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ARTICLE

# β-Glucosidase Isolated from Soybean Okara Shows Specificity toward Glucosyl Isoflavones

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A novel β-glucosidase was isolated from soybean okara in this study. Along with the β-glucosidase, a considerable basic 7S globulin of soybean was obtained in the initial extraction products. The protein samples pretreated with 130 mM dithiothreitol before the step of CM-Sepharose chromatography could greatly enhance the separation of the targeted β-glucosidase from the impurities. The purified β-glucosidase was found to be a monomer estimated to be 75 kDa by SDS−PAGE. The optimal temperature and pH for this β-glucosidase were 45 °C and 4.5, respectively. The activity of this purified β-glucosidase was completely inhibited by 1 mM $Hg^{2+}$ or 10 mM $Al^{3+}$ ion, and glucose and mannose also affected the activity. This β-glucosidase possessed strict specificity toward glucosyl isoflavones but not malonylglucosidic conjugates of isoflavones of soybean. The N-terminal amino acid sequence of the β-glucosidase was EYLKYKDPKA-, which highly matched that of glycosidases in maize (*Zea mays*) and wheat (*Triticum asetivum*).

**KEYWORDS: β-Glucosidase; okara; dithiothreitol; soybean isoflavones**

## INTRODUCTION

Soybean is rich in a group of bioactive compounds known as isoflavones. Isoflavones are called phytoestrogens because they resemble the chemical conformations of estrogens and similarly recognize estrogen receptors (*1*). Many physiological bioactivities of isoflavones have been found to be related to hormone regulation, including amelioration of postmenopausal symptoms (*2*) and increasing bone density in postmenopausal women (*3*). Isoflavones have also been found to reduce the risk of coronary heart disease in humans by lowering serum low-density lipoprotein cholesterol with their antioxidant activities (*4*,*5*). In cancer research, isoflavones have been found to reduce the incidence of breast cancer (*6*), and their usefulness in the treatment of prostate cancer has been studied (*7*).

The content of isoflavones in soybean ranges from 50 to 3,000 μg/g (*8*). Isoflavones are known to occur in 12 conjugation forms, including 3 aglycones (daidzein, genistein, and glycitein), and 9 glucosidic conjugates. Glucosidic conjugates of isoflavones consist of 3 β-glucosides (daidzin, genistin, and glycitin), 3 acetylglucosides (6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin, and 6″-*O*-acetylglycitin), and 3 malonylglucosides (6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin, and 6″-*O*-malonylglycitin). The aglycone forms of isoflavones are absorbed in the human gastrointestinal system more efficiently and to a greater extent than are the glucosidic forms (*9*, *10*). Although isoflavone glycosides are hydrolyzed in part to aglycones by saliva, then by the gut microflora, the rate of the transformation is low (*11*, *12*). Only 33% of people in western populations can metabolize daidzein glucosides to daidzein and daidzein to equol, the metabolite with stronger estrogenic effect, in the intestinal tract (*10*). Therefore, products with increased transformation of isoflavone glycosides to aglycones could be a novel addition to the food industry.

Recently, many researchers have investigated the transformation of isoflavone glycosides to aglycones by enzymatic hydrolysis and microbial fermentation. Chien et al. (*13*) studied the transformation of isoflavone phytoestrogens during the fermentation of soy milk with lactic acid bacteria and bifidobacteria. Otieno and Shah (*14*) reported on the enzymic potential of biotransforming isoflavone phytoestrogen in soy milk by selected lactic acid bacteria. Similarly, Raimondi et al. (*15*) studied the bioconversion of the soy isoflavones daidzin and daidzein by *Bifidobacterium* strains. In these studies, β-glucosidase (EC 3.2.1.21), secreted by bacteria, was considered responsible for the biotransformation of isoflavone glycosides to aglycones. In addition, the content of isoflavone aglycones was significantly higher in fermented soybean products such as miso, natto, and tempeh inoculated with *Saccharomyces rouxii*, *Bacillus subtilis* natto, and *Rhizopus oligosporus* than in nonfermented soybean (*16*, *17*). The release of aglycones from isoflavone glucosides by the enzymatic hydrolysis of β-glucosidase during the fermentation process was suggested to be responsible for the increased aglycone content of the products. The composition of isoflavone conjugates in soybean products such as tofu or soybean curds can also be changed during manufacturing (*18*).

The β-glucosidase activity of soybean has been shown to have an important role in the transformation of isoflavones in beans or roots during germination and growth (*19*−*22*). However, research into the biochemical characteristics of soybean β-glucosidase and/or the transformation of isoflavones by β-glucosidase in soybean is limited.

In this study, we discovered significant β-glucosidase activities in soybean okara, the byproduct of soy milk and tofu-making. We aimed to purify and characterize the newly discovered

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β-glucosidase and compare its biochemical properties with that of β-glucosidases previously published (*19*−*22*). We also report on the transformation of soybean isoflavones by this β-glucosidase.

