 4.3	374.4
BGL-A3	1.26 ± 0.001	213.5 ± 5.5	169.4

BGL-A1, BGL-A2, and BGL-A3 were assayed using fractions 36 (119.4 U/mg protein), 39 (91.8 U/mg protein), and 47 (194.0 U/mg protein), respectively.

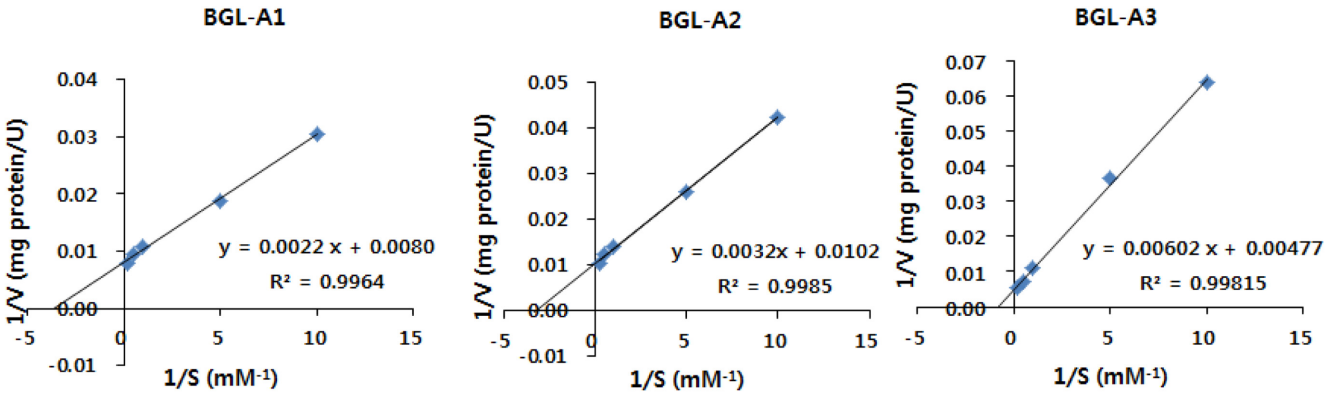

Fig. 3. Lineweaver-Burk plots of BGL-A1, BGL-A2, and BGL-A3.

of the enzymes bound to Avicel at a range of 95.4–103.3% (data not shown).

Hydrolysis of a Flavone Glycoside and Analysis of MAO-A Inhibitory Activity

When MAO-A was reacted in the presence of 10 μM acacetin or tilianin, the residual activities were 3.5% or 90.7%, respectively (Fig. 4). However, after tilianin was reacted with BGL-A1, BGL-A2, or BGL-A3, and the tilianin mixture was added to the MAO-A reaction mixtures, the residual activities of MAO-A decreased markedly to 12.8%, 14.1%, and 13.3%, respectively, closely resembling the value obtained for acacetin (Fig. 4). In this experiment, the control containing enzyme but not reacted was included, because the two reaction buffers used for glycoside hydrolysis and the MAO-A assay were different. In the controls, activities decreased to 62.8%, 59.7%, and 46.5%, probably due to the presence of reactants in the hydrolyzed glycoside mixture. However, the activities of the controls were much higher than those after the hydrolysis reaction. These results strongly suggested that BGL-A1, BGL-A2, and BGL-A3 cleaved a glycosidic linkage in the flavone glycoside, yielding an aglycone flavonoid, acacetin.

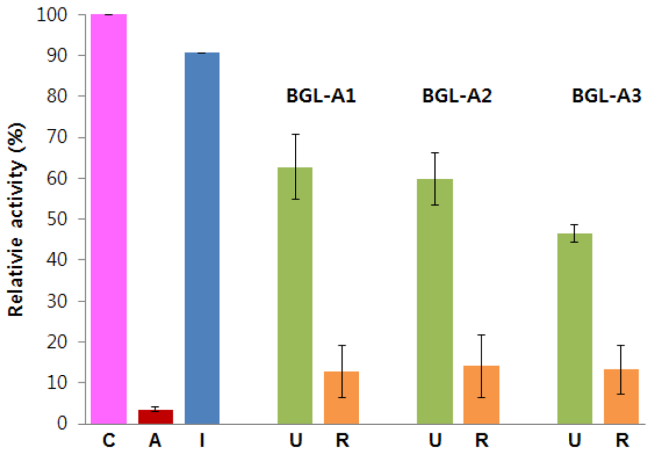

Fig. 4. Inhibition of MAO-A activity after reacting tilianin, a flavone glycoside, with BGL-A1, BGL-A2, and BGL-A3.
C, Control containing only MAO-A; I, inhibitory control in the presence of tilianin (10 μM); U, unreacted controls containing tilianin and each enzyme (*i.e.*, I + unreacted mixtures); R, reacted for 6 h at 50°C with each enzyme (*i.e.*, I + reacted mixtures). The glycoside hydrolyzing mixture (50 μl) contained a 10 times higher glycoside concentration (100 μM of tilianin) than the working concentration in the MAO-A assay mixture and approximately 0.1 U of each enzyme. After hydrolysis, each reacted mixture was added to the MAO-A assay mixtures (450 μl).

Discussion

YDJ14 isolated from compost showed greater cellulolytic activity than other isolates on the PDA plate. Based on its 18S rRNA sequence, YDJ14 was assigned to the *Aspergillus* genus and named *Aspergillus* sp. YDJ14. The *Aspergillus* sp. YDJ14 isolate grew more slowly and produced β-glucosidase later (after 21 days) than *Aspergillus* sp. YDJ216 (after 15 days) in shaking culture [15].

