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Purification and Characterization of an Isoflavone-Conjugates-Hydrolyzing β-Glucosidase from Endophytic Bacterium

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An isoflavone conjugates hydrolyzing β-glucosidase (ICHG) from endophytic bacterium, *Pseudomonas* ZD-8 was purified to homogeneity by successive ammonium sulfate precipitation, gel filtration on SephadexG-100, DEAE-sephrose CL-6B and DEAE-Sephacel chromatography. The enzyme was a monomeric protein with an apparent molecular mass of 33 kDa as determined by SDS-PAGE and gel filtration. It was optimally active at pH 6.0 and 40 °C and had a specific activity of 1485 U mg of protein^{-1} against genistin. The ICHG readily hydrolyzed ρ-nitrophenyl-β-glucoside, ρ-nitrophenyl-β-galactoside, genistin, daidzin, with K_m values of 1.64, 1.87, 0.012, 0.014 mM, respectively. The ICHG showed a pronounced specificity for glucose in the 7-position of isoflavone and flavone conjugates and hydrolyzed effectively malonyl isoflavone glucosides as well as isoflavone glucosides with similar kinetics. Glucose and glucono-δ-lactone inhibited the enzyme competitively with K_i values of 84 mM and 23 mM, respectively. The enzyme did not require divalent cations for activity, and its activity was strongly inhibited by Hg^{2+}, Ag^{+}, ρ-chloromercuribenzoate, iodoacetic acid, and *N*-ethylmaleimide while reducing agents such as β-mercaptoethanol, dithiothreitol, dithioerythritol, glutathione slightly activated the enzyme.

KEYWORDS: Endophytic bacteria; β-glucosidase; isoflavone glucosides; purification; properties; *Pseudomonas* ZD-8

INTRODUCTION

Certain phytochemicals in fruits, vegetables, and grains possess possible cancer-preventive properties that may inhibit tumor initiation, prevent oxidative damage, or affect steroid hormones or prostaglandin metabolism to block tumor promotion (*1*, *2*). Isoflavones are constitutively present in large quantities in legumes, particularly in soybean, and exist primarily as glucoside forms, 6′′-*O*-malonylglucosides and 6′′-*O*-acetylglucosides, and rarely as aglucones forms (*3*). The representative isoflavone glucosides in soybean are genistin and daidzin and their corresponding aglucones are genistein and daidzein (*4*). Many researchers have revealed that the pharmacological effects, such a cancer prophylactic effect, of isoflavone compounds are primarily attributed not to the glucosides but their aglucones, such as daidzein or genistein (*5*−*7*). Of the soybean isoflavone aglucones genistein has recently been proved particularly excellent in physiological activities, including antiosteoporosis activity, antiarteriosclerotic activity, and anticancer activities in the breast, the stomach, and the prostate (*8*−*12*).

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The isoflavone conjugates may serve as sources for the release of free aglucones (*13*). The formation of free aglucones should be associated with the enzymatic conversion, for instance, the hydrolytic enzyme, isoflavone 7-*O*-glucoside- 6′′-*O*-malonate malonesterase and isoflavone 7-*O*-glucoside β-glucosidase from soybean and chickpea have been purified and characterized (*14*−*16*). To date, there is no report on β-glucosidase with preferential activity toward isoflavone glucosides from endophytic bacterium.

Endophytes are bacterial or fungal microorganisms which colonize the healthy plant tissue inter-and/or intracellularly without causing any apparent symptoms of disease. They have been isolated from almost every host plant studied so far. The production of highly bioactive secondary metabolites was reported from several endophytes (*17*). Living inside the plant tissue, they have to cope with the toxic defense compounds of plants. An adapted potential of biodegradation and a set of specific enzymes could allow them to subsist under these environmental conditions. Up to now, only scarce research has been done in this field, and only few experimental data are available (*18*).

β-glucosidase (EC 3.2.1.21) roused considerable interest primarily because of its involvement in various biotechnological

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processes, including the production of fuel ethanol from agricultural residues, the synthesis of useful β-glucoside, the release of aromatic compounds from flavorless precursor, and improvement of the organoleptic properties in fruit juices (*19–22*). To effectively prepare the valuable drug-materials-free aglycones, it is necessary to isolate and screen new β-glucosidase producing microorganisms (*23*). We focused our attention on endophytic bacteria as a potential new source of β-glucosidase. During the course of our studies of the microbial hydrolysis of isoflavone glucoside compounds, we found a novel β-glucosidase in *Pseudomonas* ZD-8. Here, we describe the purification and characterization of this enzyme.

