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Characterization of a GH3 Family β-Glucosidase from *Dictyoglomus turgidum* and Its Application to the Hydrolysis of Isoflavone Glycosides in Spent Coffee Grounds

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ABSTRACT: A recombinant β-glucosidase from *Dictyoglomus turgidum* was purified with a specific activity of 31 U/mg by His-Trap affinity chromatography. *D. turgidum* β-glucosidase was identified as a memmber of the glycoside hydrolase (GH) 3 family on the basis of its amino acid sequence. The native enzyme existed as an 86 kDa monomer with an activity maximum at pH 5 and 85 °C with a half-life of 334 min. The hydrolytic activity of the enzyme with aryl-glycoside substrates was the highest for *p*-nitrophenyl (*p*NP)-β-D-glucopyranoside (with a K_m of 1.3 mM and a k_{cat} of 13900 1/s), followed by *o*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside, and *p*NP-β-D-galactopyranoside. However, no activity was observed for *o*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-β-L-arabinopyranoside, and *p*NP-α-L-rhamnopyranoside. The hydrolytic activity of the β-glucosidase for coffee isoflavones followed the order genistin (with a K_m of 0.67 mM and a k_{cat} of 5750 1/s) > daidzin > ononin > glycitin. The concentrations of daidzin in ground coffee and spent coffee grounds were 160 and 107 μg/g, respectively, but other isoflavones were present at low concentrations or absent. The enzyme completely hydrolyzed 1.2 mM daidzin in spent coffee grounds after 2 h, with a productivity of 0.6 mM/h. This is the first report concerning the enzymatic hydrolysis of isoflavone glycosides in spent coffee grounds.

KEYWORDS: β-glucosidase, *Dictyoglomus turgidium*, isoflavone hydrolysis, daidzein, spent coffee grounds

INTRODUCTION

The extreme thermophile *Dictyoglomus turgidum* is an anaerobic organism that grows on a broad range of carbohydrate substrates, including starch, cellulose, pectin, and carboxymethylcellulose.[1] Thus, this strain is used as a powerful enzyme source for carbohydrate hydrolysis. Recently, the whole genome of *D. turgidum* was sequenced (GenBank accession no. NC_011661), and two β-glucosidase genes were reported. One of the β-glucosidases, which belonged to the glycoside hydrolase (GH) 1 family on the basis of its amino acid sequence, was previously well characterized. However, the other β-glucosidase has not been yet characterized.

Isoflavones are one type of plant phytochemicals and the structural homologues of estrogens[2] and exist as glycoside forms most frequently found in leguminous plants. Isoflavones exhibit a variety of beneficial activities against osteoporosis, cancer, cardiovascular disease, and menopausal symptoms.[3−5] Many studies have focused on the hydrolysis of isoflavone glycosides, as isoflavone aglycones possess higher pharmaceutical activity than isoflavone glycosides.[6,7] Isoflavone aglycones have been obtained by hydrolysis reactions of microorganisms and β-glucosidases. However, microbial hydrolysis has resulted in low yield and poor productivity. Thus, the microbial β-glucosidases from *Aspergillus oryzae*,[8] *Bacillus subtilis*,[9] *Bifidobacterium lactis*,[10] *Lactobacillus acidophilus*,[10] *Lactobacillus casei*,[10] *Escherichia coli*,[11] *Paecilomyces thermophila*,[12] *Pseudomonas* sp.,[13] and *Thermotoga maritima*[14] and the leguminous β-glucosidases from *Dalbergia cochinchinensis*,[15] *Dictyoglomus nigrescens*,[15] and *Glycine max* (soybean)[16] have been proposed and applied as potential tools for the hydrolysis of isoflavone glycosides.

Generally, the enzymes originating from thermophilic microorganisms tend to be more active and stable at higher temperatures than those obtained from mesophilic microorganisms. Additionally, thermostable enzymes have many advantages in industrial applications, including high reaction velocity, resistance to chemical denaturation, reduced risk of contamination, and high substrate solubility.[17] Therefore, thermostable β-Xglucosidases have considerable industrial potential for the hydrolysis of isoflavone glycosides.

The coffee industry grows yearly and accounts for a large proportion of the agricultural products industry. Each year, a vast quantity of spent coffee grounds is produced. The hydrophobic isoflavones in the brewed coffee are eluted by water with a low yield, and much of this content remains in spent coffee grounds.[18] However, these spent grounds have been used only as an air freshener and in composts. For the recycling of spent coffee grounds, isoflavones in spent coffee grounds should be transformed into isoflavone glycosides.

In this study, a thermostable β-glucosidase from *D. turgidum* was cloned and its biochemical properties, such as optimal pH and temperature, thermostability, and substrate specificity, were investigated. The β-glucosidase was applied for the hydrolysis of the isoflavone glycoside daidzin in spent coffee grounds to the isoflavone aglycon daidzein.