## MATERIALS AND METHODS

**Materials.** Organic B0552 soybeans were obtained from a local agency in Taiwan. ρ-Nitrophenol, dithiothreitol, and electrophoresis reagents were from Sigma-Aldrich (St. Louis, MO). CM-Sepharose CL-6B cation exchanger, Hiload 16/60 Superdex 75 column, and protein standards were from GE Healthcare Bio-Sciences Co. (Piscataway, NJ, USA). All chemicals used were of analytical grade.

**Preparation of Soybean Okara with β-Glucosidase Activity.** Soybean okara was produced according to the traditional method for making soy milk. Briefly, 200 g of soybeans were soaked in 600 mL of tap water at 4 °C overnight. After draining the soaking water, the swollen soybeans were homogenized with 2 L of distilled water in a blender (Micro-Sensor Smart-Control Organic Food Processor LVT-609, Ken Ying Enterprise, Taiwan). The slurry was poured into a cotton filter bag (approximately 100-mesh pore size) and underwent vacuum filtration for separating soy milk and residues. The residue was repeatedly washed with 2 L of distilled water at least 10 times until the washed water was clear. The remaining residue was then freeze-dried by the use of a Labconco Freeze-Dry System (Lyph-Lock 18, Labconco Co., Kansas city, Missouri, USA) to obtain the okara. In total, 0.5 g of freeze-dried okara underwent extraction with 40 mL of various NaCl solutions (0.2%−10%) at 4 °C overnight, then centrifugation at 13,000*g* for 3 min. The supernatant was then analyzed for β-glucosidase activity (ρ-nitrophenyl β-D-glucopyranoside [ρNPG] assay) and protein concentration (Bradford method).

**Purification of the β-Glucosidase from Okara.** *Preparation of the Crude Extract*. The crude extract of the β-glucosidase was prepared by homogenizing 200 g of okara in 1 L of 2% (w/v) NaCl aqueous solution, then stirring at ambient temperature for 1 h. The extracted solution was separated by vacuum suction in a Buchner funnel with a filter paper (No.1, Advantec, Toyo Roshi Kaisha, Japan). The okara residue underwent extraction again in the same way. The extracted solutions were combined to total 2 L.

*Acetone Fractionation*. Two liters of extracted solution was concentrated to a final volume of 100 mL by ultrafiltration (Hollow Fiber Cartridge, MWCO 5 kDa, GE Healthcare Bio-Sciences). Cold acetone of −20 °C was slowly dropped into the concentrated solution until the acetone concentration was 40% (v/v) and then incubated at 4 °C for 30 min. The precipitated protein was collected by centrifugation (15,000 *g*, 20 min). Likewise, the remaining supernatant was sequentially fractionated by precipitation with the solution and 50%, 60%, and 80% acetone. The precipitates, obtained from acetone concentrations of 0−40%, 40%−50%, 50%−60%, and 60%−80% intervals, were dissolved with 10 mL of 50 mM, pH 4.5 phosphate buffer for the β-glucosidase activity assay.

*Dithiothreitol (DTT) Treatment*. The greatest β-glucosidase fraction (i.e., the precipitate obtained from an acetone concentration of 50%−60% interval) was added to 10 mL of 50 mM, pH 4.5, phosphate buffer containing 130 mM DTT and incubated in a 45 °C water bath for 1 h. The mixture was then centrifuged, and the supernatant was separated as the reduced enzyme solution.

*Purification by Column Chromatography*. The enzyme solution was further purified by CM-Sepharose CL-6B cation-exchange chromatography. In total, 10 mL preswollen resin was packed into a column (C 16/40, GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA) and equilibrated with 50 mM, pH 4.5, phosphate buffer. The active solutions were then loaded onto the top of the column and were eluted under the same phosphate buffer at 1.0 mL/min. The adsorbed fraction on the CM-Sepharose CL-6B was eluted with a gradient concentration of NaCl from 0 to 1 M in 50 mM, pH 4.5, phosphate buffer at a rate of 5 mM/min. Fractions with significant β-glucosidase activity were combined and concentrated to 1 mL by ultrafiltration (Amicon Ultra-15, 50 kDa MWCO, Millipore Co., MA, USA). A gel filtration column (Amersham Hiload 16/60 Superdex 75) was used for a further purification step. The active enzyme solution was loaded onto the column and eluted with 50 mM, pH 4.5 phosphate buffer at a flow rate of 0.75 mL/min. The eluate was collected in 1.5-mL fractions.

**β-Glucosidase Detection and SDS−PAGE Analysis.** β-Glucosidase activity was assayed with ρNPG used as the substrate. A mixture of 100 μL, 1 mM ρNPG (dissolved in 0.5 M, pH 4.5, phosphate buffer), and 100 μL of enzyme solution was incubated in a 45 °C water bath for 30 min, then 400 μL of 1.0 M sodium carbonate solution was added, and the absorbance was detected at 405 nm. A standard curve of 0 to 1000 μM ρ-nitrophenol solution in 100 μL, 0.5 M, pH 4.5 phosphate buffer was prepared by reacting with 400 μL of 1.0 M sodium carbonate in the same way. The unit of β-glucosidase activity was defined as the amount of enzyme releasing 1 nmol of ρ-nitrophenol per min from the substrate.