MATERIALS AND METHODS

Microorganism Isolation and Culture Conditions. *Pseudomonas* ZD-8, which was used in all experiments, was isolated from healthy *Pueraria lobata* ohwi roots. The basal medium (TY) used for production β-glucosidase consisted of 5.0 g of tryptone yeast, 3.0 g of yeast extract, and 1.0 g of $CaCl_2$ per liter of distilled water, pH 6.0. To induce the β-glucosidase activity, the TY medium supplemented with genistin (0.1%) was inoculated with *Pseudomonas* ZD-8. The culture was incubated at 30 °C in 500-ml Erlenmer flasks containing 150 mL of medium on a rotary shaker at 150 rpm and harvested in the late exponential phase (48 h), the cells were removed from the culture broth by centrifugation at 12 000*g* for 20 min. The resulting supernatant solution was used as the crude enzyme preparation for subsequent enzyme purification.

Chemicals and Reagents. Sephadex G-100, DEAE-Sepharose CL-6B, and DEAE-Sephacel were purchased from Amersham Pharmacia Biotech Co. (Buckinghamshire, UK). Isoflavones used for enzymatic analysis were purchased from Extrasynthese (Genay Cedex, France). ρ-Nitrophenyl-β-glucoside, ρ-nitrophenyl-β-galactoside, ρ-nitrophenyl-β-mannoside, ρ-nitrophenyl-α-glucoside were purchased from Sigma. All other chemicals and reagents were of analytical grade and were purchased from commercial sources, unless otherwise stated.

Isolation and Purification of Isoflavone Conjugates. Isoflavone conjugates were prepared as follows: Soybean seeds (50 g) were ground to a fine powder with a coffee grinder, and the powder was extracted with 100 mL of chloroform for 30 min with stirring. The slurry was filtered through Whatman No. 1 filter paper with suction and washed two more times with chloroform. The defatted soybean flour was extracted twice with 50 mL of distilled water. The aqueous extract (100 mL) was clarified by low speed centrifugation. The isoflavone conjugates of genistin and daidzin were partially purified using a C18 PrepSep column. The crude aqueous preparation was passed through the column, and the column was washed successively with water and 1 mL of 10% acetonitrile. The 10% eluent was discarded. Washing with 1 mL of 20% acetonitrile released bound daidzin, genistin, genistein, and daidzein.

Enzyme Purification. All the experiments described below were carried out between 0 and 4 °C unless otherwise specified.

(*a*) *Ammonium Sulfate Precipitation.* The cell free culture broth was brought to 50% ammonium sulfate saturation and stirred for 30 min, the cloudy suspension was centrifuged at 20 000*g* for 30 min, and supernatant was brought to 90% ammonium sulfate saturation. After being stirred for 30 min, the pellet obtained by centrifugation at 20 000*g* for 30 min was dissolved in the smallest possible volume 50 mM sodium phosphate buffer (pH 6.0) and dialyzed 1000-fold against 50 mM sodium phosphate buffer (pH 6.0), the supernatant was concentrated by ultrafiltration with an Amicon YM-30 membrane.

(*b*) *Size-Exclusion Chromatography.* The dialyzed enzyme solutions from (*a*) were loaded onto Sephadex G-100 column (1.8 by 100 cm) preequilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was washed at a flow rate of 20 mL/h with 400 mL of the same buffer, and 5-mL fractions were collected. Proteins were eluted in fractions 13–70, whereas the enzyme was confined to fractions 32–41. The fractions with high specific activity were then pooled and concentrated for further purification.

(*c*) *DEAE-Sepharose CL-6B Column Chromatography.* The pooled enzyme fractions from (*b*) were loaded onto DEAE-Sepharose CL-6B column (88- × 2.0-cm) previously equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was eluted with a linear gradient of NaCl from 0 to 0.5 M at a flow rate of 12 mL/h, 3-mL fractions were collected and tested for enzyme activity, active fractions that eluted with the NaCl gradient were pooled, dialyzed 1000-fold against 50 mM sodium phosphate buffer (pH 6.0), and concentrated by ultrafiltration with an Amicon YM-30 membrane.

