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Biotransformation of phenolics (isoflavones, flavanols and phenolic acids) during the fermentation of *cheonggukjang* by *Bacillus pumilus* HY1

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ABSTRACT

Changes in β-glycosidase, esterase activities, isoflavone, flavanols and phenolic acid during the fermentation of Korean whole soybean fermented food *cheonggukjang* by *Bacillus pumilus* HY1 were investigated. The levels of aglycones, flavanols and gallic acid increased, while the β-glucosidase activity, esterase activity, glycosides content (except for acetylglycosides) and flavanol gallates decreased. Total isoflavone content slightly decreased after 60 h of fermentation, while total flavanol and phenolic acid content increased. The highest levels of daidzein (aglycone type) and acetyldaidzin (glycoside type) were recorded after 48 h of fermentation. The levels of catechin, epicatechin and gallic acid also increased during fermentation. However, total contents of glycosides, malonylglycosides and flavanol gallates decreased by about 80%, 90% and 60% during 60 h of fermentation, respectively. In addition, total phenolic content increased markedly during fermentation, while levels of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity increased. Hence, it would be beneficial for the food industry if components of *cheonggukjang* could be separated and developed into functional products.

1. Introduction

Twelve isoflavones are found in soybeans and soy-containing foods. These are referred to as phytoestrogens because of their estrogenic activities and, recently, considerable attention has been focused on their physiological functions. Isoflavones are present in four chemical forms: malonylglucosides (malonyldaidzin, malonylgenistin and malonylglycitin), acetylglucosides (acetlyldaidzin, acetylgenistin and acetylglycitin), glucosides (daidzin, genistin and glycitin) and aglycones (daidzein, genistein and glycitein); however, the glucoside forms dominate (Kao & Chen, 2006). The potential health benefits of isoflavones for the prevention of atherosclerosis, osteoporosis and postmenopausal syndrome are well documented. Several epidemiological studies have suggested that a high soybean intake is related to low incidence of breast cancer and prostate cancer in Asians (Wuttke, Jarry, & Seidlová-Wuttke, 2007). Recently, several researchers have reported that isoflavones possess both antioxidant activity and metal ion-chelating properties (Kao & Chen, 2006; Wang et al., 2008). With the exception of isoflavones, few studies have examined polyphenolic classes in soybean and soybean-based products, and those few studies are from a physiological point of view (Kim, Kwon, Lee, Choung, & Moon, 2005; Pratt, Birac, Porter, & Giffee, 1981; Seo & Morr, 1984)

In general, fermentation of soybeans for the manufacture of soybean-containing foods increases the hydrolysis of isoflavone glucosides, resulting in higher concentrations of aglycones (Chien, Huang, & Chou, 2006). Bioactivity and metabolic fate of dietary soybean isoflavones differ, depending on their chemical forms. Because the structure itself is a limiting factor for absorption in the gastrointestinal tract, the chemical forms of the isoflavones and their metabolites influence the extent of absorption, with aglycones more readily absorbed and more bioavailable than highly polar conjugated species (Setchell, 2000). Following ingestion, the acetyl and malonyl glycosides are metabolized to glycosides, which are then hydrolyzed in the large intestine by bacteria, resulting in the removal of sugar moiety, to produce their respective aglycones daidzein, genistein and glycitein (Izumi, Piskula, Osawa, Obata, & Tobe, 2000).

There are several types of traditional fermented soybean foods in Korea, including *meju* (soybean cake), *doenjang* (soybean paste), *kanjang* (soybean sauce) and *cheonggukjang* (soybean cook). Here, we focus on *cheonggukjang* which is made from cooked whole

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soybeans fermented with microorganisms, including *Bacillus* spp., over two to three days. It is regarded as a good source of protein, hydrolyzed peptides and lipids, and is consumed commonly by Koreans for its health benefits, like reduction of arterial stiffness. *Cheonggukjang* is also characterized by its unique flavour and sticky, fibrous texture, due to γ-aminobutyric acid formed during fermentation (Yang, Chang, & Lee, 2006). Recently, the isoflavone profiles during *cheonggukjang* fermentation were reported (Jang et al., 2006; Yang et al., 2006). However, other functional components that are likely to be present in *cheonggukjang* remain unknown. It would be beneficial to the health food industry if beneficial components in *cheonggukjang* could be separated and developed as distinct functional products. The objectives of this study were to determine the content of β-glycosidase and esterase activities, isoflavones, flavanols and phenolic acid during the fermentation of *cheonggukjang* by *B. pumilus* HY1.

2. Materials and methods

2.1. Materials

Soybeans were purchased from the local market in Chinju, Korea and an isolate of strain HY1 was obtained from Korean soybean sauce (*kanjang*) collected from Daegok in Chinju, Korea. The isolated HY1 was classified as *B. pumilus*, based on phylogenetic analysis of the 16S rDNA sequence and designated as *B. pumilus* HY1. Three standard isoflavones, including daidzein, genistein and glycitein, were purchased form Sigma Chemical Co. (St. Louis, MO, USA); three standards, namely, β-genistin, β-daidzin, β-glycitin, were obtained from Indofine (Hillsborough, NJ, USA), and six standard compounds, including, acetyl-β-daidzin, acetyl-β-glycitin, acetyl-β-gensitin, malonyl-β-daidzin, malonyl-β-glycitin and malonyl-β-genistin, were purchased from LC Laboratories (Woburn, MA, USA). Seven standard flavanols, including catechin, catechin gallate, epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate and caffeine were from Sigma Chemical Co. (St. Louis, MO, USA). Also, eight standard phenolic acids (gallic acid, *p*-hydroxylbenzoic acid, protocatechuic acid, vanillic acid, *p*-coumraic acid, caffeic acid, ferulic acid and tannic acid), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC-grade H_2O, methanol, acetonitrile and glacial acetic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). Folin–Cicalteu phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH**.**), *p*-nitrophenol-β-D-glucopyranoside (*p*-NPG), *p*-nitrophenol-β-butyric acid (*p*-NPB) and *p*-nitrophenol (*p*-NP), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Cheonggukjang preparation