SDS−PAGE was performed as described by Laemmli (*23*) in a Hoefer Mighty Small II SE-250 mini vertical gel electrophoresis unit. Analysis was carried out under nonreducing and reducing (with 10% β-mercaptoethanol) conditions. A mixture of markers obtained from Amersham Pharmacia Biotech was used as the protein weight reference. Protein gels were visualized with silver staining as described (*19*).

**Characterization of the Purified β-Glucosidase.** To measure the effects of temperature and pH on the purified β-glucosidase activity, assays were carried out at different temperatures (20−70 °C) and different pH values (pH 3−10), with acetate (pH 3−5), phosphate (pH 6−8), and glycine-NaOH (pH 9−10) used as buffers. The effect on the purified enzyme was expressed as relative activity as compared with that determined at 45 °C and pH 4.5 for 60 min. The effect of metallic salts and carbohydrates on the purified β-glucosidase was determined under the following conditions. Aliquots of 50 μL of diluted enzyme solutions were added to an equal volume of 50 mM, pH 4.5 phosphate buffer, each containing 2 or 20 mM of one of the following compounds: the metallic salts $CoCl_2$, $MnCl_2$, $NiCl_2$, $ZnCl_2$, $BaCl_2$, $AlK(SO_4)_2$, $Li_2SO_4$, $CaCl_2$, $MgCl_2$, and $HgCl_2$, and the carbohydrates fructose, galactose, glucose, glucuronic acid, glucosamine, and mannose. Enzyme mixtures were incubated with 100 μL of 1 mM ρNPG at 45 °C for 30 min. The residual activity was measured as described above, and inhibition or activation was expressed as a percentage of the activity without the effector.

**Tests for Substrate Specificity of the Purified β-Glucosidase toward Soybean Isoflavones.** To study the substrate specificities of the isolated β-glucosidase, partially purified soybean isoflavones were prepared as the isoflavone-substrate for the enzyme assay. Soybeans were soaked overnight in 3 volumes (v/w) of 4 °C tap water. After draining the soaking water, the same volume of distilled water was added to the swollen soybeans for homogenization in a blender. The soybean slurry was lyophilized and ground to pass through a 40-mesh sieve. In total, 100 g of lyophilized powder was extracted with 1 L of 60% methanol solution under shaking for 2 h and filtered by vacuum suction. The residue was washed twice with 200 mL of 60% methanol. The filtrates were combined, and the solvents were removed under reduced pressure to obtain the crude extracts of isoflavones. The crude extracts were then resuspended with a small amount of distilled water and loaded onto a column with 20 g of Diaion HP20 resin (Mitsubishi Chemical Corp., Tokyo, Japan). After extensive washing with 2 L of distilled water to remove impurities, the adsorbed fractions were eluted with 70% methanol and then evaporated to dryness under vacuum. The dried residue, namely, the partially purified soybean isoflavones, was used for the analysis of isoflavones by high-performance liquid chromatography (HPLC) and was used as the substrate for testing the specificity of the purified β-glucosidase. An amount of 0.1 g of the partially purified soybean isoflavones was dissolved in 1 mL of 0.5 M, pH 4.5 phosphate buffer and equilibrated under a water bath at 45 °C. Catalytic reaction of the purified β-glucosidase was initiated by adding 1 mL of the purified enzyme solution and mixing well. After incubation at 45 °C for 1 h, 2 mL of methanol containing 1000 ppm benzoic acid was added to stop the reaction. The reaction mixture underwent centrifugation to remove insoluble substances, and the supernatant underwent composition analysis of isoflavones. A linear HPLC gradient was composed of 0.1% glacial acetic acid in $H_2O$ (solvent A) and 0.1% glacial acetic acid in acetonitrile (solvent B). Following the injection of 20 μL of sample, solvent B was increased from 10% to 35% over 60 min, then increased to 90% within the next 3 min, and finally to 10% within 2 min and held at that percentage for the next 12 min. The solvent flow rate was 1 mL/min. The HPLC system involved Thermo Separation Product ConstaMeric 3200 and 3500 gradient pumps equipped with a Thermo Separation Product SpectroMonitor 3200 digital UV/vis detector. A reverse-phase analytical column (YMC-pack ODS-AM, 4.6 × 250 mm, 5 μm, YMC Co., Ltd., Kyoto, Japan) was used for separation. The eluted components were detected at 254 nm.

**N-Terminal Sequence Analysis of the β-Glucosidase.** The N-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation with use of the Procise 494 protein sequencer