(*d*) *DEAE-Sephacel Column Chromatography.* The pooled enzyme fractions from (*c*) were applied to a DEAE-Sephacel column (30- × 2.5-cm) equilibrated with 50 mM sodium phosphate buffer (pH 6.0). The column was washed with 250 mL of the same buffer, and proteins were eluted with a linear gradient of NaCl from 0 to 1 M, fractions (2.5 mL) were collected every 6 min and screened for enzyme activity, fractions that eluted with the running buffer and exhibited enzyme activity were combined, dialyzed overnight, concentrated by ultrafiltration with an Amicon YM-30 membrane and lyophilized. The fraction was used as the purified enzyme for subsequent analysis.

Determination of Molecular Mass and pI. The molecular mass of the denatured protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of the native protein by gel filtration on a Superose 12HR 5/30 column. Isoelectric point (pI) was estimated by PAGE with 6.25% Ampholine (pH 3.5 ∼ 10) in a gel rod (0.5- × 10-cm) using a kit for Isoelectric Focusing Calibration (Pharmacia LKB) according to recommendations by the supplier.

Determination of Protein. Protein concentrations were determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard as described by Bradford. For the eluates from the chromatographic columns, the protein concentrations were determined by measuring the absorbance at 280 nm (*24*).

Enzyme Assay. β-Glucosidase activity was routinely determined by using a reaction mixture (1 mL) containing 4 mM genistin, 50 mM sodium phosphate buffer (pH 6.0), and appropriately diluted enzyme solution in the same buffer. After incubation at 40 °C for 30 min, the reaction was stopped by adding ice-cold 0.5 M Na_2CO_3 (1 mL), and the activity was measured by quantifying the release of aglucone genistein by HPLC analysis. One unit of enzymatic activity (U) was defined as that the amount of enzyme that produced 1 μmol of product per minute in the reaction mixture under these assay conditions. Specific activity was expressed as enzyme units/mg protein.

Determination of Substrate Specificity and Kinetic Parameters. The activity of the purified β-glucosidase was measured under standard conditions with various substrates at 2 mM final concentration unless otherwise noted. Daidzein 7-*O*-glucoside, daidzein-7-*O*-glucosyl 6′′-*O*-malonate, genistein 7-*O*-glucoside, and genistein-7-*O*-glucosyl 6′′-*O*-malonate were assayed at 0.2, 0.18, 0.79, and 1.15 mM. The appropriate concentration of the β-glucosidase used for each substrate was determined graphically from the linear portions of initial reaction velocity. All substrates were dissolved in 50 mM sodium phosphate buffer (pH 6.0), except that genistein 7-*O*-glucoside was dissolved in ethylene glycol monoethyl ether at 50 mM and then diluted to final concentration in the same buffer. Depending on different substrates, activity was measured either spectrophotometrically or by HPLC (*16*, *21*). All K_m and V_{max} values were calculated by the Lineweaver-Burk equation by using the microcal origin software program, and the k_{cat} value is equal to V_{max} divided by the total enzyme concentration (in molarity) which was calculated using M_r of 33 kDa for the purified β-glucosidase. The kinetic parameters reported are the mean values of three independent determinations.

HPLC Analyses. HPLC analyses were performed on a Lichrospher 100 Rp-18 column (250- × 4-mm), using a linear gradient of acetonitrile from 15 to 35% containing constant 0.1% acetic acid in 50 min. The solvent flow rate was 0.8 mL/min and the absorption was measured at 254 nm. The instrument used was an HP1100 HPLC system. To examine the changes of isoflavone aglucones content, 100 μL of soybean extract was incubated with ICHG for 30 min at 40 °C and then used for HPLC.

Table 1. Purification of ICHG from *Pseudomonas* ZD-8

purifn step	vol (mL)	total protein (mg)	total activity (units)	sp act (units mg^{-1})	yield (%)	purifn
crude extract	2836	351	16497	47	100	1
$(NH_4)_2SO_4$	721	67	15013	224	91	4.8
SephadexG-100	87	8.3	5444	614	33	13
DEAE-Sepharose CL-6B	14	1.9	1815	933	11	20
DEAE-Sephacel	1.8	0.75	1105	1485	6.7	31.6

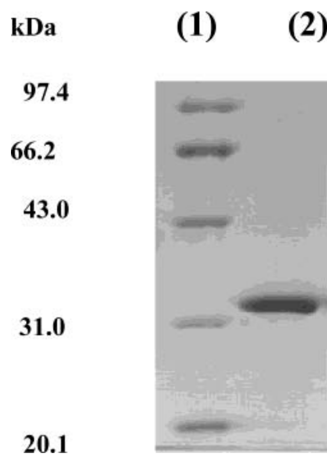

Figure 1. SDS-PAGE analysis of the purified ICHG (lane 2) and molecular weight markers (lane 1) stained with Coomassie blue.