MATERIALS AND METHODS

Materials. The isoflavone standards, including genistin, daidzin, glycitin, ononin, daidzein, glycitein, genistein, and formononein, and the

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aryl-glycoside substrates, including, *p*-nitrophenyl (*p*NP)-β-D-glucopyranoside, *o*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside, *o*NP-β-D-galactopyranoside *p*NP-α-D-glucopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-β-L-arabinopyranoside, and *p*NP-α-L-rhamnopyranoside, were purchased from Sigma (St. Louis, MO). The spend coffee grounds were purchased from a local Starbucks. HPLC grade acetonitrile and acetic acid were purchased from Fisher Scientific (Hanover Park, IL). All of the restriction enzymes were purchased from New England Biolabs (Hertfordshire, U.K.). All other regents were purchased from Sigma.

Bacterial Strains and Plasmid. The genomic DNAs from *D. turgidum* DSMZ 6724 (DSMZ, Braunschweig, Germany), *E. coli* ER2566 (New England Biolabs, Hertfordshire, UK), and pET28a(+) plasmid (Novagen, Madison, WI) were used as the source of β-glucosidase gene, host cells, and expression vector, respectively. The gene encoding for β-glucosidase was amplified by PCR using the genomic DNA isolated from *D. turgidum* as a template. The sequence of the oligonucleotide primers used for gene cloning was based on the DNA sequence of *D. turgidum* β-glucosidase (GenBank accession no. YP_002352162). Forward (5′- GCTAGCATGAGTGTGGATATAAAAAAGCTCA-3′) and reverse primers (5′- CTCGAGTTAGCTATTAAGTTCTCTCAGTAGGT-3′) were designed to introduce the *Nhe*I and *Xho*I restriction sites (underlined), respectively, and were synthesized by Bioneer (Daejon, Korea). The PCR-amplified DNA fragments were purified and inserted into the pET28a(+) vector digested with the same restriction enzymes. The ER2566 strain of *E. coli* was transformed with the ligation mixture using an electroporator (MicroPulser, Bio-Rad, Hercules, CA) and plated on Luria−Bertani (LB) agar containing 25 μg/mL kanamycin. A kanamycin-resistant colony was selected, and plasmid DNA from the transformant was isolated using a plasmid purification kit (Promega, Madison, WI). DNA sequencing was carried out using a DNA analyzer (ABI Prism 3730xl, Perkin-Elmer, Waltham, MA). Gene expression was evaluated by both SDS-PAGE and enzyme activity assay.

Enzyme Preparation. *E. coli* cells containing the β-glucosidase/pET28a(+) gene from *D. turgidum* were cultivated in a 2000 mL flask containing 500 mL of LB medium and 25 μg/mL kanamycin at 37 °C with shaking at 200 rpm. When the optical density of bacteria reached 0.6 at 600 nm, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM to induce β-glucosidase expression, after which the culture was incubated for 4 h with shaking at 150 rpm at 16 °C as the conditions for obtaining the highest expression level.

The grown cells were subsequently harvested and disrupted by sonication on ice for 2 min in 50 mM phosphate buffer (pH 8) containing 300 mM NaCl with the addition of 1 mg/mL lysozyme. The unbroken cells and cell debris were removed by centrifugation at 13000*g* for 20 min at 4 °C, and the supernatant obtained was used as a crude extract. The enzyme solution of the supernatant was applied to a His-Trap affinity chromatography column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM phosphate buffer (pH 8). The bound protein was subsequently eluted at 4 °C with the same buffer containing 250 mM imidazole at a flow rate of 1 mL/min. The active fractions were collected and dialyzed at 4 °C for 16 h against 50 mM citrate/phosphate buffer (pH 5). The resulting solution was used as a purified enzyme. The purification step using the column was conducted using a fast protein liquid chromatography system (Bio-Rad) in a cold room at 4 °C.

Molecular Mass Determination. The subunit molecular mass of *D. turgidum* β-glucosidase was assessed by SDS-PAGE under denaturing conditions, using the proteins of a prestained ladder (MBI Fermentas, Hanover, MD) as reference proteins. All protein bands were stained with Coomassie blue for visualization. The molecular mass of the native enzyme from *D. turgidum* was determined by gel filtration chromatography using a Sephacryl S-300 HR 16/60 preparative grade column (Amersham Biosciences). The purified enzyme solution of 10 mg/mL obtained from the elution of the His-Trap affinity chromatography column as above-described was applied to the Sephacryl S-300 HR 16/60 column and eluted with 50 mM citrate/phosphate buffer (pH 5) containing 150 mM NaCl at a flow rate of 1 mL/min. The retention time of *D. turgidum* β-glucosidase was measured during the elution process. The standard molecular mass proteins at 10 mg/mL such as thyroglobulin (molecular mass of 669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), albumin (66 kDa), and chymotrypsin (29 kDa) as reference proteins (Amersham Biosciences) were applied to the gel filtration chromatography column and eluted under the same elution conditions of the β-glucosidase. The five reference proteins were identified by the retention time (migration length). The molecular mass of the native enzyme was calculated by comparison with the retention time (migration length) of reference proteins.

Effects of Metal Ions, pH, and Temperature. To investigate the effect of metal ions on enzyme activity, an enzyme assay was carried out after treatment with 1 mM ethylenediaminetetraacetic acid (EDTA) at 4 °C for 1 h or after addition a 1 mM concentration of each metal ion such as Co^{2+}, Mn^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, or Ca^{2+}. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5) containing each metal ion at 85 °C.