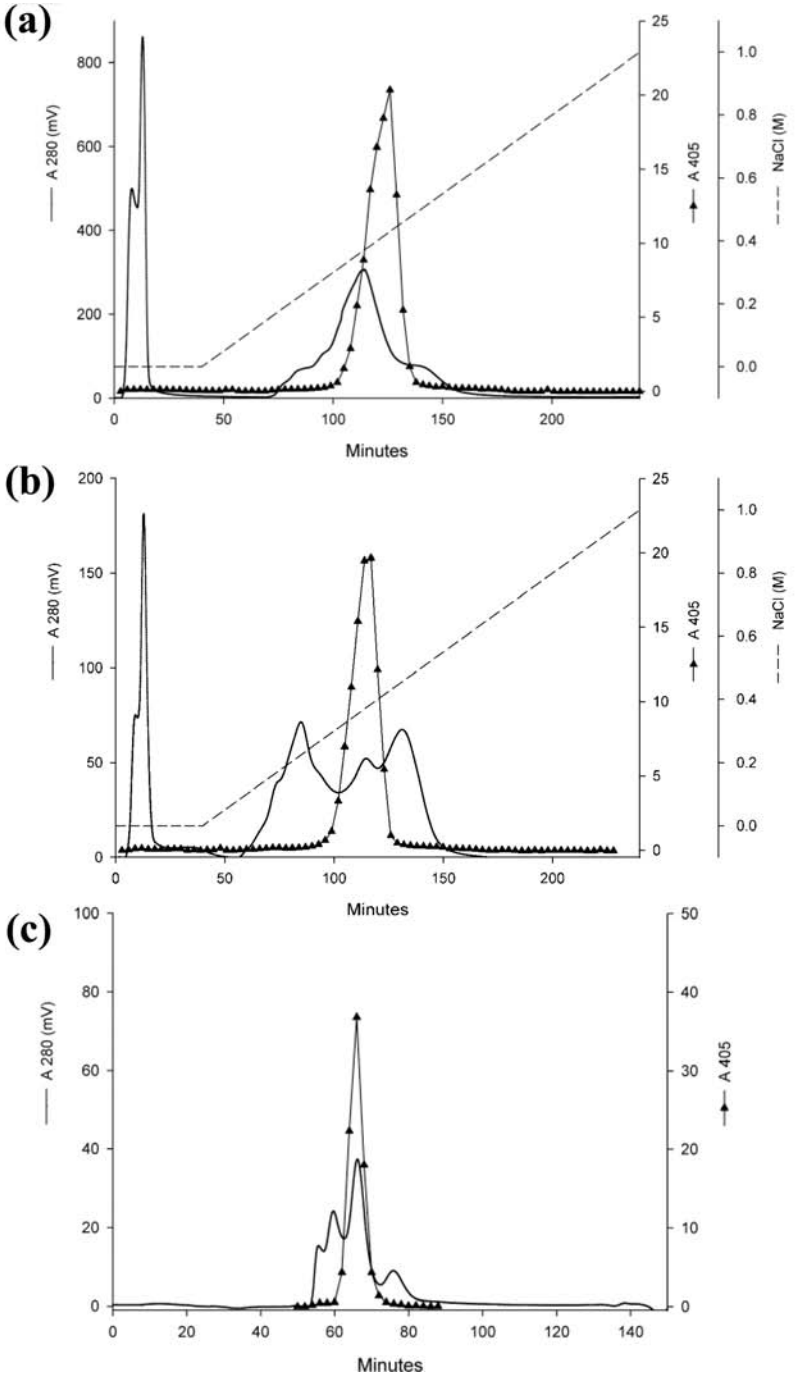


**Figure 1.** Purification of the $\beta$-glucosidase obtained from soybean okara. The chromatograms obtained by CM-Sepharose CL-6B cation-exchange chromatography without (**a**) and with dithiothreitol treatment (**b**), and gel filtration on a Superdex 75 column (**c**).

(Applied Biosystems, Foster city, CA, USA). Sequences were compared with known grain $\beta$-glucosidases found by a BLAST search of the NCBI database.

## RESULTS

**Extraction of $\beta$-Glucosidase from Soybean Okara.** Soybean okara was prepared by homogenizing 200 g of soybeans in 10 volumes (v/w) of distilled water. After extensive washing with distilled water, 217 g of wet okara was obtained. After lyophilization, 43 g of dry okara (21.5% yield) was recovered. The moisture content of wet okara was approximately 80%. The optimal NaCl concentration for extracting $\beta$-glucosidase from okara was examined by extracting with 0.2−10% (w/v) NaCl solution. Only little $\beta$-glucosidase activity was detected when the extraction solution contained NaCl less than 1%. The $\beta$-glucosidase activity was highest with 2.0% NaCl extraction. Increasing the salt concentration resulted in decreased specific $\beta$-glucosidase activity (unit/mg protein) in the extract. For instance, the specific activities with 5.0%, 7.5%, and 10.0% NaCl were only respectively 80%, 65%, and 40% of the activity with 2.0% NaCl.

**Purification of $\beta$-Glucosidase from Soybean Okara.** The $\beta$-glucosidase was purified from okara as described. About 87% of $\beta$-glucosidase activity was recovered from the precipitate with the 50%−60% acetone interval. Subsequently, the active acetone fractions with or without DTT treatment were loaded onto a cation-exchange CM-Sepharose CL-6B column. The elution patterns from chromatography are shown in **Figure 1a** and **b**. DTT treatment gave better separation, with a minor protein peak,

