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Hydrolysis of soybean isoflavone glycosides by a thermostable β-glucosidase from *Paecilomyces thermophila*

Shaoqing Yang [a], Lijun Wang [a], Qiaojuan Yan [b], Zhengqiang Jiang [a,*], Lite Li [a]

[a] Department of Biotechnology, College of Food Science and Nutritional Engineering, China Agricultural University, P.O. Box 294, No. 17 Qinghua Donglu, Haidian District, Beijing 100083, China
[b] Bioresource Utilization Laboratory, College of Engineering, China Agricultural University, Beijing 100083, China

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ABSTRACT

The β-glucosidase from *Paecilomyces thermophila* J18 was found to be capable of hydrolysing daidzin and genistin in a previous study. This report further evaluated the thermostability and hydrolysis of soybean isoflavone glycosides. The enzyme was found to be very stable at 50 °C, and retained more than 95% of its initial activity after 8 h at 50 °C. It converted isoflavone glycosides, in soybean flour extract and soybean embryo extract, to their aglycones, resulting in more than 93% of hydrolysis of three isoflavone glycosides (namely, daidzin, genistin and glycitin) after 4 h of incubation. Also, addition of the β-glucosidase greatly increased the contents of isoflavone aglycones in the suspended soybean flour and soymilk. The results indicate that the thermostable β-glucosidase may be used to increase the isoflavone aglycones in soy products. This is the first report on the potential application of fungal β-glucosidases for converting isoflavone glycosides to their aglycones in soy products.

1. Introduction

Isoflavones are diphenolic secondary metabolites of plants, and are frequently found to be rich in soybeans. The main isoflavones found in soybeans consist of 12 chemical forms, including three aglycones (i.e. daidzein, genistein and glycitein) and their glycosides, acetyl-, malonyl-, and β-glycosides (Kudou et al., 1991). Soybean isoflavones have attracted considerable attention for their health benefits in the prevention of cancer, osteoporosis, postmenopausal syndromes and hypercholesterolaemia (Nielsen & Williamson, 2007; Setchell et al., 2002). The content and composition of these isoflavones vary in soybean foods, depending on the soybean varieties and processing techniques, such as heat treatment and fermentation (Anderson & Wolf, 1995; Barbosa, Lajolo, & Genovese, 2006; Chun, Kim, & Kim, 2008; Coward, Smith, Kirk, & Barnes, 1998; Riedl, Zhang, Schwartz, & Vodovotz, 2005; Yamabe, Kobayashi-Hattori, Kaneko, Endo, & Takita, 2007).

Bioavailability of soy isoflavones differs between isoflavone aglycones and glycosides. However, study of bioavailability of soy isoflavones has remained controversial (Izumi et al., 2000; Kawakami, Tsurugasaki, Nakamura, & Osada, 2005; Nielsen & Williamson, 2007; Setchell et al., 2002). Numerous studies have revealed that isoflavone aglycones are superior to isoflavone glycosides in various bioactivities, due to their effective absorption (Izumi et al., 2000; Kawakami et al., 2005; Nielsen & Williamson, 2007; Piskula, Yamakoshi, & Iwai, 1999; Walsh et al., 2007). There is interest in increasing the amounts of isoflavone aglycones in soy products. Some authors have reported that the isoflavone glycosides were hydrolysed into their corresponding aglycones during the fermentation of soybean foods, such as tempeh, soy bread, soymilk, miso and douchi (Anderson & Wolf, 1995; Chun et al., 2008; Kuo, Cheng, Wu, Huang, & Lee, 2006; Riedl et al., 2005; Wang et al., 2007; Yamabe et al., 2007). There are many reports of the deglycosylation of isoflavone glycosides during fermentation by *Saccharopolyspora erythraea*, lactic acid bacteria, *Bifidobacteria*, basidiomycetes and *Bacillus subtilis* through their β-glucosidase activities in soy products (Chun et al., 2008; Hessler, Larsen, Constantinou, Schram, & Weber, 1997; Kuo et al., 2006).

β-1,4-Glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21) mainly catalyse hydrolysis of the β-1,4-glycosidic linkage in various disaccharides, oligosaccharides, alkyl- and aryl-β-D-glucosides (Bhatia, Mishra, & Bisaria, 2002). Studies on these β-glucosidases have increased in recent years, due to their important roles in a variety of fundamental biological processes (Bhatia et al., 2002; Chuankhayan, Rimlumduan, Svasti, & Cairns, 2007; Ismail & Hayes, 2005).