Whole soybeans (1000 g) were washed and soaked with three volumes of tap water at 20 ± 2 °C for 12 h, and steamed for 30 min at 121 ± 1 °C. The steamed soybeans were left to stand for 1 h at 37 °C to cool down. Then, the cooked soybeans were inoculated with 5% (w/w) strain HY1 (7.43 log cfu/ml) and fermented for 60 h at 37 ± 2 °C in an incubator and sampled at 0, 12, 24, 36, 48 and 60 h.

2.3. pH and viable cell number

Ten grams of *cheonggukjang* samples were dissolved in 90 ml of distilled water to determine the pH (MP 220, pH meter, UK) over 12 h. One gramme of sample was mixed with 9 ml of 0.85% NaCl solution and the diluted suspension (0.1 ml portions) was spread on an TSA plate. The plates were incubated at 37 °C for 24 h, after which colony counts were carried out.

2.4. Isoflavone extraction

The method described by Chien et al. (2006) was modified to extract isoflavone from *cheonggukjang*. Briefly ground *cheonggukjang* was freeze-dried and dry powder (10 g) was extracted with 100 ml of 80% methanol by shaking (160 rpm) at 30 °C for 12 h and filtered through Whatman no. 42 filter paper. The filtrate was dried under vacuum. Dried samples were stored at −40 °C in the dark prior to further use. The dried materials were redissolved in 10 ml of 80% methanol and filtered through a 0.45 μm Minipore PVDF filter (Schleicher & Schuell, GmbH, Dassel, Germany). The filtrate was used for the HPLC analysis. All samples were analyzed with three replications.

2.5. HPLC analysis

2.5.1. Isoflavone analysis

Isoflavones were analyzed using HPLC (Agilent 1100 series, Agilent Co., Forest Hill, Vic, Australia), with a Lichrophore 100 RP C18 column (4.6 × 250 mm, 5 μm, Merck, Germany) and absorbances of the separated isoflavones were measured at 259 nm and quantified using 12 isoflavones standard stock solutions by a diode array UV-visible detector (Agilent 1100 series, Agilent Co., Forest Hill, Vic, Australia). A linear HPLC gradient was composed of 0.1% glacial acetic acid (solution A) and 100% acetonitrile (solution B). Samples (20 μl) were injected onto the column, solvent B was maintained at 10%, 20%, 25% and 35% for 20, 10, 10 and 10 min, respectively. Solvent flow rate was maintained at 1 ml min^{-1} at 30 °C.

2.5.2. Flavanol analysis

Flavanols were analyzed on a TSKgel ODS-100Z column (4.6 × 250 mm, 5 μm, Tosoh Corp., Tokyo, Japan) by reverse-phase HPLC (Perkin–Elmer 200 series, Perkin–Elmer Co., Norwalk, CT, USA). A linear gradient, from 60 to 100%, was performed for 15 min using aqueous 10 mM KH_2PO_4 (pH 2.5) (solution A) and aqueous 100% methanol (solution B), applied at a flow rate of 1 ml min^{-1} at 30 °C. Samples (20 μl) were injected onto the column and the absorbances of the separated flavanols were measured at 270 nm and quantified using seven flavonol standard stock solutions by UV detector (Perkin–Elmer UV 200 series, Perkin–Elmer Corp., Norwalk, CT, USA).

2.5.3. Phenolic acid analysis

Phenolic acids were analyzed on a XTerra™ RP C8 column (4.6 × 250 mm, 5 μm, Waters Corp., Milford, MA, USA) by reverse-phase HPLC (Perkin–Elmer 200 series, Perkin–Elmer Corp., Norwalk, CT, USA). A linear gradient of 60–100% for 40 min, using aqueous 0.5% glacial acetic acid as solution A and aqueous 100% methanol as solution B, was applied at a flow rate of 1 ml min^{-1} at 30 °C. Samples (20 μl) were injected onto the column and the absorbances of the separated phenolic acids were measured at 280 nm and quantified using eight phenolic acid standard stock solutions by UV detector (Perkin–Elmer UV 200 series, Perkin–Elmer Corp., Norwalk, CT, USA).

2.5.4. Calibration curves of the individual phenolic compounds

The calibration curves for 27 phenolic compounds were made from the serial dilutions of the samples dissolved in 80% methanol. The linear range and the equation of linear regression were obtained sequentially at 1.0, 2.5, 5, 10, 20, 40, 60, 80 and 100 μg/ml. Mean areas ($n = 3$) generated from the standard solution were plotted against concentration to establish the calibration equation. A high linearity of $R^2 > 0.998$ was obtained for each calibration curve.