**Table 1.** Summary of the Procedures for Purification of the $\beta$-Glucosidase from Soybean Okara

| step | total protein (mg) | total activity (units) | activity recovery (%) | specific activity (unit/mg) | purification (fold) |
|---|---|---|---|---|---|
| crude extract | 1580 | 67800 | 100 | 43.0 | 1.0 |
| acetone fractionation | 42.3 | 11909 | 17.6 | 282 | 6.5 |
| dithiothreitol treatment | | 13100 | 19.3 | | |
| CM-Sepharose CL-6B chromatography | 0.8 | 2845 | 4.2 | 3570 | 83 |
| Superdex 75 gel filtration | 76 $\mu$g | 593 | 0.88 | 7850 | 183 |

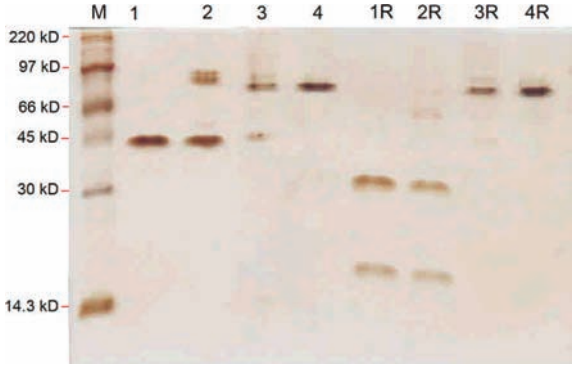


**Figure 2.** SDS−PAGE analysis (12.5% acrylamide) of protein samples from each step of purification. Lane M, protein markers; lane 1, crude enzyme extract; lane 2, 50%−60% precipitated acetone fraction; lane 3, activity peak from CM-Sepharose CL-6B chromatography; lane 4, activity peak from Superdex 75 gel filtration. Lanes 1R−4R: protein samples were as described above for lanes 1−4 but were treated with $\beta$-mercaptoethanol for SDS−PAGE.

which was eluted out under the conditions of elution buffers containing more than 0.3 M NaCl, reflecting $\beta$-glucosidase activity. The active fractions were pooled and dialyzed to remove NaCl, then concentrated by ultrafiltration for gel filtration. The elution pattern for Superdex 75 gel filtration is shown in **Figure 1c**. After Superdex 75 gel filtration, the $\beta$-glucosidase from okara was purified to homogeneity. The purification steps and yields are in **Table 1**. In this purification process, 593 units of the purified $\beta$-glucosidase were obtained. The recovery was 0.88%, with a specific activity of 7850 U/mg and a 183-fold purification efficiency.

The SDS−PAGE profiles of the $\beta$-glucosidase-related fractions, including the crude extract, by acetone fractionation, CM-Sepharose CL-6B chromatography, and Superdex 75 gel filtration are in **Figure 2**. SDS−PAGE of the purified $\beta$-glucosidase under denaturing conditions revealed a single protein band of estimated 75 kDa (**Figure 2**, lane 4R). In addition, the purified $\beta$-glucosidase on treatment with and without $\beta$-mercaptoethanol showed the same migration distances in the 12.5% acrylamide gel (containing 0.1% SDS), which indicated that the purified $\beta$-glucosidase was a monomer and constituted one polypeptide chain (**Figure 2**, lanes 4, 4R). The $\beta$-glucosidase we isolated from soybean okara differed from the homodimeric $\beta$-glucosidase C (36 kDa) reported by Matsuura et al. (*21*).

**Characterization of the Purified $\beta$-Glucosidase.** The optimal temperature and pH for the activity of the purified $\beta$-glucosidase were 45 °C and pH 4.5, respectively (**Figure 3**). Under pH 4.5, the relative activity of $\beta$-glucosidase at 30 and 60 °C was about 44% and 40% of its activity determined at 45 °C. In addition, about 80% of the relative activity at 45 °C was retained at both pH 4.0 and 5.0. However, the relative activity dropped markedly to only about 5% at pH 3.5. This point would be critical in application. The $\beta$-glucosidase was stable at pH 4−6 and at temperatures below 70 °C. After incubation at 70 °C and pH 4.5 for 1 h, $\beta$-glucosidase still retained about 85% of its original activity.

The effects of metallic ions, carbohydrates, and chemicals on the activity of $\beta$-glucosidase are in **Table 2**. Mercury cation $Hg^{2+}$ is