RESULTS AND DISCUSSION

All the experiments were carried out at least in triplicate, and the results were expressed as means of those data. Standard deviation were determined and reported.

Enzyme Production and Purification. *Pseudomonas* ZD-8 was aerobically cultivated at 30 °C in TY with genistin**.** ICHG activity was detected at the late log phase and reached the maximum level 2 days after the start of cultivation. ICHG was purified from the culture supernatant of *Pseudomonas* ZD-8 by ammonium sulfate precipitation, gel filtration on SephadexG-100, DEAE-sephrose CL-6B, and DEAE-Sephacel chromatography. The data on the purification are summarized in (**Table 1**). The enzyme was purified 31.6-fold to a specific activity of 1485 U/mg protein from the cell with a yield of 6.7%. The purified enzyme gave a single band in SDS-PAGE. This indicated that the purified sample was electrophoretically homogeneous under the dissociating conditions. The molecular mass of the purified enzyme estimated was estimated by SDS-PAGE analysis was approximately 33 kDa (**Figure 1**). The relative molecular mass of native enzyme estimated by gel filtration on a calibrated column of Sephacryl 200 HR was around 32 kDa. Hence, it is assumed that the native ICHG is a monomer. The pI value was estimated to be 5.2.

Effects of pH and Temperature on the Activity and Stability of the Enzyme. The pH optimum was determined at pH values between 3.5 and 8.5. The pH stability was measured under standard conditions after incubation of the purified β-glucosidase for 2 h at 30 °C. The pH-activity profile of the enzyme was bell shaped, with maximum values at pH 6.0 (**Figure 2**), and the enzyme was found to be stable in the pH range between 5.0 and 8.0. The temperature optimum was determined at the range of 20−70 °C. Temperature stability was measured under standard conditions after incubation of the purified β-glucosidase in 50 mM sodium phosphate buffer (pH 6.0) for 30 min at different temperatures (20−70 °C). The optimal temperature for the enzyme was 40 °C (**Figure 3**). The enzyme was fairly stable at up to 45 °C and had 44% of its activity at 50 °C. It was completely inactivated at 65 °C.

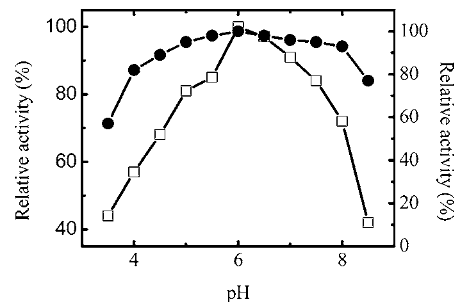

Figure 2. Effect of pH on activity (□) and stability (●) of ICHG from *Pseudomonas* ZD-8.

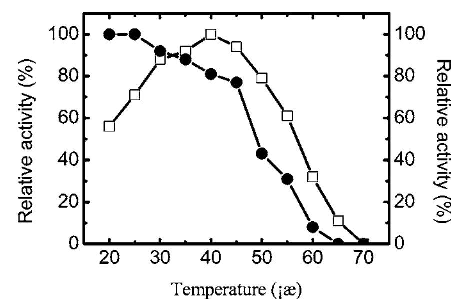

Figure 3. Effect of temperature on activity (□) and stability (●) of ICHG from *Pseudomonas* ZD-8.