Abbreviations: ADin, 6″-O-acetyl daidzin; AGin, 6″-O-acetyl genistin; AGlin, 6″-O-acetyl glycintin; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; MDin, 6″-O-malonyl daidzin; MGin, 6″-O-malonyl genistin; MGlin, 6″-O-malonyl glycitin; MES, 2-(N-morpholino)ethane sulfonic acid; *p*NP, *p*-nitrophenyl; *p*NPG, *p*NP-β-D-glucopyranoside.

* Corresponding author. Tel.: +86 10 62737689; fax: +86 10 82388508.
E-mail address: zhqjiang@cau.edu.cn (Z. Jiang).

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In addition to being applied for cellulose degradation, β-glucosidases could also be used to hydrolyse phenolic compounds and phytoestrogen glycosides to improve their biological activity (Bhatia et al., 2002). Isoflavones are the most well-known phytoestrogen glycosides. Some β-glucosides have been reported to hydrolyse isoflavones (Chuankhayan et al., 2005; Hsieh & Graham, 2001; Ismail & Hayes, 2005; Suzuki et al., 2006; Yang, Ning, Shi, Chang, & Huan, 2004). The effects of various microorganisms on conversion of isoflavone glycosides to their aglycones have been extensively studied during recent decades (Chun et al., 2008; Hessler et al., 1997; Kuo et al., 2006; Otieno & Shah, 2007). However, the effects of β-glucosidases on hydrolysis of soybean isoflavone glycosides have not been extensively characterised (Chuankhayan et al., 2007; Kuo et al., 2006; Pandjaitan, Hettiarachchy, & Ju, 2000; Yang et al., 2004). Two β-glucosidases from the legumes, *Dalbergia cochinchinensis* and *Dalbergia nigrescens*, were compared for their ability to hydrolyse isoflavone glycosides from soybean (Chuankhayan et al., 2007). The newly isolated thermophilic fungus, *Paecilomyces thermophila* J18, could produce extracellular β-glucosidase. The purified β-glucosidase was found to have the ability to hydrolyse soybean isoflavone glycosides, namely, daidzin and genistin, and has potential to increase the isoflavone aglycones in soy products (Yang, Jiang, Yan, & Zhu, 2008). Thus, the main purpose of the present study was to further evaluate the thermostability of the β-glucosidase and its potential for deglycosylation of isoflavone glycosides in soy products.

2. Materials and methods

2.1. Materials

Five varieties of soybeans (Zhonghuang-13, 17, 22, 27, 35 and Zhongdou-27) were provided by the Center of Soybean Research, China Agricultural Academy (Beijing, China). All of these varieties, which were produced and harvested in 2007, were analysed for isoflavone contents. Daidzin, genistin, glycitin, daidzein, genistein, and glycitein were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6″-O-Malonyl daidzin (MDin), 6″-O-malonyl genistin (MGin), 6″-O-malonyl glycintin (MGlin), 6″-O-acetyl daidzin (ADin), 6″-O-acetyl genistin (AGin) and 6″-O-acetyl glycintin (AGlin) were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). All other chemicals used were analytical grade reagents unless otherwise stated.

2.2. Enzyme and protein assays

β-Glucosidase from almonds (WA 10531) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). β-Glucosidase, from *P. thermophila* J18, was purified as previously described (Yang et al., 2008). β-Glucosidase activity was determined at 50 °C or 37 °C (for almond β-glucosidase) using 5.0 mM *p*NPG (*p*-nitrophenyl-β-D-glucopyranoside) as substrate in 50 mM MES (2-(N-morpholino)ethane sulfonic acid) buffer (pH 6.2). After 10 min of incubation, the reaction was stopped by adding three volumes of saturated sodium tetraborate solution and the absorbance was read at 405 nm. One unit of β-glucosidase activity was defined as the amount that produced 1 μmol of *p*-nitrophenol per minute. Protein concentrations were measured by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951) with BSA (bovine serum albumin) as the standard.

2.3. Thermostability of the β-glucosidase

Thermostability of the β-glucosidase from *P. thermophila* J18 was evaluated at different temperatures. All assays were performed in 50 mM MES buffer (pH 6.2) in the absence of substrate. At the appropriate time intervals, residual β-glucosidase activity was immediately measured, using the standard assay described above. The percentage of remaining activity was expressed relative to the residual β-glucosidase activity of a control β-glucosidase sample kept on ice.

