Effect of Isoflavone Aglycone Content and Antioxidation Activity in Natto by Various Cultures of Bacillus Subtilis During the Fermentation Period

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Abstract

This study investigated the isoflavone aglycones content and antioxidant activities (total phenol, 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging activity and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical scavenging activity) of cooked soybeans fermented by various cultures of Bacillus subtilis, including B. subtilis N205 (BS205), B. subtilis BCRC14718 (BS14718) and a co-culture of B. subtilis N205 with B. subtilis BCRC 14718 (BSCO) at 42°C for 48 h. Fermentation by the various B. subtilis strains produced with varying contents of isoflavone aglycones, arranged in increasing concentration as follows: BS14781 (172.94 mg/100 g dry wt.) > BSCO (117.20 mg/100 g dry wt.) > BS205 (34.20 mg/100 g dry wt.). However, the total phenolic content, DPPH free radical scavenging activity and ABTS free radical scavenging activity in Natto fermented by BSCO was higher than those produced by BS205 and BS14718 after fermentation for 48 h.

Materials and Methods

Materials

The isoflavone standards, daidzein and genistein, were purchased from Sigma Chemical (St. Louis, MO, USA). Glycitein standards were purchased from LC Laboratories (Woburn, MA, USA). Trifluoroacetic acid, LC grade acetonitrile, methanol, and n-hexane were purchased from Merck (Darmstadt, Germany).

Bacteria

The B. subtilis N205 strain possesses high nattokinase activity and was isolated from commercial natto products. Commercial natto products were purchased from supermarkets in Taiwan. The nattokinase activities of the strains identified by the API 50 CHB computerized identification system (bioMérieux, Nürtingen, Germany), and analyzed by the APILAB PLUS 3.3.2 software. A pure culture of B. subtilis BCRC 14718 was obtained from the Biorepositories Collection and Research Center (Hsinchu, Taiwan). The pure culture and isolated culture were stored at 80°C. Two cultures were activated using antibiotics instead of nutrient agar (Oxoid CM3) at 37°C for 24 h, and the activated cultures were again inoculated under the same conditions to serve as the pre-cultures to the inoculum for the preparation of fermented soybean.

Preparation of fermented natto

Soybeans were prepared according to previously described

Keywords: Isoflavone aglycones; Antioxidant activity; Bacillus subtilis; Natto

Introduction

Isoflavones are diphenolic secondary metabolites of plants, and are frequently found to be rich in soybeans. Isoflavones possess both antioxidant activities and metal ion-chelating properties [1,2]. They also exhibit strong antioxidant potency in liposomes challenged with UV exposure, peroxyl, and hydroxyl free radicals in vitro [3].

The main isoflavones found in soybeans consist of aglycones (daidzein, glycitein and genistein) [4]. Several researchers have reported that cooked soybeans fermented with B. subtilis enhance the antioxidant activities in chungkookjang (in Korea), natto (in Taiwan and Japan), kimera (in India) [5-7] and temphe (in Indonesian) [8]. Specific of the isoflavone aglycones of genistein and daidzein have been suggested to be responsible for these observed benefits [9].

Most researchers have been interested in the fermentation of soybeans with B. subtilis because it increases the hydrolysis of isoflavone glucosides, resulting in higher concentrations of aglycones [10-13]. We have previously reported that B. subtilis BCRC 14718 is more effective in converting isoflavone glucosides to aglycones in prepared natto [10]. The fermentation process changes the isoflavone content, and its composition causes hydrolysis by β-glucosidase derived from microorganisms, thereby increasing the concentration of isoflavone aglycones in fermented cooked soybean [14,15].

Traditionally natto is a popular fermented soybean produced by Bacillus subtilis that provided a potent nattokinase activity. Nattokinase activity also plays an indirect part in promoting fibrinolysis [16]. In this study, we used the B. subtilis N205 (BS205) isolated from commercial natto, which exhibited the high nattokinase activity, and the B. subtilis BCRC14718 (BS14718) produced high isoflavone aglycone content in fermentation processing [13]. We try to develop a high antioxidant activity natto production which simultaneously provided the high nattokinase activity and isoflavone aglycone content. Thus, this study was to compare of isoflavone aglycones content and their antioxidant activities after cooked soybeans fermented by a single culture of BS205 or BS14718, or a co-culture of BS205 with BS14718 (BSCO).