Table 2. Effect of the Various Substances on Relative Activity of ICHG from *Pseudomonas* ZD-8

substances	relative activity (%)	substances	relative activity (%)
none	100	10 mM 1,10 phenanthroline	93 ± 5.2
1 mM $MgCl_2$	93 ± 5.8	1 mM PCMB	0
1 mM $CuSO_4$	91 ± 4.6	1 mM IAA	8 ± 0.5
1 mM $ZnSO_4$	95 ± 5.7	1 mM NEM	0
1 mM $MnCl_2$	96 ± 6.1	1 mM β-mercaptoethanol	113 ± 6.8
1 mM $AgNO_3$	12 ± 0.7	1 mM Dithiothreitol	128 ± 7.1
1 mM $HgCl_2$	6 ± 0.3	1 mM Dithioerythritol	120 ± 6.4
10 mM EDTA	95 ± 5.4	1 mM Glutathione	106 ± 5.7

Effects of Various Compounds and Metal Ions on Enzyme Activity. The effects of various chemicals on the enzyme activity were investigated by addition of the tested compounds into the reaction mixture at the final concentration. The activity was then measured under standard assay conditions and expressed as a percentage of the activity obtained in the absence of the added compound (**Table 2**). The presence of Hg^{2+} and Ag^{+} caused a complete inhibition at 1.0 mM, while less pronounced effects (5 to 10% inhibition) were observed in the presence of the remaining divalent cations. The enzyme activity was strongly inhibited by ρ-chloromercuribenzoate (PCMB), iodoacetic acid (IAA), and *N*-ethylmaleimide (NEM), while reducing agents such as β-mercaptoethanol, dithiothreitol, dithioerythritol, and glutathione slightly activated the enzyme. The cheating agent EDTA and phenanthroline showed little effect on the enzyme activity.

Substrate Specificity. Relative rates of hydrolysis of various substrates by the ICHG were studied in (**Table 3**). It is clear that the enzyme acted specificially on the aryl-β-glucosidic bond in substrate molecule, while α-glucosides as well as disaccharides were not hydrolyzed at all. A comparison of the enzyme

Table 3. Apparent Kinetic Constants for Hydrolysis of ICHG from *Pseudomonas* ZD-8

substrate	relative activity (%)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
ρ-nitrophenyl-β-glucoside	52	1643 ± 77	0.87 ± 0.04	0.53
ρ-nitrophenyl-β-galactoside	37	1873 ± 86	0.62 ± 0.02	0.33
ρ-nitrophenyl-β-mannoside	32	1942 ± 93	0.32 ± 0.01	0.16
ρ-nitrophenyl-α-glucoside	0	0	0	0
salicin	67	225 ± 12	1.32 ± 0.07	5.8
esculin	71	211 ± 11	1.57 ± 0.08	7.4
arbutin	63	286 ± 13	1.06 ± 0.04	3.7
genistein-7-*O*-glucoside (genistin)	100	12 ± 0.8	5.31 ± 0.3	443
daidzein-7-*O*-glucoside (daidzin)	97	14 ± 0.9	5.12 ± 0.3	366
genistein-7-*O*-glucosyl 6″-*O*-malonate	88	48 ± 3	2.68 ± 0.1	56
daidzein-7-*O*-glucosyl 6″-*O*-malonate	84	57 ± 3	2.39 ± 0.1	42
apigenin 7-*O*-β-glucoside	94	23 ± 1	3.92 ± 0.2	170
genistein-4-*O*-glucoside	0	0	0	0
rutin	0	0	0	0
isoquercitrin	0	0	0	0

activities on various aryl-glycosides confirmed that the enzyme has specificity for mono-β-glycosides, with a preference for glucose at the nonreducing end. The enzyme could also efficiently hydrolyze several natural glucosides, such as salicin, esculin, and arbutin. Even more surprisingly, the enzyme was more active toward the 7-*O*-substituted isoflavone and flavone glucosides than those described above. The reaction kinetics of the purified enzyme were determined from Lineweaver−Burk plots under optimal conditions (30 min, 40 °C, pH 6.0). The enzyme had apparent K_m values of 1.64, 1.87, 1.94, 0.225, 0.211, 0.286, 0.012, 0.014, 0.048, 0.057, and 0.023 mM for hydrolysis of ρ-nitrophenyl-β-glucoside, ρ-nitrophenyl-β-galactoside, ρ-nitrophenyl-β-mannoside, salicin, esculin, arbutin, genistin, daidzin, genistein-7-*O*-glucosyl 6″-*O*-malonate, daidzein-7-*O*-glucosyl 6″-*O*-malonate, and apigenin 7-*O*-β-glucoside, respectively. All the determined kinetic parameters are summarized in **Table 3**.

The study of inhibition by glucose and glucono-δ-lactone was performed with genistin as the substrate. Glucose and glucono-δ-lactone acted as competitive inhibitors of genistin hydrolysis with inhibition constant (K_i) values of 84 mM and 23 mM, respectively, which were obtained from the intersections of the lines on Dixon plot analysis.