To assess the effects of pH and temperature on β-glucosidase activity, pH values were varied from 4.0 to 6.5 using 50 mM citrate/phosphate buffer at 85 °C, and temperatures were varied from 70 to 95 °C at pH 5. The influence of temperature on enzyme stability was monitored as a function of incubation time by placing the enzyme solution at five different temperatures (70, 75, 80, 85, and 90 °C) in 50 mM citrate/phosphate buffer (pH 5). Samples were withdrawn at time intervals and were assayed in 50 mM citrate/phosphate buffer (pH 5) containing 1 mM *p*NP-β-D-glucopyranoside at 85 °C for 5 min. The half-life of the enzyme was calculated using Sigma Plot 9.0 software (Systat Software, San Jose, CA).

Hydrolytic Activity. The hydrolytic activity of *D. turgidum* β-glucosidase was determined using *p*NP-β-D-glucopyranoside, *o*NP-β-D-glucopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-β-L-arabinopyranoside, *o*NP-β-D-galactopyranoside, *p*NP-α-L-rhamnopyranoside, *p*NP-α-D-glucopyranoside, genistin, daidzin, ononin, and glycitin. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5) containing 1 mM aryl-glycoside or 1 mM isoflavone glycoside and 0.02 U/mL enzyme for 5 min at 85 °C. One unit (U) of enzyme activity for aryl-glycoside or isoflavone glycoside was defined as the amount of enzyme required to liberate 1 μmol of *p*NP or 1 μmol of isoflavone aglycone per minute at 85 °C and pH 5, respectively. The specific activity (U/mg) was defined as the produced amount of NP or isoflavone aglycone as a product per enzyme amount per unit reaction time. The productivity (mM/h) was defined as the increase in the concentration of isoflavone aglycone as a product per unit reaction time.

Kinetic Parameters. Various concentrations of *p*NP-β-D-glucopyranoside, *o*NP-β-D-glucopyranoside, genistin, daidzin, ononin, and glycitin (0.1−3 mM) were used to determine the kinetics parameters of the enzyme. The reactions were performed in 50 mM citrate/phosphate buffer (pH 5) at 85 °C for 5 min. The amounts of isoflavone aglycones were detected by the HPLC assay. The K_m (mM) and k_{cat} (1/s) were determined by the Lineweaver−Burk plot from the Michaelis−Menten equation. To calculate the catalytic constant, k_{cat}, the amount of protein was divided by the subunit molecular mass of 85830 Da. Protein concentrations were determined according to the Bradford method using bovine serum albumin as a standard protein.[19]

Hydrolysis of Daidzin to Daidzein. The hydrolysis of daidzin to daidzein by β-glucosidase from *D. turgidum* was assessed using reagent grade daidzin and spent coffee grounds extract. The extraction method for spend coffee grounds was modified on the basis of the extraction method for soy flour.[14] The extraction conditions were optimized by varying solvent kind (methanol, ethanol, and DMSO), methanol concentration

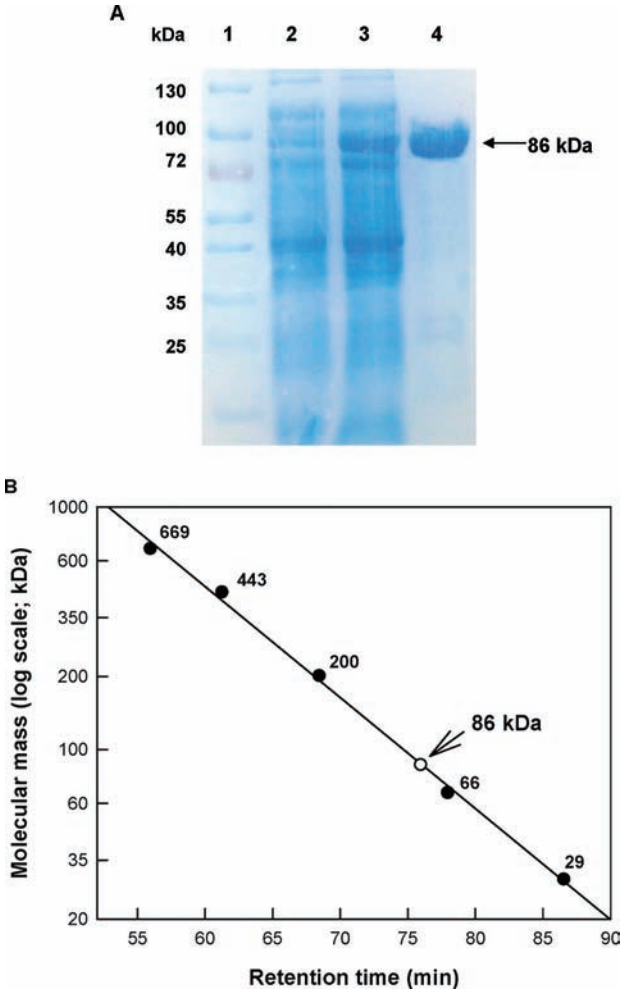

Figure 1. (A) SDS-PAGE of β-glucosidase from *D. turgidum*. Lanes: 1, prestained marker protein (170, 130, 100, 72, 55, 40, 35, and 25 kDa); 2, control strain with plasmid pET 28a(+); 3, crude extract; 4, purified enzyme. (B) Determination of the total molecular mass of β-glucosidase from *D. turgidum* by gel filtration chromatography. Thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), albumin (66 kDa), and chymotrypsin (29 kDa); β-glucosidase from *D. turgidum*.