The proteins were separated by High-Q chromatography, and major and minor activity peaks were analyzed by SDS-PAGE. SDS-PAGE of the major peak revealed two protein bands, which appeared in a gradient manner before and behind the maximum peak. The molecular masses of the proteins in fractions 37 and 40 corresponded to 100 (BGL-A1) and 45 kDa (BGL-A2), respectively (Fig. 2B). The molecular mass of the protein in the minor peak corresponded to 40 kDa (BGL-A3). In total, three β-glucosidase isoenzymes were separated from this strain.

Molecular masses of extracellular β-glucosidases are diverse [6]. Similar to this *Aspergillus* sp. YDJ14 isolate, previous studies reported isoenzymes of β-glucosidase produced from several strains of *Aspergillus* species, including isozymes of approximately 78 and 43 kDa from *A. ochraceus* MTCC 1810 [11]; 130, 120, and 100 kDa from *A. oryzae* [12]; 125 and 50 kDa from *A. nidulans* [31]; and 97 and 45 kDa from *Aspergillus* sp. YDJ216 [15]. BGL-A3 is the smallest isozyme (40 kDa) of the reported fungal β-glucosidases, close to that (43 kDa) from *A. ochraceus* [11].

As shown by the zymogram analysis of fractions in the gel, BGL-A3 was active, suggesting that it may be more resistant to denaturation/renaturation than BGL-A1 and BGL-A2. However, contrary to this idea, the MUG-hydrolyzing activity in a liquid assay of BGL-A1 was greater than that of BGL-A2 and BGL-A3. Although the reason for this finding is unclear, some factor(s) may possibly be lost or modified during SDS-PAGE and the denaturation/renaturation step [15].

In the present study, BGL-A1 and BGL-A2 had the same optimum pH (*i.e.*, pH 4.0), whereas the optimum pH of BGL-A3 was different (*i.e.*, pH 5.0). These values are similar to those of most microbial β-glucosidases reported thus far [5]. The optimum temperature of BGL-A3 (50°C) was different from that of BGL-A1 and BGL-A2 (60°C), but similar to or higher than the temperatures reported for most other fungal β-glucosidases. The optimum temperature of BGL-A3 was lower than those (65–70°C) of *A. niger* and *A. terreus* β-glucosidases, and those (~70°C) of β-glucosidases from other species, including *Trichoderma reesei* [2, 6].

BGL-A1, BGL-A2, and BGL-A3 showed absolute substrate specificity for *p*NPG but negligible activities for other substrates, including cellobiose. This finding suggests BGL-A1, BGL-A2, and BGL-A3 are typical aryl β-glucosidases, like those from *A. sydowii* BTMFS 55 [32], *Neosartorya fischeri* NRRL181 [33], *Phialophora* sp. G5 [34], and *Aspergillus* sp. YDJ216 [15]. The specificity of aryl β-glucosidases may be caused by their affinity for the aglycone moiety and their high electrophilicity, which would increase the stability of the intermediate generated during catalysis, as suggested by Plant *et al*. [35].

The K_m and V_{max} of BGL-A3 for *p*NPG differed from those of BGL-A1 and BGL-A2. Among the three β-glucosidases, the specificity constant expressed by V_{max}/K_m of BGL-A3 was the lowest. However, there was no difference in the binding properties of the three β-glucosidases to a carbohydrate, Avicel.

The antioxidant, anticancer, anti-hypercholesterolemia, anti-inflammatory, and antihypertensive activities of microbial β-glucosidases via hydrolysis of flavonoid glycosides have been investigated [4]. The hydrolysis of soybean isoflavones, ginsenosides, and geniposides by β-glucosidases from several strains, including *A. terreus* [13], *Neosartorya fischeri* [19], *A. niger* [14, 36], *Neurospora crassa* [37], *Paecilomyces* Bainier sp. 229 [38], and *A. versicolor* [39], have been reported.

However, little information is available on the improvement of inhibitory activity by glycoside hydrolysis against pharmaceutical enzymes, especially MAO. In this study, we investigated the hydrolysis of a flavone glycoside, tilianin, by BGL-A1, BGL-A2, and BGL-A3, and the subsequent effect on the inhibition of MAO-A activity. After hydrolyzing tilianin with the three β-glucosidases, MAO-A was significantly inhibited following the addition of the reacted mixtures. The hydrolysis and identification of the reaction products require further study. We suggest that the production of acacetin, which potently inhibits MAO-A (IC_{50} = 0.19 μM), may be responsible for the inhibitory activity against MAO-A, and that the three β-glucosidases from *Aspergillus* sp. YDJ14 efficiently cleave the glycosidic linkage of the flavone glycoside.

In this study, three extracellular β-glucosidases, BGL-A1, BGL-A2, and BGL-A3, were separated from *Aspergillus* sp. YDJ14. The molecular masses of BGL-A1, BGL-A2, and BGL-A3 were 100, 45, and 43 kDa, respectively, and all three enzymes were strict aryl β-glucosidases. These isozymes can be investigated in further study by analysis of N-terminal sequences and cloning of their genes. BGL-A1 and BGL-A2 showed many similar enzymatic properties, such as optimum pH, temperature, thermostability, and K_m values for *p*NPG, whereas the enzymatic properties of BGL-A3 differed from those of the other two enzymes. The three β-glucosidases effectively hydrolyzed tilianin, a flavonoid glycoside, and the reaction products showed inhibitory activity against MAO-A. The results suggest that hydrolysis of the flavone glycoside by these enzymes offers a means of improving inhibition of the pharmaceutical enzyme.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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