Hydrolysis of isoflavone glucosides in soybean extract by the ICHG was followed by HPLC, as showed in (**Figure 4**). The reaction rate of the two isoflavone glucosides, genistin, and daidzin from the extract by the enzyme was extremely rapid; therefore, after 30 min, the contents of genistein and daidzein were over 12 times more than the initial contents of soybean extract.

There have been a number of reports on β-glucosidases from plants, bacteria, and fungi, and they played important roles in many biotransformation processes. However, few attempts have been made to investigate the enzyme hydrolysis of isoflavone glucosides from microbes. This is the first report to our knowledge on production, purification, and properties of a novel β-glucosidase from endophytic bacteria. The specific activity of the purified enzyme preparation under optimal conditions was 1485 U mg of protein^{-1} on genistin, whereas the specific activities reported for β-glucosidases toward isoflavone conjugates from plant origins from 3.6 to 1370 U mg of protein^{-1} (*14*, *16*, *19*). As a monomeric 33 kDa protein, ICHG is also among the smallest known β-glucosidases compared to those from plant origins whose molecular masses range from 81 kDa to 165 kDa (*14*, *16*). The pI of the ICHG was lower than that of β-glucosidases from plant origins. The pH optimum of the

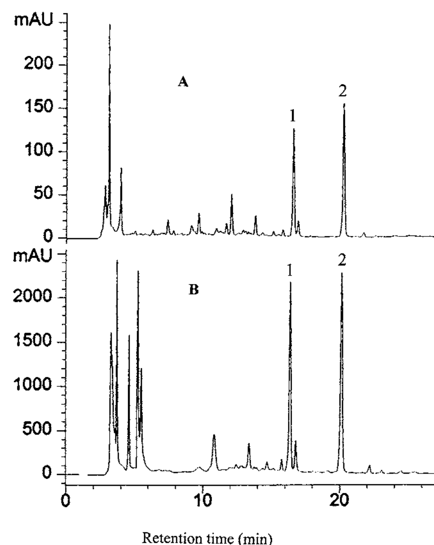

Figure 4. HPLC patterns of soybean extracts after incubation with ICHG from *Pseudomonas* ZD-8 for (**A**) 0 min and (**B**) 30 min. 1, daidzein; 2, genistein. See Materials and Methods for HPLC conditions.

ICHG was equal to that recorded for β-glucosidase from *Glycine max* roots (*16*). However, it was higher than those reported β-glucosidases from soybean seeds and lower than that recorded for β-glucosidases from *Cicer arietinum* L (*14*, *19*). The optimal temperature of 40 °C is lower than those reported for β-glucosidases from soybean seeds and higher than that reported for β-glucosidases from *Glycine max* roots (*14*, *16*, *19*).

The effects observed in the presence of potential inhibitors or activators of the purified enzyme activity were investigated as shown in (**Table 2**). We found that the purified enzyme was significantly affected by sulfhydryl oxidant metals (Hg^{2+}, Ag^{+}), while other metal ions did not have remarkable effect on the activity. This indicated that thiol may be involved in active catalytic site, and furthermore, activity was completely inhibited by thiol-modifying reagents such as ρ-chloromercuribenzoate (PCMB), iodoacetic acid (IAA) and *N*-ethylmaleimide (NEM), but reducing agents such as β-mercaptoethanol, dithiothreitol, dithioerythritol, and glutathione slightly activated the enzyme, therefore suggesting again that sulfhydryl groups may be involved in the catalytic center of the enzyme (*19*, *21*). The importanance of SH-groups in ICHG is consistent with the fact that cysteine residues are involved in stability and enzyme activity of β-glucosidase, although most β-glucosidase in family 1 require two essential carboxylates for their catalytic activity, contributing to the general acid base and nucleophilic catalysis. The chelating agents, such as ethylenediamine tetraacetate (EDTA) and 1,10-phenanthroline did not affect activity, indicating that divalent cations are not required for enzyme activity (**Table 2**).