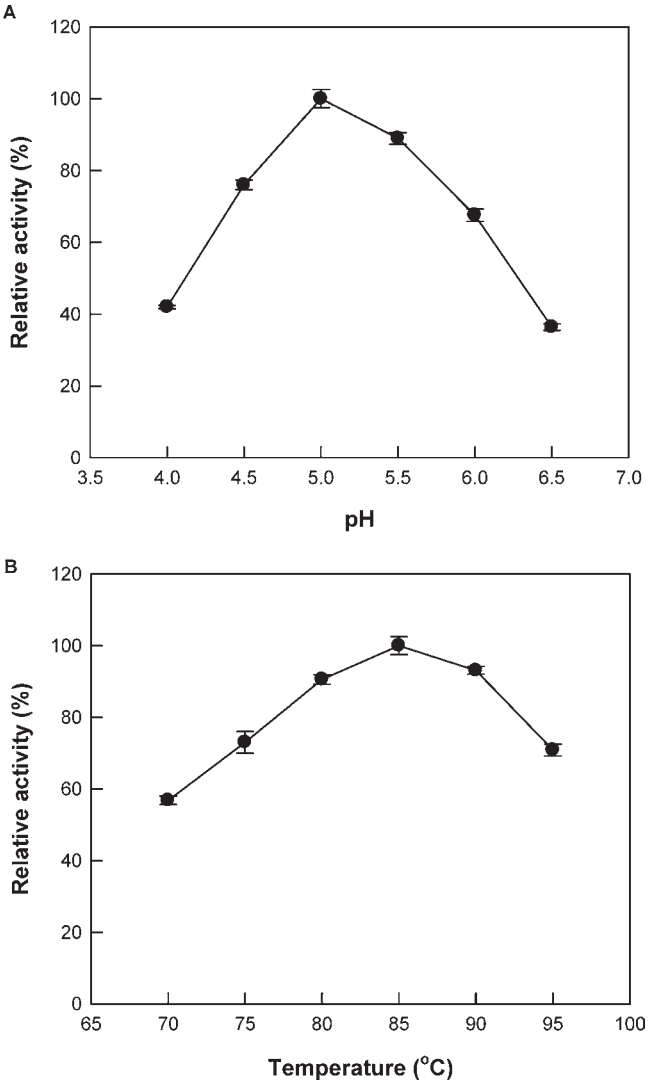

Figure 2. Effects of (A) pH and (B) temperature on the activity of β-glycosidase from *D. turgidum*. The reactions for pH experiments were performed with 1 mM *p*NP-β-D-glucopyranoside and 0.02 U/mL of enzyme at 85 °C for 5 min. The reactions for temperature experiments were performed in 50 mM citrate/phosphate buffer (pH 5) containing 1 mM *p*NP-β-D-glucopyranoside and 0.02 U/mL of enzyme at for 5 min. Data represent the mean of three experiments, and error bars represent standard deviation.

(50−100%), temperature (60−90 °C), and extraction time (1−5 h), and the highest amount of isoflavones was obtained after extraction with 6 volumes of 80% methanol by stirring for 3 h at 80 °C for 3 g of spent coffee grounds. Thus, these conditions were used for extracting spent coffee grounds. The hydrolytic reactions were performed at 85 °C in 50 mM citrate/phosphate buffer (pH 5) containing 16 U/mL of enzyme and 1 mM reagent grade daidzin for 100 min or 62 U/mL of enzyme and 5% (w/v) spent coffee grounds extract for 140 min.

Analytical Methods. Isoflavones were assayed using an HPLC system (Agilent 1100, Santa Clara, CA) equipped with a UV detector at 254 nm and a C18 column (50 × 4.6 mm, YMC, Kyoto, Japan). The column was eluted at 35 °C with a gradient of solvent A (3% acetic acid in water) and solvent B (3% acetic acid in mixture of water and acetonitrile of 50:50 (v/v)) from 75:25 to 40:60 for 12 min and then from 40:60 to 75:25 for 3 min. The flow rate was 1.5 mL/min. The substrates daidzin, glycitin, genistin, and ononin were detected with retention times of 3.4, 3.8, 4.3, and 5.1 min, respectively. The products daidzein, glycitein, genistein, and formononein were detected with retention times of 5.4, 5.8, 7.2, and 8.5 min, respectively. The isoflavones in the reaction samples formed from different substrates and spent coffee grounds were identified as the same retention times with the isoflavone standards. The amounts of isoflavones in spent coffee grounds were determined using linear calibration curves relating the peak areas to the concentrations of isoflavone standards.