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procedures [13]. First, 10 g of soybeans was soaked in water for 12 h at room temperature. After decanting the water, the soaked soybeans were placed into a glass jar and autoclaved at 121°C for 45 min. The surface of the sterilized soybeans was sprayed with 1% suspensions of inoculum (approximately 10^6 CFU/g) described above by microtips and the mixture was then fermented at 42°C for 48 h. The inocula included BS205, BS14718, or BSCO with the same proportions of B. subtilis N205 with B. subtilis BCRC 14718 inoculated in the fermented steamed soybeans. The prepared natto samples were stored at 20°C for isoflavone extraction and analysis.

**Extraction and analysis of isoflavones**

The isoflavone extraction and analysis methods used have been previously described [17]. 1 g of the freeze-dried samples was extracted with 20 ml of 80% methanol in a flask and was stirred for 1 h at 60°C. The extracted solutions were centrifuged at 5000×g for 10 min, and the supernatants were dried by evaporation. The dried extracts were dissolved in 5 ml of 50% methanol and treated with 20 ml of n-hexane four times to remove the lipids. Following the evaporation of aqueous methanol, the insoluble residue was dissolved in 10 ml of 80% methanol and filtered through a 0.45 μm filter membrane prior to HPLC analysis.

Reversed phase HPLC analysis was carried out with a Hitachi Model L-6200 (Hitachi Ltd., Tokyo, Japan) equipped with an ODS-AM-303 column (250 mm×4.6 mm i.d.; 5 μm; YMC Inc.) and an ultraviolet spectrophotometer at 254 nm. The mobile phase was composed of 0.1% (v/v) glacial acetic acid in acetonitrile (A) and 0.1% (v/v) glacial acetic acid in water (B). The following steps occurred for the HPLC analysis: 10 μl of the sample was injected onto the column starting at 15% (A) and held for 20 min; the mobile phase was then increased to 24% (A) within 10 min and was held for 10 min; the gradient consisted of an increase to 35% (A) within 4 min and was held for 8 min; finally, it was decreased back to 15% (A) for another 5 min. The flow rate was 1.0 ml/min. Quantitative data for each isoflavone was obtained by comparison to known standards.

**Determination of total polyphenol content**

The total polyphenol concentrations in the fermented natto were determined as previously described [18]. The methanolic extract (0.5 μl) was mixed with 0.5 μl of Folin-Ciocalteau phenol reagents, and 200 μl of 5% Na₂CO₃ was added and allowed to react for 1 h at room temperature. The absorbance was measured at 750 nm using an automated microplate reader and compared to a gallic acid standard.

1. **1-Diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging assay**

   The DPPH radical scavenging effect was estimated as previously described [19]. A 0.5 mM DPPH solution in methanol was prepared and 200 μl of the DPPH solution was then added to 25 μl of each test sample. After a 90-min incubation period at ambient temperature, the absorbance at 517 nm was measured. The inhibitory percentage of DPPH was calculated according to the following equation: Scavenging effect (%)=[1- absorbance of sample/absorbance of control]×100%.

2. **2’,3’-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical-scavenging assay**

   The total antioxidant activity of the seed extracts was measured by the ABTS’ radical cation decolorization assay involving preformed ABTS’ radical cations [20]. ABTS was dissolved in 0.01 M sodium phosphate buffer to a 7 mM concentration. The ABTS was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12 to 16 h before use. The stock solutions of the soybean extracts in methanol were diluted so that a 2 μl aliquot of each dilution was used in the assay. After the addition of 200 μl of the diluted ABTS’ solution to the antioxidant compounds or Trolox standards in methanol, samples were taken at 37°C exactly 7 min after initial mixing. The absorbance of the resulting solution was measured at 734 nm.

   The inhibitory percentage of the ABTS’ was calculated according to the following equation:

   Scavenging effect (%)=[1- absorbance of sample/absorbance of control]×100%.