Competitive inhibition by glucose is a common characteristic of β-glucosidases that limits their use in enzymatic hydrolysis of plant products. Its high resistance to glucose inhibition is surely what makes the newly purified β-glucosidase of great interest for biotechnological applications. The higher specific β-glucosidase toward isoflavone conjugates from *Cicer arietinum* L. was competitively inhibited by glucose, with an inhibition constant (K_i) of 20 mM. Most microbial β-glucosidases have a glucose K_i ranging from as low as 0.5 mM to no more than 100 mM, therefore, the K_i calculated here, 84 mM, gives ICHG an outstanding position among all the β-glucosidases, which indicates that the enzyme is highly tolerant to glucose (*14*, *25*). As in case of other β-glucosidases, ICHG was

competitively inhibited by glucono-δ-lactone. The higher specific β-glucosidases toward isoflavone conjugates from *Cicer arietinum* L., *Glycine max* roots and soybean seeds have glucono-δ-lactone iK_i ranging from 0.5 μM to 24 mM (*14*, *16*, *19*). The K_i calculated here, 23 mM, gives also ICHG an outstanding position among the β-glucosidases.

The most unusual property that could make ICHG from *Pseudomonas* ZD-8 an attractive biocatalyst for applied biotransformation is lower Michaelis constant (K_m) and higher k_{cat} values for genistin and daidzin compared to those reported from *Cicer arietinum* L, soybean seeds, soybean roots, and commercial almond emulsin. Therefore, the currently purified ICHG has higher specific activity toward isoflavone conjugates. The comparison of K_m and k_{cat} values and their ratios revealed that ICHG has about 117-fold higher affinity toward daidzin than does ρ-nitrophenyl-β-glucoside and can hydrolyze the former substrate about 700-fold faster than the latter. The catalytic efficiencies (k_{cat}/K_m) are considered as a measurement of the enzyme's specificity among these substrates, indicating that genistin clearly is the preferred substrate. The feature is similar to those previously observed by Hosel et al and Hsieh et al and different from β-glucosidases from soybean seeds and commercial almond emulsin (*14*, *16*). In addition, the ICHG showed a pronounced specificity for glucose in the 7-position of isoflavone and flavone conjugates and had no measurable activity with the other positions-substituted isoflavone and flavone conjugates. Furthermore, the purified ICHG effectively hydrolyzed malonyl isoflavone glucosides as well as isoflavone glucosides with similar kinetics, while most β-glucosidases did not cleave the modified terminal glucose residues (*19*).

In conclusion, despite the fact that there are some reports on β-glucosidases purified to homogeneity with higher specificity from plant origins, the purified enzyme described here differs from those reported hitherto in at least one of the following aspects: molecular mass, pI, sensitivity to many kinds of reagents, higher tolerance to glucose, and substrate specificity (*14*−*16*, *19*). Thus, *Pseudomonas* ZD-8 is a promising candidate for enzymatic production. Furthermore, the producing origanism is well suited for molecular and classical genetic manipulations so as to obtain an abundant and economical source of isoflavone aglucones as an added-value coproduct by enzymatic hydrolysis.

LITERATURE CITED

(1) Caragay, A. B. Cancer-preventive foods and ingredients. *Food Technol*. **1992**, *46*, 65−68.

(2) Boone, S. W.; Kelloff, G. J.; Malone, W. E. Identification of candidate cancer chemopreventive agents and their evaluation in animal models and human clinical trails: a review. *Cancer Res.* **1990**, *50*, 2−9.

(3) Messina, M. J. Legumes and soybeans: overview of their nutritional profiles and health effects. *Am. J. Clin. Nutr*. **1999**, *70 (suppl)*, 439S-450S.

(4) Wang, H. J.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem*. **1994**, *42*, 1666−1673.

(5) Kaufman, P. B.; Duke, J. A.; Brielmann, H.; Boik, J.; Hoyt, J. E. A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: implications for human nutrition and health. *J. Altern. Complement. Med*. **1997**, *3*, 7−12.

(6) Yamakoshi, J.; Piskula, M. K.; Izumi, T.; Tobe, K.; Saito, M.; Kataoka, S.; Obata, A.; Kikuchi, M. Isoflavone Aglycone-Rich Extract without Soy Protein Attenuates Atherosclerosis Development in Cholesterol-Fed Rabbits. *J. Nutr*. **2000**, *130*, 1887−1893.

(7) Coward, L.; Barnes, N. C.; Setchell, K. D. C.; Barnes, S. Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *J. Agric. Food Chem*. **1993**, *41*, 1981−1987.

(8) Ohno, S.; Nakajima, Y.; Inoue, K.; Nakazawa, H.; Nakajin, S. Genistein administration decreases serum corticosterone and testosterone levels in rats. *Life Sci*. **2003**, *26*, 733−742.