RESULTS AND DISCUSSION

Gene Cloning, Purification, and Molecular Mass Determination of β-Glucosidase from *D. turgidum*. A 2247 bp gene encoding for a GH3 family domain protein from *D. turgidum*, with the same sequence as that reported in Genbank (YP_002352162), was cloned and subsequently expressed in *E. coli*. The amino acid sequence of the expressed enzyme evidenced 92, 66, 64, 64, and 33% identities with the GH3 family β-glucosidases from *Dictyoglomus thermophilum* (YP_002251757), *Thermobaculum terrenum* (YP_003321925), *Clostridium stercorarium*

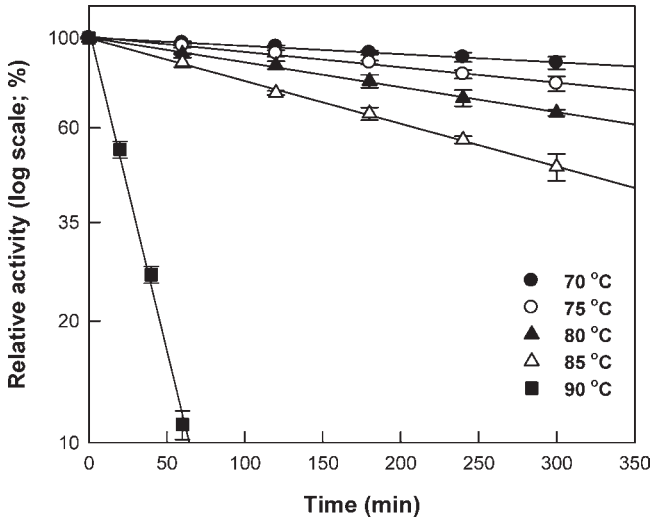

Figure 3. Thermal inactivation of the activity of β-glucosidase from *D. turgidum*. The enzymes were incubated at 70, 75, 80, 85, and 90 °C for various periods of time. A sample was withdrawn at each time interval, and the relative activity was determined. Data represent the mean of three experiments, and error bars represent standard deviation.

(YP_001037681), *Caldicellulosirputor saccharolyticus* (YP_001179905), and *Thermobaculum martima* (NP_227841), respectively. However, β-glucosidases from *D. thermophilum*, *T. terrenum*, and *C. saccharolyticus* have not been yet characterized.

The putative recombinant β-glucosidase from *D. turgidum*, which belongs to the GH3 family, was purified as a soluble protein from crude extract by His-Trap affinity chromatography with a purification of 6.7-fold, a yield of 53%, and a specific activity of 31.1 U/mg. The molecular mass of the purified enzyme analyzed by SDS-PAGE was approximately 85 kDa (Figure 1A), which was consistent with the calculated value of 85830 kDa based on the 755 amino acid residues and 6 histidine residues as determined using the Compute pI/Mw tool. On the basis of the molecular masses of the reference proteins, the native enzyme existed as a monomer with a molecular mass of 86 kDa as determined by gel filtration chromatography (Figure 1B). The total molecular masses and association forms of the GH3 family β-glucosidases from *T. martima*,[20] *Flavobacterium meningosepticum*,[21] and *Clostridium sterrarium* [22] were 78−81 kDa and monomers. However, the GH3 family β-glucosidase from *A. niger* was a dimer with a total molecular mass of approximately 200 kDa.[23]

Effects of Metal Ions, pH, and Temperature on β-Glucosidase Activity from *D. turgidum*. The effect of metal ions such as Co^{2+}, Mn^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, or Ca^{2+} on enzyme activity was assessed. The putative β-glucosidase from *D. turgidum* was not activated by monovalent or divalent cations and was not inhibited by EDTA. Therefore, the enzyme was determined as metal-independent (data not shown). A GH3 family β-glucosidase from *T. maritima* was shown to be metal-independent.[20]

The enzyme activity was examined over a pH range of 4.0−6.5. The maximal activity was observed at pH 5. At pH values of 4.5 and 6.5, the activity was approximately 70% of the maximum (Figure 2A). The effect of temperature on the enzyme activity was assessed, and the maximum activity was observed at 85 °C (Figure 2B). The activities of the GH3 family β-glucosidases from *T. maritima*,[20] *Thermobaculum neopolitana*,[24] *C. sterrarium*,[22] and *A. oryzae*[25] were maximal at 80 °C and pH 7.0, at 90 °C and pH 5.6, at 65 °C and pH 5.5, and at 60 °C and pH 5.0, respectively. The thermostability of the putative β-glucosidase from *D. turgidum* was examined by measuring the activity over time (Figure 3). The enzyme displayed first-order kinetics for thermal inactivation, and the half-lives of the enzyme at 70, 75, 80, 85, and 90 °C were 1575, 854, 524, 334, and 20 min, respectively. Previously, the highest thermostability was observed with β-glucosidase from the GH3 family β-glucosidase (Tm-BglB) from *T. maritima* with a half-life of approximately 90 min at 70 °C. However, the half-life of *D. turgidum* β-glucosidase examined in this study was approximately 18-fold higher than that of *T. maritima* β-glucosidase (Tm-BglB).[26] These results demonstrate that the putative β-glucosidase from *D. turgidum* is the most thermostable enzyme among the GH3 family β-glucosidases, which may prove useful in a variety of industrial fields.