2.4. Analysis of soy isoflavones by high performance liquid chromatography (HPLC)

The contents and compositions of isoflavones were determined quantitatively by HPLC, as previously described (Wang et al., 2007). The HPLC system used was a Shimadzu HPLC (Kyoto, Japan), consisting of an LC-10AT pump, a UV detector (SPD-10AVVP), and a Dikma Diamonsil C_{18} column (4.6 × 250 mm) (Dima Co., Ltd., Orlando, FL). The mobile phase for HPLC consisted of solvent (A) i.e. 0.1% (v/v) acetic acid in filtered MilliQ water, and (B), solvent i.e. 0.1% (v/v) acetic acid in acetonitrile. The following gradient for solvent B was applied: 15–25% over 35 min, 25–26.5% over 12 min, and 26.5–50% over 30 s, followed by isocratic elution for 14.5 min. The flow rate was 1.0 ml/min. The column temperature was 40 °C and the absorbance was measured at 254 nm. The isoflavone content of the samples was calculated by interpolation of the calibration curves prepared by various concentrations of the isoflavone standards. The hydrolysis effect of the β-glucosidases was evaluated by the following equations: hydrolysis activity (%) for control samples = 100−100 × (isoflavone content in control samples/isoflavone content in raw samples); hydrolysis activity (%) for enzyme-hydrolysed samples = 100−100 × (isoflavone content in enzyme-hydrolysed samples/isoflavone content in control samples without enzyme).

2.5. Isoflavones extraction from soybean flour and soybean embryo

Five varieties of soybeans were ground to fine powders using a coffee grinder and the powders were defatted, using petroleum ether, by a Soxhlet extractor. The defatted soybean flour (5 g) was extracted with 20 ml of 80% methanol by stirring overnight (12 h) at room temperature. The mixtures were centrifuged at 11,000 × *g* for 15 min, and the supernatants were filtered through a 0.45 μm filter for the quantification of isoflavones using HPLC. The isoflavone contents and compositions in soybeans were expressed as mg per 100 g of sample.

Zhongdou-27 soybeans (50 g) were washed and then soaked in tap water (200 ml) for 8 h at room temperature. The soybean embryo was separated by hand and dried in an oven at 50 °C. The crushed soybean embryo was extracted with 80% methanol by stirring overnight at room temperature. The slurry was centrifuged at 11,000 × *g* for 15 min, and the supernatant was filtered through a 0.45 μm filter.

2.6. Hydrolysis of soybean flour extract and soybean embryo extract

Soybean flour extract and soybean embryo extract (50 μl) aliquots were hydrolysed with 0.01 U/ml of individual β-glucosidase in 450 μl of 50 mM MES buffer (pH 6.2). The reaction mixtures were incubated at 50 °C (for *P. thermophila* β-glucosidase) or 37 °C (for almond β-glucosidase) for 10 min and 4 h, respectively, and the reactions were stopped by boiling for 5 min. Control reactions of extract samples without enzymes were set up in the same manner. The hydrolysed samples (20 μl) were diluted with a mixture of acetic acid-acetonitrile (final concentration 15% acetonitrile and 0.1% acetic acid) and determination of isoflavones was done by HPLC.

2.7. Hydrolysis of soybean flour suspension and soy milk

The defatted soybean flour (40 mg) was suspended in 400 μl of 50 mM MES buffer (pH 6.2). The mixtures were hydrolysed with

0.02 U/ml of each β-glucosidase at 50 °C (for *P. thermophila* β-glucosidase) or 37 °C (for almond β-glucosidase) for 10 min and 4 h, respectively, and the reactions were terminated by boiling for 5 min. The hydrolysed solutions were centrifuged at 11,000 × *g* for 15 min to collect the supernatants and pellets, respectively. The pellets were then extracted with 80% methanol, and the methanol extract was collected by centrifugation. The reaction supernatant and methanol extract of pellet were considered as soybean flour suspension isoflavones in supernatant and in pellet, respectively. Two kinds of samples (20 μl) were diluted by the solution of acetic acid-acetonitrile, followed by determining the isoflavones by HPLC.

Soybeans were washed and soaked overnight in distilled water. Soymilk was prepared by grinding the swollen soybeans, using a FSM-100 grinder (Shenyang, China). The ratio of dry soybean to water used for grinding was 1:10 (w/v). The raw soymilk was boiled for 10 min and cooled to room temperature. The mixtures were hydrolysed with 0.01 U/ml of β-glucosidase at 50 °C (for *P. thermophila* β-glucosidase) or 37 °C (for almond β-glucosidase) for 10 min and 4 h, respectively, and the reactions were terminated by boiling for 5 min. The hydrolysed samples (20 μl) were diluted with a mixture of acetic acid-acetonitrile and isoflavones content was determined by HPLC.