(9) Hwang, T. C.; Koeppe, R. E.; Andersen, O. S. Genistein can modulate channel function by a phosphorylation-independent mechanism: importance of hydrophobic mismatch and bilayer mechanics. *Biochemistry* **2003**, *25*, 13646−13658.

(10) Wei, H.; Saladi, R.; Lu, Y.; Wang, Y.; Palep, S. R.; Moore, J.; Phelps, R.; Shyong, E.; Lebwohl, M. G. Isoflavone genistein: photoprotection and clinical implications in dermatology. Isoflavone genistein: photoprotection and clinical implications in dermatology. *J Nutr.* **2003,** *133*, 3811S-3819S.

(11) Cotter, A.; Cashman, K. D. Genistein appears to prevent early postmenopausal bone loss as effectively as hormone replacement therapy. *Nutr. Rev*. **2003**, *61*, 346−351

(12) Cappelletti, V.; Fioravanti. L.; Miodini, P., Di Fronzo, G. Genistein blocks breast cancer cells in the G(2)M phase of the cell cycle. *J. Cell Biochem*. **2000**, *14*, 594−600.

(13) Naim, M.; Gestetner, B.; Zilkah, S.; Birk, Y.; Bondi, A. Soybean isoflavones: characterization, determination, and antifungal activity. *J. Agric. Food Chem*. **1974**, *22*, 806−810.

(14) Hosel, W.; Barz, W. β-glucosidases from *Cicer Arietinum L.*: purification and properties of isoflavone 7-O-glucoside β-glucosidases. *Eur. J. Biochem*. **1975**, *57*, 607−616.

(15) Hinder, W.; Koster, J.; Barz, W. Purification and properties of a specific isoflavone 7-O-glucoside-6″-O-malonate malonesterase from roots of chickpea (*Cicer Arietinum L.*). *Arch. Biochem. Biophys*. **1986**, *248*, 570−578.

(16) Hsieh, M. C.; Graham, T. L. Partial purification and characterization of a soybean β-glucosidase with high specific activity towards isoflavone conjugates. *Phytochemistry* **2001**, *58*, 995−1005.

(17) Tan, R. X.; Zou, W. X. Endophytes: A rich source of functional metabolites. *Nat. Prod. Rep.* **2001**, *18*, 448−459.

(18) Zikmundova, M.; Drandarov, K.; Bigler, L.; Hesse, M.; Werner, C. Biotransformation of 2-benzoxazolinone and 2-hydroxy-1,4-benzoxazin-3-one by endophytic fungi isolated from Aphelandra tetragona. *Appl. Environ. Microbiol*. **2002**, *68*, 4863−4870.

(19) Matsuura, M.; Sasaki, J.; Murao, S. Studies on β-glucosidases from soybeans that hydrolyze daidzin and genistin: isolation and characterization of an isozyme. *Biosci. Biotech. Biochem*. **1995**, *59*, 1623−1627.

(20) Matsuura, M.; Obata, A. β-glucosidases from soybeans hydrolyze daidzin and genistin. *J. Food Sci.* **1993**, *58*, 144−147.

(21) Oh, K. B.; Kazu, H.; Mikako, S.; Lee, H, J. Isolation and properties of an extracellular β-glucosidase from a filamentous fungus, *Cladosporium resinae*, isolated from kerosene. *Biosci. Biotech. Biochem*. **1999**, *63*, 281−287.

(22) Yan, T. R.; Lin, C. L.; Purification and characterization of a glucose-tolerant β-glucosidase from *Aspergillus niger* CCRC31494. *Biosci. Biotech. Biochem*. **1997**, *61*, 965−970.

(23) Hessler, P. E.; Larsen, P. E.; Constantinou, A. I.; Schram, K. H.; Weber, J. M. Isolation of isoflavones from soy-based fermentations of the erythromycin-producing bacterium *Saccharopolyspora erythraea*. *Appl. Microbiol. Biotechnol*. **1997**, *47*, 398−404.

(24) Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **1976**, *72*, 248−254.

(25) Christine, R.; Salmon, J. M.; Vallier, M. J.; Gunata, Z. Purification, characterization, and substrate specificity of a novel highly glucose-tolerant β-glucosidase from *Aspergillus oryzae*. *Appl. Environ. Microbiol*. 1998, *64*, 3607−3614.

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