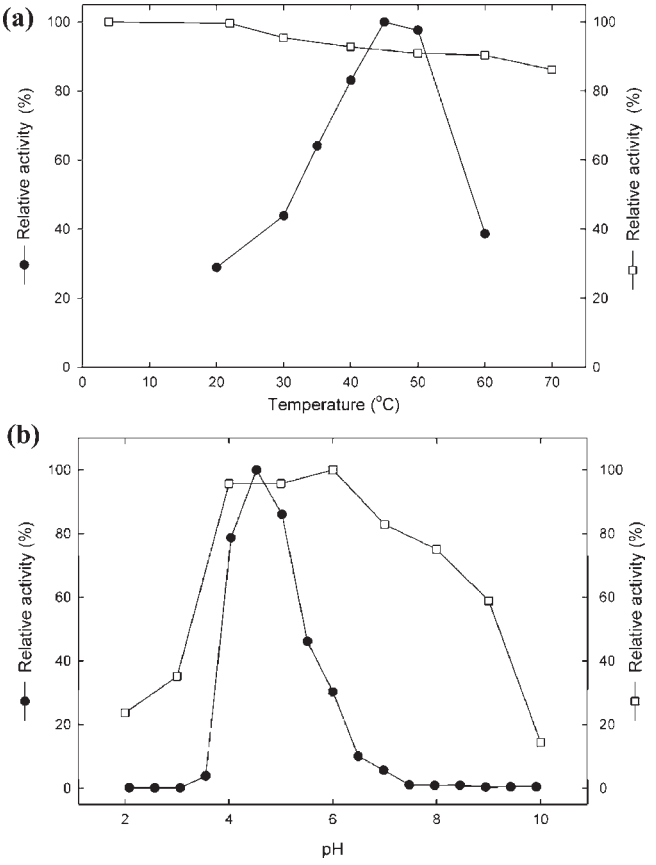


**Figure 3.** Effect of temperature and pH on the activity of the $\beta$-glucosidase from soybean okara. Effect of temperature (**a**) and pH (**b**) on the activity (●) and stability (□) of the purified $\beta$-glucosidase. Activity was relative to that detected at 45 °C and pH 4.5. Stability tests were performed at various pH values or temperatures by incubating for 60 min.

known to have unfavorable effects on the −SH groups of proteins. In addition, $Al^{3+}$ had a negative effect on the purified $\beta$-glucosidase. In this study, the activity of $\beta$-glucosidase was completely inhibited by 1 mM $Hg^{2+}$ or 10 mM $Al^{3+}$. In addition, among the tested carbohydrates, glucose and mannose substantially inhibited the activity of $\beta$-glucosidase. Approximately, 50% activity was inhibited by 10 mM glucose or mannose, whereas only 20% activity was inhibited by 10 mM galactose. No inhibition effects were observed with 10 mM fructose. In addition, glucuronic acid was the most inhibitive monosaccharide against $\beta$-glucosidase activity. Less than 10% of activity was left in the presence of 10 mM glucuronic acid. Thus, the substrate selectivity of the $\beta$-glucosidase was sensitive to the configurations of a hydroxyl group at C5 and C6 but less sensitive with one at C2. Although both glucose and mannose similarly inhibited the activity of this enzyme, no inhibition was found with the same concentration of glucosamine. Therefore, the availability of a C2 hydroxyl group might be essential to the selective catalysis of this $\beta$-glucosidase.

We found no inhibitive effects on enzyme activities with reducing reagents, 65 mM DTT or 730 mM $\beta$-mercaptoethanol.

**Table 2.** Effects of Metallic Salts and Reagents on the Activity of the Purified $\beta$-Glucosidase from Soybean Okara[a]

| reagent | mM | residual activity (%) | reagent | mM | residual activity (%) |
|---|---|---|---|---|---|
| $CoCl_2$ | 10 | 91 | glucuronic acid | 10 | 8 |
| $MnCl_2$ | 10 | 98 | | 1 | 91 |
| $NiCl_2$ | 10 | 105 | galactose | 10 | 80 |
| $ZnCl_2$ | 10 | 80 | fructose | 10 | 100 |
| $BaCl_2$ | 10 | 91 | glucose | 10 | 50 |
| $AlK(SO_4)_2$ | 10 | 0 | glucosamine | 10 | 100 |
| | 1 | 82 | mannose | 10 | 50 |
| $Li_2SO_4$ | 10 | 92 | $Na_2 \cdot EDTA$ | 10 | 100 |
| $CaCl_2$ | 10 | 100 | dithiothreitol | 65 | 110 |
| $MgCl_2$ | 10 | 92 | iodoacetamide | 65 | 96 |
| $HgCl_2$ | 1 | 0 | $\beta$-mercaptoethanol | 730 | 90 |

[a] The $\beta$-glucosidase was incubated with metallic salts or reagents (as indicated) at 45 °C for 30 min, and then the residual activity was measured. Data was expressed as a percentage of the activity without the effector.

The results supported the lack of intermolecular disulfide linkage in the protein molecule of $\beta$-glucosidase, as was confirmed by SDS−PAGE results (**Figure 2**). However, complete inhibition by 1 mM $Hg^{2+}$ indicated the possible existence of an intramolecular sulfhydryl group. In addition, iodoacetamide had little influence on the $\beta$-glucosidase activity (**Table 2**), probably because the sulfhydryl group located in active sites was wrapped inside and kept unharmed.

**Substrate Specificity of the $\beta$-Glucosidase.** We used the partially purified soybean isoflavones containing glucosidic isoflavones, malonylglucosidic conjugates, and aglycones as the substrates of the isolated $\beta$-glucosidase. The peaks of daidzin and genistin in the hydrolytic product of the substrate were much reduced in $\beta$-glucosidase as compared with the experimental control (**Figure 4**). However, after 60 min of hydrolysis, the amount of aglycones daidzein and genistein were formed remarkably. The results suggested that glucosyl-conjugated isoflavones were hydrolyzed into their corresponding aglycones, namely, daidzin and genistin, and were converted to daidzein and genistein by the enzyme. However, malonylglucosidic isoflavones were not hydrolyzed at all and remained intact. The results in **Table 3** indicate that the amount of daidzein and genistein increased with reduced daidzin and genistin because of the hydrolysis of the $\beta$-glucosidase. Accordingly, the $\beta$-glucosidase was considered to have strict substrate specificity toward glucosyl isoflavones but not malonyl conjugates of isoflavones.