2.8. Statistical analysis

All sample analyses were done in duplicate and statistical analysis was done using SAS (version 6.12), developed by the SAS Institute Inc. (Cary, NC, USA). Duncan's multiple range tests were used to estimate significant differences amongst means at a probability level of 5%.

3. Results and discussion

3.1. Thermostability of β-glucosidase at different temperatures

Thermostability of *P. thermophila* β-glucosidase was evaluated in 50 mM MES buffer (pH 6.2) by measuring the residual activity of β-glucosidase incubated for different time periods at different temperatures (Fig. 1). Results indicated that the enzyme was highly thermostable at 50 °C, and retained more than 95% of the initial activity after 8 h. Even at 60 °C, it retained more than 70% of the initial activity after 8 h.

The β-glucosidase from *P. thermophila*, with a temperature optimum of 75 °C, was very thermostable at 50 °C, which makes it highly suitable for industrial processes. The thermostability is higher than that reported for many β-glucosidases which hydrolyse soybean isoflavones (Hsieh & Graham, 2001; Kuo & Lee, 2008; Matsuura & Obata, 1993; Suzuki et al., 2006; Yang et al., 2004). In contrast, the optimal temperatures for soybean β-glucosidases are reported to be 30 °C and 45 °C (Hsieh & Graham, 2001; Matsuura & Obata, 1993). However, the β-glycosidase from *D. nigrescens* Kurz had a temperature optimum at 65 °C (Chuankhayan et al., 2005). The β-glucosidase from *Pseudomonas* ZD-8 was fairly stable up to 45 °C and had 44% of its activity at 50 °C (Yang et al., 2004). The recombinant β-glucosidase from the roots of soybean seedlings was stable at 20 °C for 8 h (Suzuki et al., 2006). The recombinant β-glucosidase from *B. subtilis* natto retained 80% of its activity when incubated at 45 °C for 2 h, but was completely inactivated when incubated at 50 °C for 1 h (Kuo & Lee, 2008).

3.2. Isoflavone contents and compositions in five varieties of soybeans

The content and composition of isoflavones in five varieties of soybeans are presented in Table 1. The total isoflavone contents ranged from 190 to 446 mg/100 g. Especially, Zhongdou-27 had the highest level of isoflavones and contained 69.1 mg of daidzin, 42.4 mg of genistin, 0.49 mg of daidzein, 4.91 mg of genistein, 159 mg of MDin and 150 mg of MGin on a dry basis (per 100 g). Its total isoflavone content was 1.29–2.85 times higher than that in the other varieties. Isoflavone glycosides accounted for 16.9–28.4% and the ratio of aglycones was very low (0.9–3.2%) in all varieties. The predominant soybean isoflavone forms were malonyl glycosides, in the range of 101–309 mg/100 g. The malonyl, glycoside and aglycone forms corresponded to approximately 73.2%, 25.6% and 1.2% of the total isoflavone content in Zhongdou-27. Isoflavone contents in different varieties have been reported to vary from 126 to 444 mg/100 g of grains, depending on the varieties and environmental conditions (Yamabe et al., 2007). Regarding the composition of isoflavones in soybeans, malonyl glycosides were the major components (Kudou et al., 1991; Yamabe et al., 2007), accounting for 57.2–72.2%. Thus, Zhongdou-27 was selected for use in the subsequent experiments.

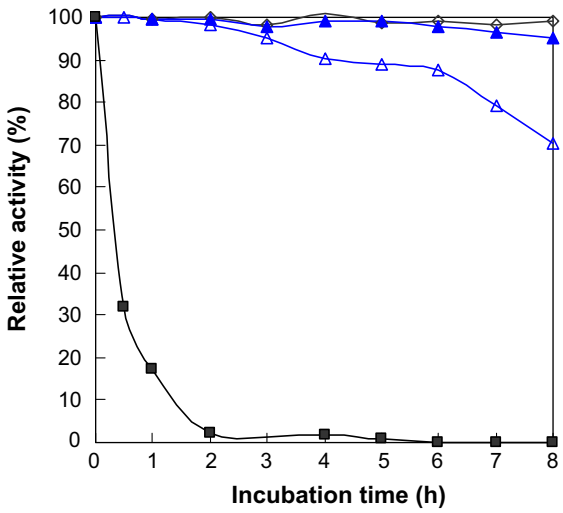

Fig. 1. Thermostability of *P. thermophila* β-glucosidase at different temperatures in 50 mM MES (pH 6.2). Results are the means of three determinations. Symbols: (◇) 40 °C; (▲) 50 °C; (△) 60 °C; (■) 70 °C.