Table 1. Substrate Specificity of β-Glucosidase from *D. turgidum*

substrate	specific activity[a] (U/mg)
*p*NP-β-D-glucopyranoside	31.1 ± 1.5
*o*NP-β-D-glucopyranoside	7.9 ± 0.2
*p*NP-β-D-xylopyranoside	0.9 ± 0
*p*NP-β-D-fucopyranoside	0.6 ± 0
*p*NP-β-D-galactopyranoside	0.2 ± 0
*o*NP-β-D-galactopyranoside	ND
*p*NP-β-D-mannopyranoside	ND
*p*NP-β-L-arabinopyranoside	ND
*p*NP-α-L-rhamnopyranoside	ND
*p*NP-α-D-glucopyranoside	ND
genistin	21.3 ± 0.98
daidzin	16.7 ± 0.35
ononin	9.3 ± 0.17
glycitin	5.7 ± 0.02

[a] Data represent the mean and standard deviation from three separate experiments. ND, not detected.

Table 2. Kinetic Parameters of β-Glucosidase from *D. turgidum*[a]

substrate	K_m (mM)	k_{cat} (1/s)	k_{cat}/K_m (1/(mM s))
*p*NP-β-D-glucopyranoside	1.30 ± 0.01	13900 ± 449	10400 ± 554
*o*NP-β-D-glucopyranoside	1.90 ± 0.01	5890 ± 58	3110 ± 159
genistin	0.67 ± 0.02	5750 ± 122	8590 ± 314
daidzin	0.83 ± 0.01	3660 ± 156	4440 ± 205
ononin	0.78 ± 0.01	877 ± 24	1130 ± 34
glycitin	1.03 ± 0.07	725 ± 31	709 ± 57

[a] Data represent the mean and standard deviation from three separate experiments. The standard error was recorded to be <5%.

Substrate Specificity of β-Glucosidase from *D. turgidum*. The specific activity of the putative β-glucosidase from *D. turgidum* was evaluated with aryl-glycosides and isoflavone glycosides. The enzyme exhibited the highest activity for *p*NP-D-glucopyranoside, followed by *o*NP-β-D-glucopyranosides (Table 1). The activity for *p*NP-β-D-xylopyranoside, *p*NP-β-D-fucopyranoside, and *p*NP-β-D-galactopyranoside was <3% of *p*NP-β-D-glucopyranoside activity. However, no activity was observed for *p*NP-β-D-mannopyranoside, *p*NP-β-L-arabinopyranoside, *o*NP-β-D-galactopyranoside, and *p*NP-α-L-rhamnopyranoside. These results

Table 3. Isoflavone Contents in Ground Coffee and Spent Coffee Grounds

	isoflavone contents[a] (μg/g)	
	ground coffee	spent coffee grounds
daidzin	160 ± 3.8	107 ± 2.7
daidzein	14.8 ± 0.30	12.1 ± 0.61
ononin	0.15 ± 0.003	0.10 ± 0.002
genistin	0.02 ± 0.001	0.01 ± 0.001
glycitin	ND	ND

[a] Data represent the mean and standard deviation from three separate experiments. ND, not detected.