**N-Terminal Sequence Analysis of the $\beta$-Glucosidase.** The N-terminal amino acid sequence was determined to be Glu-Tyr-Leu-Lys-Tyr-Lys-Asp-Pro-Lys-Ala-. A BLAST search of the sequence in the NCBI database did not reveal a relevant sequence of *Glycine max* and whether this partial sequence belonged to a highly conserved sequence of glycosidases (EC 3.2.1.*). **Figure 5** shows the sequence to have high similarity to those of glucan hydrolases from *Zea mays* (exoglucanase precursor), *Triticum asetivum* ($\beta$-D-glucan exohydrolase), *Gossypium hirsutum* (endo-α-1.4-glucanase), *Hordeum vulgare* subsp. *Vulgare* ($\beta$-D-glucan exohydrolase), *Lilium longiflorum* (exo-1,3-$\beta$-glucosidase), *Nicotiana tabacum* ($\beta$-D-glucan exohydrolase), *Phillyrea latifolia* ($\beta$-1,3-glucanase), and *Oryza sativa* ($\beta$-glucosidase precursor).

## DISCUSSION

In this study, we isolated a novel $\beta$-glucosidase from soybean okara by a standard procedure of protein purification but with dithiothreitol (DTT) treatment. DTT treatment was an important and helpful step to eliminate the contaminant proteins from the proteins obtained from acetone fractionation and increase the efficiency of consequent purification. According to Bourne et al. (*24*), dry

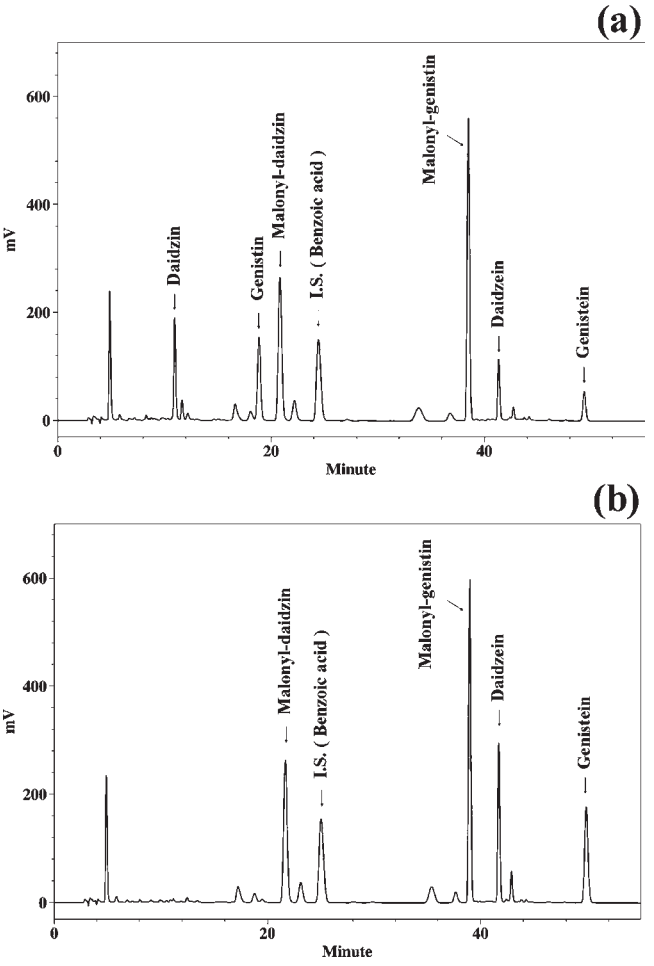


**Figure 4.** High-performance liquid chromatography of isoflavones in the substrate material after enzymatic reaction with the $\beta$-glucosidase of soybean okara at 45 °C, pH 4.5, for 60 min. (**a**) Incubation with the inactivated enzyme solution; (**b**) incubation with the native enzyme.

okara contains protein up to 18.2%−32.2% protein, 6.9%−22.2% crude fat, and 9.1%−18.6% crude fiber. Because most soybean seed proteins are salt-soluble globulins and the targeted $\beta$-glucosidase was obtained from okara with 10 volumes (v/w) of 2% NaCl solution, we speculated that a considerable amount of saline extractable globulins would exist in the products of the initial extraction. The Asano et al. (*25*) study of the proteins of okara revealed that 90% of saline extractable proteins were the basic 7S globulin. Basic 7S globulin is a cysteine-rich glycoprotein that consists of 27- and 16-kDa subunits linked by disulfide bonding and accounts for ∼3% of the total protein in mature soybean seeds (*26*−*29*). Our SDS−PAGE results revealed that 27- and 16-kDa proteins produced with 2-mercaptoethanol treatment originated from soybean basic 7S globulin (**Figure 2**, lanes 1R and 2R). Therefore, we considered that the most abundant protein in the crude extract was the basic 7S globulin.