3.3. Hydrolysis of soybean flour extract and soybean embryo extract

Defatted soybean flour and soybean embryo were extracted with methanol, and two kinds of extracts were separated and analysed by HPLC, as shown in Fig. 2B and D. The peaks of daidzin, glycitin, genistin, daidzein, MDin, MGin and genistein were identified in the soybean flour extract (Fig. 2B) by comparison with commercial standards (Fig. 2A). AGlin was not detected (Fig. 2A). Results suggest that daidzin, genistin, MDin, and MGin were the predominant isoflavones in the soybean flour extract, whereas the peaks for their aglycones (daidzein and genistein) were very small (Fig. 2B).

Two β-glucosidases were compared for hydrolysis of soy isoflavone glycosides in extracts (Table 2). The extents of conversion of isoflavone glycosides in soybean flour extract by *P. thermophila* β-glucosidase to their aglycones, were found to be 98.0%, 95.8% and 99.3% of hydrolysis of daidzin, glycitin and genistin, respectively in 4 h. The peaks of daidzin, glycitin, and genistin were hardly visible (Fig. 2C). However, malonyl glycosides (MDin and MGin) were hardly hydrolysed by *P. thermophila* β-glucosidase (Fig. 2C). Conversely, 39.5%, 50.4%, and 41.5% of daidzin, glycitin and genistin in soybean flour extract were converted into their aglycones by almond β-glucosidase. As shown in Fig. 2D, the difference with soybean flour extract was that daidzin, glycitin, MDin,

Table 1
Isoflavone contents and compositions of five varieties of soybeans.

Varieties	Isoflavone (mg/100 g)[a]												
	β-Glycoside			Aglycone			Malonyl glycoside			Acetyl glycoside			Total
	Din	Glin	Gin	Dein	Glein	Gein	Din	Glin	Gin	Din	Glin	Gin	
Zhonghuang-13	38.7	6.28	13.4	0.6	0	2.53	103	Nd[b]	161	11.7	7.61	Nd	345
Zhonghuang-17	20.1	0.65	23.1	0.69	0.11	3.73	39.0	Nd	62.7	3.74	2.7	Nd	157
Zhonghuang-22	16.2	1.78	21.8	1.13	0	4.9	47.3	Nd	85.2	8.59	3.58	Nd	190
Zhonghuang-27	31.4	7.4	22.7	3.04	0.05	3.53	39.6	Nd	96.8	9.73	2.42	Nd	217
Zhonghuang-35	18.9	2.64	15.2	1.09	0.07	3.1	50.5	Nd	100	8.55	4.69	Nd	205
Zhongdou-27	69.1	2.45	42.4	0.49	0.04	4.91	159	Nd	150	6.16	11.2	Nd	446

[a] Values expressed in dry weight are means of determinations in three independent experiments.
[b] Not detected in 5 g of defatted soybean flour used to extract isoflavones.