**Statistical analysis**

Each test was performed in triplicate. Data were analyzed using an

| Time (h) | Daidzin (mg 100 g⁻¹) | Glycitin | Genistin | Daidzein (mg 100 g⁻¹) | Glycitein | Genistein |
|----------|-----------------------|----------|----------|-----------------------|-----------|-----------|
| B. subtilis N205 | 0  | 132.67 ± 0.66 | 51.24 ± 0.79 | 157.00 ± 0.35 | 11.82 ± 0.16 | 1.49 ± 0.19 | 9.26 ± 0.35 |
| | 12 | 113.61 ± 2.34 | 53.61 ± 1.02 | 116.57 ± 2.04 | 14.96 ± 0.26 | 1.38 ± 0.02 | 11.15 ± 1.58 |
| | 24 | 99.10 ± 1.32 | 56.18 ± 1.36 | 100.45 ± 1.69 | 17.16 ± 0.09 | 1.07 ± 0.53 | 15.97 ± 0.58 |
| | 36 | 107.08 ± 0.47 | 56.06 ± 0.52 | 98.21 ± 1.18 | 27.55 ± 0.86 | 1.37 ± 0.15 | 21.59 ± 0.50 |
| | 48 | 98.82 ± 1.06 | 55.66 ± 0.57 | 104.00 ± 0.74 | 37.20 ± 0.97 | 1.48 ± 0.12 | 21.15 ± 0.86 |
| B. subtilis BCRC14718 | 0  | 132.67 ± 0.66 | 51.24 ± 0.79 | 157.00 ± 0.35 | 11.82 ± 0.16 | 1.49 ± 0.19 | 9.26 ± 0.35 |
| | 12 | 56.71 ± 1.30 | 59.58 ± 4.34 | 78.88 ± 2.21 | 103.62 ± 0.01 | 1.53 ± 0.04 | 38.03 ± 1.84 |
| | 24 | 40.83 ± 1.06 | 30.18 ± 0.08 | 60.76 ± 0.33 | 133.91 ± 2.74 | 1.19 ± 0.06 | 37.85 ± 1.55 |
| | 36 | 48.49 ± 2.95 | 27.89 ± 0.45 | 43.24 ± 2.68 | 127.96 ± 3.48 | 1.34 ± 2.06 | 45.59 ± 0.83 |
| | 48 | 42.42 ± 0.77 | 37.17 ± 0.01 | 54.6 ± 1.50 | 106.63 ± 1.41 | 1.92 ± 0.26 | 23.06 ± 2.41 |
| Co-culture B. subtilis N205 with B. subtilis BCRC14718 | 0  | 132.67 ± 0.66 | 51.24 ± 0.79 | 157.00 ± 0.35 | 11.82 ± 0.16 | 1.49 ± 0.19 | 9.26 ± 0.35 |
| | 12 | 113.61 ± 2.86 | 53.61 ± 1.14 | 106.57 ± 4.19 | 33.76 ± 0.09 | 1.44 ± 0.05 | 12.45 ± 2.16 |
| | 24 | 89.10 ± 5.42 | 46.18 ± 3.49 | 90.45 ± 0.49 | 88.29 ± 2.77 | 1.07 ± 0.14 | 27.84 ± 0.53 |
| | 36 | 55.57 ± 0.87 | 49.94 ± 0.42 | 57.92 ± 2.18 | 108.26 ± 1.31 | 1.46 ± 0.01 | 36.23 ± 0.25 |
| | 48 | 58.82 ± 1.06 | 45.66 ± 1.03 | 84.28 ± 1.63 | 77.14 ± 1.78 | 1.82 ± 0.22 | 22.88 ± 1.93 |

Means standard deviation; n=3. Means in the same column with different letters are significantly different (P<0.05).

Table 1: Isoflavone content in cooked soybean fermented with various cultures of Bacillus subtilis at 42°C for 48 hrs.
ANOVA and the SAS software (version 6.03, SAS Institute Inc., Cary, NC). Duncan's multiple range tests were utilized to analyze differences between treatments (the significance level was P<0.05).

Results

Isoflavone aglycone content in fermented natto

The total isoflavone aglycone content in cooked soybeans fermented by BS14718 was significantly increased (P<0.05) compared to those fermented by BS205 and BSCO (Table 1). The total isoflavone glucoside concentrations in those fermented product were significantly exhibited the opposite results (Table 1). After fermentation for 24 h, the total isoflavone aglycone concentration was significantly increased in cooked soybeans fermented by BS14718 (172.94 mg/100 g), followed by BSCO (117.20 mg/100 g) and BS205 (34.20 mg/100 g) (Table 1). After fermenting for 48 h, the isoflavone aglycone content was observed to be 131.61 mg/100 g for BS14718, 101.84 mg/100g for BSCO and 59.83 mg/100 g for BS205. The major composition of the isoflavone aglycones daidzein and genistein exhibited a significant increase in content after fermentation for 48 h (Table 1). The concentration of daidzein was significantly increased from 11.82 mg/100 g to 106.63 mg/100 g, 77.14 mg/100 g and 37.20 mg/100 g in cooked soybeans fermented by BS14718, BSCO and BS205, respectively (P<0.05) (Table 1). However, a similar increase in concentration was observed for genistein from 9.26 mg/100 g to 23.06 mg/100 g, 22.88 mg/100 g and 21.15 mg/100 g, respectively (P<0.05), when the cooked soybeans were fermented with BS14718, BSCO and BS205 (Table 1).