The DTT treatment also enlightened the results of SDS−PAGE. Treatment with the reducing agent could reduce the basic 7S globulin into the corresponding subunits. Therefore, we used DTT treatment before ion-exchange chromatography separation to increase the performance of purification and also tested the effects of DTT on $\beta$-glucosidase activity of crude extracts. Fortunately, the activity of the $\beta$-glucosidase was resistant to reducing treatment with DTT (**Table 2**) and facilitated the separation of $\beta$-glucosidase from the impurities of basic 7S globulin in soybean okara.

**Table 3.** Contents of the Main Isoflavones in the Isoflavone Substrate and the Hydrolytic Product by Hydrolysis of the β-Glucosidase[a]

| | μmol/0.1 g of sample | | | | | |
|---|---|---|---|---|---|---|
| sample | Daidzin | Genistin | Malonyldaidzin | Malonylgenistin | Daidzein | Genistein |
| isoflavone-substrate | 20.6 | 19.8 | 29.6 | 47.8 | 21.8 | 19.1 |
| hydrolytic product | | | 30.7 | 49.2 | 44.2 | 41.3 |

[a] Experimental details are described in the text.

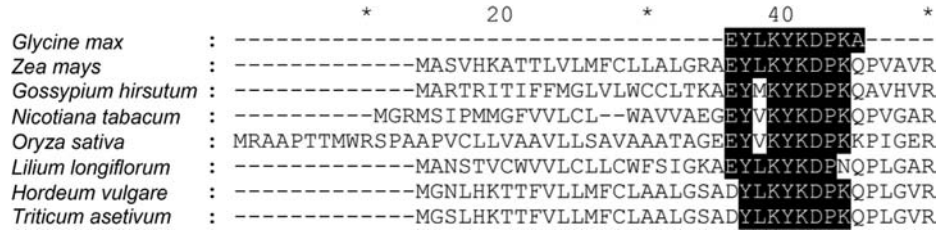


**Figure 5.** Alignment of N-terminal amino acid sequence of the purified β-glucosidase and some selected carbohydrate hydrolases from a BLAST search of the NCBI database. *Zea mays* (exoglucanase 1, accession no. NP_001130296.1); *Gossypium hirsutum* (endoalpha-1,4-glucanase, accession no. AAZ15705.1); *Nicotiana tabacum* (β-D-glucan exohydrolase, accession no. BAA33065.1); *Oryza sativa* (β-glucosidase precursor, accession no. NP_001051274.1); *Lilium longiflorum* (exobeta-glucanase, accession no. AAR14129.1); *Triticum aestivum* (β-D-glucan exohydrolase, accession no. AAM13694.1); *Hordeum vulgare* (β-D-glucan exohydrolase, accession no. AAC49170.1).

Several reports describe glucosidase activity in soybean, but only a few have given the biochemical characteristics of β-glucosidase in soybean. Especially in the past 2 decades, only 4 relevant papers have been published. Suzuki et al. (*22*) reported on an isoflavone conjugate-hydrolyzing β-glucosidase, which was synthesized abundantly in the roots of *Glycine max* seedlings but only negligibly in the hypocotyl and cotyledon. This enzyme was a homodimer of an estimated subunit of 58 kDa and with the N-terminal amino acid sequence of Asp-Ser-Val-Pro-Leu (*22*). Hsieh and Graham (*19*) reported that a partially purified β-glucosidase from soybean roots was most likely a dimer (approximately 165 kDa) with potential subunits of 80 and/or 75 kDa and that the optimal pH and temperature were pH 6.0 and 30 °C, respectively. Matsuura and Obata (*20*), and Matsuura et al. (*21*) detected 3 potent β-glucosidases (A, B, and C) in soybean cotyledons, but only β-glucosidase-C was isolated and characterized. This enzyme was estimated to be 81 and 36 kDa by gel filtration and SDS−PAGE, respectively. The enzyme showed optimal activity at pH 4.5 and 45 °C, with a pH range of action of 3.5−7.0. Our comparison of the molecular mass and biochemical properties of the newly purified β-glucosidase, a monomer of 75 kDa, differed from those of previously reported β-glucosidases in soybeans. In addition, this β-glucosidase possessing strict specificity toward glucosyl isoflavones but not malonylglucosidic isoflavones also differed from the characteristics of β-glucosidases in soybean roots, which were reported to catalyze the hydrolysis of malonylglucosidic isoflavones (*19*, *22*). Malonylglucoside-hydrolyzable β-glucosidases were not found in cotyledons or germs of dormant soybeans but were highly expressed in roots during germination.

Isoflavone aglucones were previously considered to be responsible for the objectionable flavor of soy milk. Matsuura et al. (*30*) reported that the quantities of daidzein and genistein in soy milk paralleled the objectionable aftertaste, and the authors provided a method to make soy milk with minimal objectionable aftertaste. However, along with increasing interest in phytochemicals in the diet, scientific publications have reported isoflavone aglycones with remarkable function related to the reduced risk of cardiovascular disease, and prostate, breast and colon cancers, and improved bone health. The results of this study would be very useful in the application of β-glucosidases on increasing the content of isoflavone aglycones of soybean products as well as in biochemical research.

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