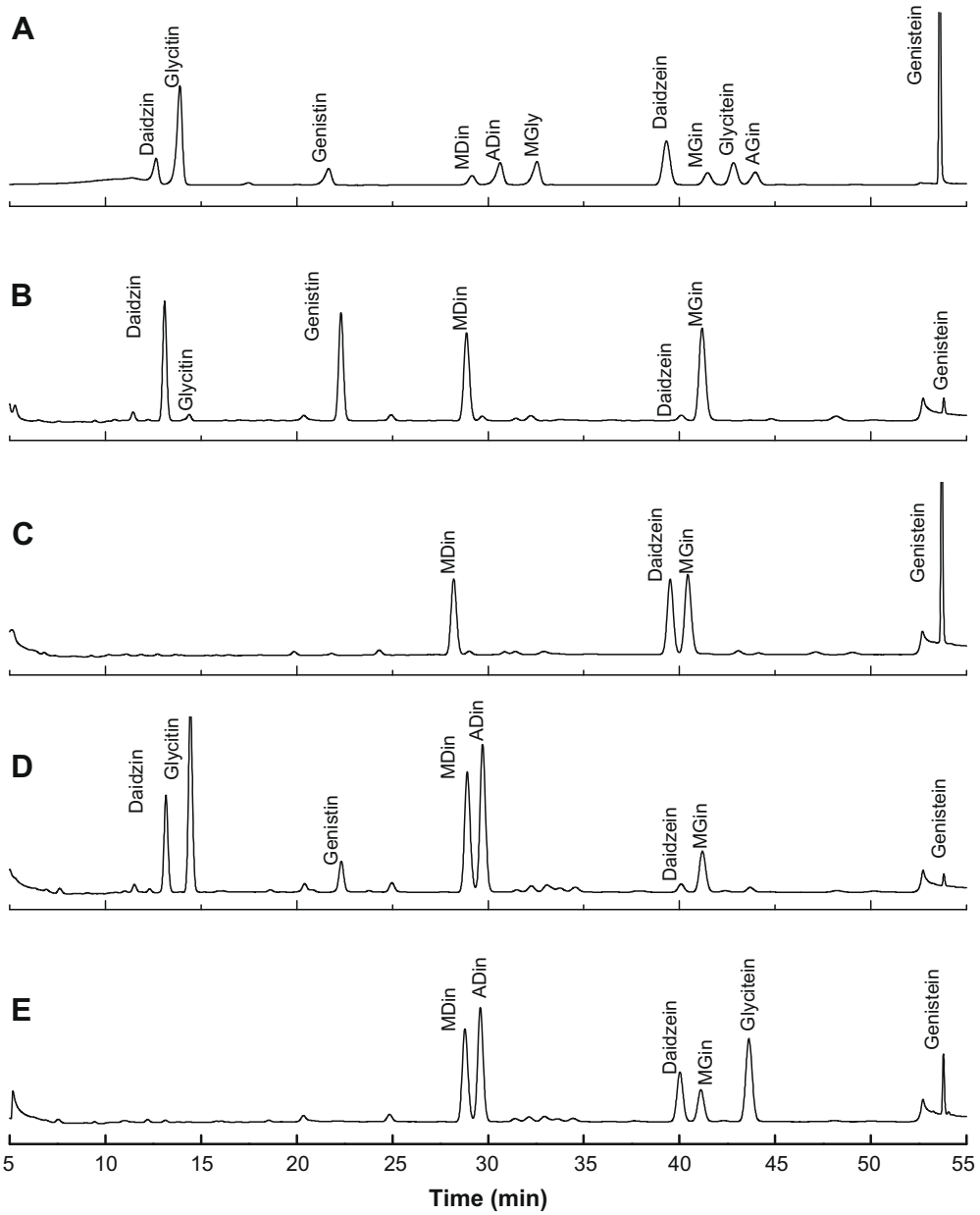

Fig. 2. HPLC chromatograms of isoflavones. (A) Standards: MDin, 6″-O-malonyl daidzin; MGin, 6″-O-malonyl genistin; MGlin, 6″-O-malonyl glycitin; ADin, 6″-O-acetyl daidzin; AGin, 6″-O-acetyl genistin; (B) Isoflavones in soybean flour extract; (C) Soybean flour extract hydrolysed with 0.01 U/ml of *P. thermophila* at 50 °C for 4 h; (D) Isoflavones in soybean embryo extract; (E) Soy embryo extract hydrolysed with 0.01 U/ml of *P. thermophila* at 50 °C for 4 h.

ADin and MGin were the predominant isoflavones in the soybean embryo extract, the peak for glycitin being very large, and ADin was identified. *P. thermophila* β-glucosidase hydrolysed nearly all of the soybean isoflavone β-glycosides (97.3% of daidzin, 93.0% of

Table 2
Comparison of soy isoflavones hydrolysed by two β-glucosidases.