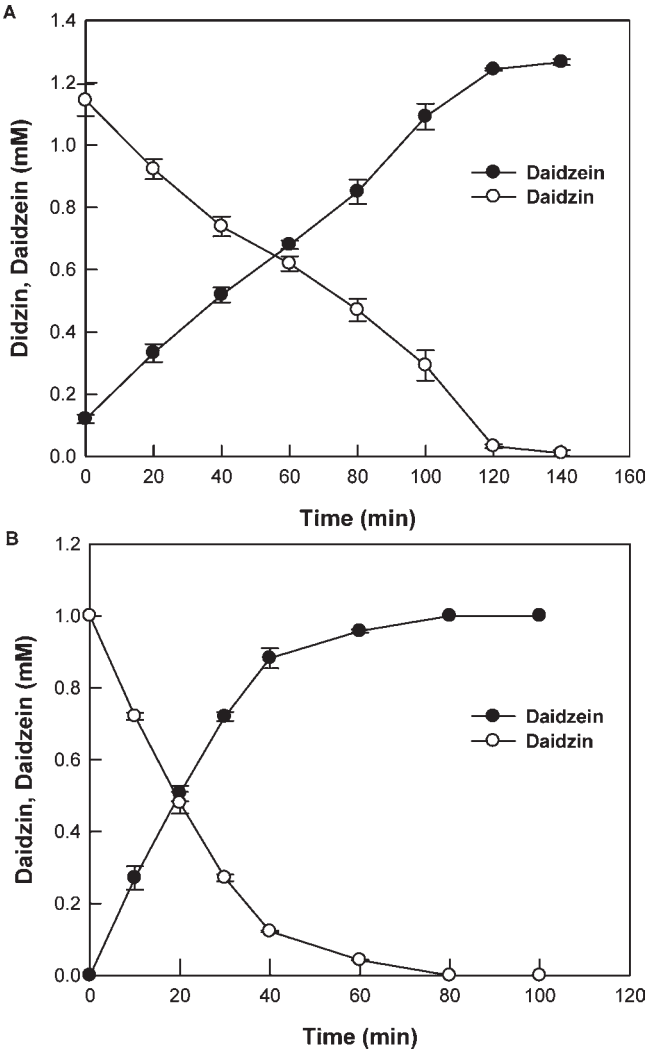

Figure 4. Conversion of daidzin to daidzein by β-glucosidase from *D. turgidum* using (A) spent coffee grounds extract and (B) reagent grade daidzin. The reactions were performed at 85 °C in 50 mM citrate/phosphate buffer (pH 5) containing 16 U/mL enzyme and 1 mM reagent grade daidzin for 100 min or 62 U/mL enzyme and 5% (w/v) spent coffee grounds extract for 140 min. Data represent the mean of three experiments, and error bars represent standard deviation.

indicated that this enzyme is a β-glucosidase and that the enzyme has a higher activity for β-1,4 linkage than for β-1,2 linkage.

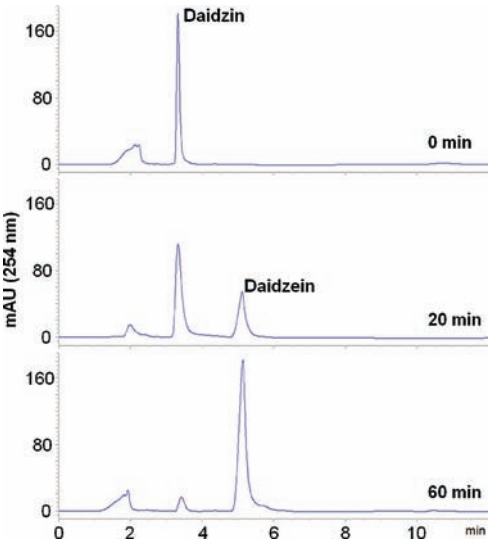

Figure 5. HPLC profiles for the conversion of daidzin into daidzein. The reactions were performed at 85 °C in 50 mM citrate/phosphate buffer (pH 5.0) containing 16 U/mL enzyme and 1 mM reagent grade daidzin for 60 min.

A GH3 family β-glucosidase from *C. stercorarium* evidenced hydrolytic activity for *p*NP-β-D-glucopyranoside and *p*NP-β-D-xylopyranoside but not for *p*NP-β-D-mannopyranoside.[22] The substrate specificity of β-glucosidase from *D. turgidum* followed the order genistin > daidzin > ononin > glycitin. The substrate specificity of other GH3 family β-glucosidases from *P. thermophila*,[12] *Pseudomonas* sp.,[13] *T. maritima*,[14] and *D. cohinchinensis*[15] was higher for genistin as a substrate than for daidzin. These results indicated that the substrate specificity of β-glucosidase from *D. turgidum* was similar to that of other GH3 family β-glucosidases.

The Michaelis−Menten constants (K_m), turnover numbers (k_{cat}), and catalytic efficiencies (k_{cat}/K_m) for *p*NP-β-D-glucopyranoside, *o*NP-β-D-glucopyranoside, genistin, daidzin, ononin, and glycitin are provided in Table 2. Among the kinetic parameters, only the K_m values of the GH3 family β-glucosidases from *A. oryzae*,[25] *F. meningosepticum*,[21] *C. stercorarium*,[22] and *T. neapolitana*[24] for *p*NP-β-D-glucopyranoside have been previously reported (0.29, 0.68, 0.8, and 0.11 mM, respectively). The K_m of *D. turgidum* β-glucosidase for *p*NP-β-D-glucopyranoside was 1.3 mM, which was the highest among the reported K_m values of the GH3 family β-glucosidases. The orders of k_{cat} and k_{cat}/K_m of *D. turgidum* β-glycosidase (genistin > daidzin > ononin > glycitin) were the same as those noted in regard to specific activity, whereas the order of K_m was glycitin > daidzin > ononin > genistin. The most preferred substrate of the β-glucosidase among isoflavone aglycones was genistin. Previously, the highest reported k_{cat} for genistin was 700 1/s for a β-glucosidase from *D. nigrescens*.[15] The k_{cat} of *D. turgidum* β-glucosidase for genistin (5750 1/s) was 8.2-fold higher than that of *D. nigrescens* β-glucosidase, indicating that the k_{cat} of *D. turgidum* β-glucosidase for genistin is the highest reported thus far. The k_{cat} of *D. turgidum* β-glucosidase for *p*NP-β-D-glucopyranoside (13900 1/s) was 9.0-fold higher than that of the GH3 family *T. maritima* β-glucosidase (Tm-BglB) (1550 1/s). These results indicate that this enzyme is an efficient producer of isoflavone aglycones.

Hydrolysis of Daidzin in Spent Coffee Grounds to Daidzein. We extracted isoflavones in Arabica roasted coffee from Starbucks "HouseBlend" and spent Arabica coffee grounds, and

Table 4. Conversion of Daidzin to Daidzein by β-Glucosidases

substrate	source	daidzin (mM)	daidzein (mM)	mole yield (%)	productivity (mM/h)	ref
soybean milk	soybean	0.15	0.04	27	0.013	28
soybean flour	*Thermotoga maritima*	NR	0.35[a]	86	NR	14
soybean flour extract	almond	NR	NR	81	NR	29
	Aspergillus oryzae	2.5	1.8	73	0.61	27
	Aspergillus oryzae	10	8.4	84	0.17	8
	Paecilomyces thermophila	NR	NR	98	NR	12
spent coffee grounds extract	*Dictyoglomus turgidum*	1.2	1.2	100	0.60	this study
daidzin	*Escherichia coli*	0.01	0.0095	95	0.002	11
	Dictyoglomus turgidum	1.0	1.0	100	1.0	this study

[a] Daidzein (mg)/soybean flour (g).

the amount of daidzin in the spent coffee grounds was measured to be 67% of that detected in the roasted coffee (Table 3). The large contents of daidzin were found both in the roasted coffee (160 μg/g) and in the spent coffee grounds (107 μg/g), and the contents of daidzein were approximately 10% of daidzin contents. However, other isoflavone glycosides such as genistin and ononin were detected at low concentrations of <0.1% of daidzin contents (<0.2 μg/g) in the roasted coffee and spent coffee grounds, and no contents were observed for glycitin. Daidzin, a hydrophobic compound, in roasted coffee is eluted by hot water with a low yield, and the remaining daidzin can be used as a valuable material. Following acid hydrolysis of Brazilian Arabica roasted coffee from local markets, the contents of daidzein, formononetin, and genistein produced as isoflavone aglycones were reported as 7.5−21.5, 8.0−11.9, and 1.7−2.4 μg/g, respectively.[18] These results may be attributable to differences in coffee species.

The production of daidzein by the GH3 family β-glucosidases has been performed using reagent grade daidzin, whole soy isoflavone, or processed soy product (soybean flour). However, the GH3 family β-glucosidases have not been applied for the hydrolysis of isoflavone glycosides in food industrial waste. Thus, the GH3 family β-glucosidase from *D. turgidum* in the present study was applied first to the hydrolysis of daidzin in spent coffee grounds extract, as food industrial waste, to daidzein at optimal temperature and pH of 85 °C and pH 5.0. The concentration of spent coffee grounds extract was adjusted to 5%, which yielded daidzin and daidzein concentrations of 1.2 and 0.12 mM, respectively. The enzyme at 62 U/mL completely hydrolyzed 1.2 mM daidzin in spent coffee grounds extract to daidzein within 120 min, corresponding to a productivity of 0.6 mM/h (Figure 4A). The enzyme at 16 U/mL completely also hydrolyzed 1.0 mM reagent grade daidzin to daidzein within 80 min, corresponding to a productivity of 0.75 mM/h (Figure 4B). The HPLC profiles for the conversion of reagent grade daidzin into daidzein are shown in Figure 5. With increasing reaction time from 0 to 60 min, the substrate daidzin decreased, whereas the product daidzein increased. Daidzein production was investigated by varying the enzyme concentration from 1 to 30 U/mL for 1.0 mM reagent grade daidzin as a substrate and from 1 to 124 U/mL of enzyme for 5% spent coffee grounds extract for 120 min (data not shown). Daidzein production increased with increasing enzyme concentrations up to 16 U/mL for 1.0 mM reagent grade daidzin and up to 62 U/mL for 5% spent coffee grounds extract and reached plateaus at enzyme concentrations above 16 and 62 U/mL, respectively. Therefore, the optimal enzyme concentration for 1.0 mM reagent grade daidzin was 16 U/mL and that for 5% spent coffee grounds extract was 62 U/mL.

The conversion of daidzin from reagent and complex substrate to daidzin by β-glucosidases is summarized in Table 4. The previous highest molar yield and productivity of daidzein from reagent grade daidzin were 95% and 0.002 mM/h, respectively, using a β-glucosidase from *E. coli*.[11] These values were 5% and 250-fold lower, respectively, than those of *D. turgidum* β-glucosidase. Although the experimental conditions of the previous works were different from the present one and the components of isoflavone glycosides from soybean extract were exactly different from spent coffee grounds extract, the previous highest molar yield of daidzein from daidzin in soybean flour extract was 98% for *P. thermophila* β-glucosidase,[12] and the previous highest reported productivity using 2.5 mM daidzin in soybean flour extract was 0.61 mM/h for *A. oryzae* β-glucosidase.[27] *D. turgidum* β-glucosidase completely hydrolyzed daidzin in spent coffee grounds extract to daidzein, and its productivity was almost the same as the productivity using *A. oryzae* β-glucosidase from daidzin in spent soybean flour extract. Thus, *D. turgidum* β-glucosidase is the potent daidzin-hydrolyzing enzyme.

In conclusion, in this study we cloned and characterized a thermostable GH3 family β-glucosidase from *D. turgidum* and used the enzyme in the complete hydrolysis of daidzin in spent coffee grounds to daizein. These results suggest that this enzyme may prove useful in the industrial hydrolysis of isoflavone glycosides and also show that spent coffee grounds may prove to be a valuable material for the production of isoflavone aglycon.

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