Soybean glycoside	Hydrolytic activity (%)[a]					
	Control		*P. thermophila* β-glucosidase		Almond β-glucosidase	
	10 min	4 h	10 min	4 h	10 min	4 h
Soybean flour extract isoflavones						
Daidzin	0.0	2.1 ± 0.9	63.8 ± 7.4E	98.0 ± 1.0^{A}	34.4 ± 7.0^{A}	39.5 ± 1.5^{DEF}
Glycitin	0.0	0.0	12.3 ± 9.5^{F}	95.8 ± 6.0^{A}	38.0 ± 0.5^{A}	50.4 ± 0.8^{BC}
Genistin	0.0	0.4 ± 0.3	63.9 ± 7.4^{E}	99.3 ± 1.1^{A}	34.1 ± 5.3^{A}	41.5 ± 1.8^{DE}
Soybean embryo extract isoflavones						
Daidzin	0.0	0.3 ± 0.3	85.3 ± 0.4^{B}	97.3 ± 0.8^{A}	11.4 ± 1.1^{E}	34.4 ± 1.9^{GF}
Glycitin	0.0	1.7 ± 0.8	91.8 ± 0.7^{A}	93.0 ± 3.0^{AB}	15.4 ± 4.1^{DE}	31.8 ± 1.2^{G}
Genistin	0.0	2.9 ± 2.0	81.8 ± 0.9^{C}	93.2 ± 5.9^{AB}	22.2 ± 2.1^{DC}	39.3 ± 1.0^{DEF}
Soybean flour suspension isoflavones in supernatant						
Daidzin	0.0	1.0 ± 0.2	74.2 ± 11.3^{D}	84.0 ± 1.0^{BC}	17.2 ± 0.8^{DE}	48.7 ± 3.3^{BC}
Glycitin	0.0	0.0	70.6 ± 8.4^{D}	80.0 ± 4.1^{C}	31.5 ± 1.7^{AB}	57.5 ± 1.5^{A}
Genistin	0.0	0.4 ± 0.4	74.8 ± 0.4^{D}	92.6 ± 4.2^{AB}	25.2 ± 0.8^{BC}	37.8 ± 3.3^{GEF}
Soybean flour suspension isoflavones in sediment						
Daidzin	0.0	2.1 ± 0.5	79.8 ± 4.6^{BC}	84.9 ± 6.2^{BC}	19.1 ± 0.9^{DCE}	53.8 ± 7.7^{AB}
Glycitin	0.0	0.0	93.5 ± 9.2^{A}	96.2 ± 5.4^{A}	14.3 ± 4.8^{DE}	45.1 ± 2.0^{DC}
Genistin	0.0	0.5 ± 0.4	82.8 ± 1.5^{B}	85.4 ± 5.1^{BC}	19.8 ± 1.7^{DC}	53.4 ± 2.1^{AB}
Soymilk isoflavones						
Daidzin	0	1.4 ± 0.2	88.4 ± 2.8^{AB}	95.4 ± 0.8^{A}	29.5 ± 1.7^{AB}	45.4 ± 1.8^{DC}
Glycitin	0	0	93.7 ± 6.3^{A}	100^{A}	34.6 ± 0.8^{A}	40.4 ± 1.2 ^{DE}
Genistin	0	1.1 ± 0.3	89.4 ± 0.9^{AB}	94.6 ± 2.8^{A}	32.9 ± 2.4^{AB}	44.4 ± 1.1^{DC}

[a] Values represent the means ± standard deviation; $n = 2$. Values in a column with different superscripts were significantly different ($p < 0.05$).

glycitin and 93.2% of genistin) that appeared in soybean embryo extract in 4 h (Table 2). However, the peaks of MDin, MGin and ADin remained constant (Fig. 2E), suggesting that they were hardly hydrolysed by *P. thermophila* β-glucosidase.

In soybean flour extract and soybean embryo extract, hydrolysed by *P. thermophila* β-glucosidase, there were 38.3 and 30.6 fold increases in the concentrations of three isoflavone aglycones, respectively, after 4 h of incubation. By comparison, the concentrations of three isoflavone aglycones only increased by a maxima of 14.7- and 11-fold, respectively, in soybean flour extract and soybean embryo extract upon hydrolysis by almond β-glucosidase (Table 3). Thus, *P. thermophila* β-glucosidase showed higher deglycosylation of isoflavone glycosides than did the commercial almond β-glucosidase in soybean flour extract and soybean embryo extract. After hydrolysis of isoflavone glycosides in soybean extract by β-glucosidase from *Pseudomonas* ZD-8, the contents of genistein and daidzein were found to have increased by 12-fold (Yang et al., 2004). Essentially no hydrolysis was observed in control reactions without enzymes (Table 2), demonstrating that no active endogenous β-glucosidase was present in the methanol extracts. Small amounts of isoflavone glycosides hydrolysed in control samples were possibly due to non-enzyme hydrolysis after 4 h of incubation. Similar results were observed by other researchers (Chuankhayan et al., 2007; Ismail & Hayes, 2005).

Table 3
Comparison of the contents of isoflavone aglycones in the total isoflavones of soybean flour extract, soybean embryo, soybean flour suspension and soymilk, with or without β-glucosidases.

	Content of isoflavone aglycones (%) in the total isoflavones[a]				
	Not treated	*P. thermophila* β-glucosidase		Almond β-glucosidase	
		10 min	4 h	10 min	4 h
Soybean flour extract	1.2 ± 0.1	36.0 ± 0.1	45.9 ± 2.4	13.3 ± 0.2	17.6 ± 1.1
Soybean embryo extract	1.4 ± 0.3	35.9 ± 0.4	42.8 ± 1.8	7.2 ± 0.1	15.4 ± 0.3
Soybean flour suspension (supernatant)	1.1 ± 0.1	28.1 ± 0.1	38.9 ± 0.4	10.7 ± 0.1	19.8 ± 0.5
Soybean flour suspension (pellet)	1.2 ± 0.1	36.2 ± 4.5	40.9 ± 0.5	7.8 ± 0.4	21.1 ± 1.7
Soymilk	4.2 ± 0.6	36.3 ± 2.2	43.5 ± 1.3	12.2 ± 0.2	20.5 ± 0.5

[a] Values represent the means ± standard deviation; $n = 2$.

3.4. Hydrolysis of soybean flour suspension and soymilk

The results of hydrolysis of soybean flour suspension and soymilk are summarised in Tables 2 and 3, respectively. On comparision of commercial almond β-glucosidase, *P. thermophila* β-glucosidase was found to be better for hydrolysing isoflavone glycosides in soybean flour suspension (Table 2). This hydrolysis resulted in a 35.4-fold increase in three isoflavone aglycones in solution and in a 34.1-fold increase of three isoflavone aglycones in the pellet, respectively (Table 3). In soymilk treated with *P. thermophila* β-glucosidase, percent hydrolyses of three isoflavone glycosides were 95.4% of daidzin, 100% of glycitin and 94.6% of genistin (Table 2). Moreover, percent hydrolyses of the three glycosides in soymilk by commercial almond β-glucosidase were significantly lower than those of three glycosides in soymilk with *P. thermophila* β-glucosidase. After 4 h of incubation, the contents of three isoflavone aglycones were over 10.4 times more than the initial contents of soymilk (Table 3). Thus, addition of *P. thermophila* β-glucosidase greatly increased the amount of isoflavone aglycones in soybean flour suspension and soymilk.

β-Glucosidases are widely distributed in plants, bacteria and fungi and play significant roles in many biotransformation processes, such as the degradation of cellulosic biomass, cyanogenesis, and the cleavage of isoflavone glycosides (Bhatia et al., 2002; Kuo & Lee, 2008). Some β-glucosidases (especially of plant origin) show high specific activity towards soybean isoflavones (Hsieh & Graham, 2001; Kuo & Lee, 2008; Suzuki et al., 2006; Yang et al., 2004). But, few attempts have been made to investigate the hydrolysis of soybean isoflavones by β-glucosidases from microorganisms (Yang et al., 2004). There are differences in hydrolysis of soybean isoflavones by β-glucosides, which depend on their specificity towards various chemical forms of isoflavones. However, most of them are not effective in hydrolysing the conjugated isoflavones (malonyl and acetyl glycosides) to their respective aglycones (Ismail & Hayes, 2005; Suzuki et al., 2006). The partially

purified soybean β-glucosidase with high specificity for isoflavone conjugates seems to hydrolyse isoflavone malonyl glycosides as well as isoflavone glycosides (Hsieh & Graham, 2001). The β-glucosidase from the endophytic bacterium, *Pseudomonas* ZD-8, showed a pronounced specificity for glucose in the 7-position of isoflavone and flavone conjugates and effectively hydrolysed malonyl isoflavone glycosides, as well as isoflavone glycosides (Yang et al., 2004). The recombinant β-glucosidase from the roots of soybean seedlings was highly specific for isoflavone conjugates, but had low activities for daidzin (17%) and genistin (9%) (Suzuki et al., 2006). The endogenous β-glucosidase activity in soybean can convert isoflavone to aglycones during proofing. The level of malonyl glycosides decreased 3–15% whereas that of acetyl glycosides was fairly constant (Riedl et al., 2005). Most commonly, previous studies suggest that the unstable malonyl glycosides can easily be transformed into less conjugated forms by a thermal process (Barbosa et al., 2006; Coward et al., 1998). In the study, the isoflavone glycosides (daidzin, glycitin and genistin) could be hydrolysed effectively by the thermostable β-glucosidase from *P. thermophila* into their corresponding aglycones. However, both *P. thermophila* β-glucosidase and almond β-glucosidase could not hydrolyse the isoflavone conjugates (malonyl and acetyl glycosides), which is consistent with the fact that most β-glucosidases only cleave non-modified terminal glucose residues (Ismail & Hayes, 2005).

4. Conclusions

The thermostable β-glucosidase from *P. thermophila* could more effectively hydrolyse the glycoside forms of soybean isoflavones to their aglycones than could commercial almond β-glucosidase. The study suggests that the β-glucosidase is an attractive candidate for use in converting isoflavone glycosides to their aglycones, thus enhancing nutritional value in soy products. It can also be applicable in the process of isoflavones deglycosylation and production of isoflavone aglycones.

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