Total phenolic content in fermented natto

The total phenolic content in the methanolic extract of cooked soybeans fermented by the three cultures of B. subtilis at 42°C for 48 h was significantly higher than non-fermented soybeans (Figure 1). The total phenolic content in the different extractions of natto fermented for 48 h, the isoflavone aglycone was observed to be 102 mg/100 g for BS14718, 101.84 mg/100g for BSCO and 59.83 mg/100 g for BS205. After fermentation for 48 h, the total phenolic content was significantly exhibited to be 117.20 mg/100 g for BS14718, 101.84 mg/100g for BSCO and 59.83 mg/100 g for BS205. The total isoflavone aglycone content in cooked soybeans fermented by various cultures of B. subtilis at 42°C for 48 h.

DPPH free radical activity in fermented natto

The DPPH free radical activity of the extraction of cooked soybeans fermented by the three cultures of B. subtilis at 24 h was in the range of 43%-53%, and that for the non-fermented soybeans were in the range of 43%-49% (Figure 2). The inhibition of DPPH absorption for the methanolic extracts of fermented soybeans by BSCO (43%-61%) and BS205 (43%-54%) was more effective than that for cooked soybeans fermented by BS14718 (43%-48%) and the non-fermented cooked soybeans (Figure 2). After 48 h of fermentation, antioxidant activity of DPPH showed BSCO (IC_{50}=32.39 mg/ml) higher than BS205 (IC_{50}=51.46 mg/ml). However, the methanolic extract from the cooked soybeans fermented by BS 14718 did not have an obvious change in its scavenging effect compared with the non-fermented soybeans (Figure 2). Therefore, the cooked soybeans fermented by the various strains may affect the DPPH radical scavenging activity differently.

ABTS free radical activity in fermented natto

The ABTS free radical scavenging effect was significantly increased in the methanolic extracts from the three types of fermented soybeans fermented by B. subtilis fermented BSCO (43%–61%) and BS205 (43%-54%) was more effective than that for cooked soybeans fermented by BS14718 (43%-48%) and the non-fermented cooked soybeans (Figure 2). After 48 h of fermentation, antioxidant activity of DPPH showed BS14718 (IC_{50}=32.39 mg/ml) higher than BS205 (IC_{50}=51.46 mg/ml). However, the methanolic extract from the cooked soybeans fermented by BS 14718 did not have an obvious change in its scavenging effect compared with the non-fermented soybeans (Figure 2). Therefore, the cooked soybeans fermented by the various strains may affect the DPPH radical scavenging activity differently.

Discussion

In this study, cooked soybeans fermented by a single culture of BS14718 exhibited a greater increase in isoflavone aglycone content, as previously reported [13], compared to those fermented by a single culture of BS 205 or a co-culture of BSCO. This is consistent with the
The ABTS scavenging activity during fermentation periods in this study. Scavenging activity than those produced by either BS205 or BS14718 during the fermentation period; however, a large increase in isoflavone aglycone content in BS14718-Natto did not produce the most improved antioxidant activities, including total phenolic content, DPPH free radical scavenging ability and ABTS free scavenging ability. The BSCO-Natto showed higher antioxidant activity, specific for ABTS free radical scavenging ability during the fermentation periods. Furthermore, another mechanism that may have higher antioxidant activity caused the increase in peptides or free amino acids by microbial protease activity during soybean fermentation [28]. Fan et al. [26] demonstrated that the douch antioxidant activity was dependent on the increase in peptides with fermentation rather than on the increase in isoflavone aglycones. This interesting result suggests that the effect of antioxidant activity in fermented natto was not only due to the isoflavone aglycone content but also due to the content of another metabolism component, i.e., peptides in soybeans fermented by a different microorganism [29]. Thus, total phenolic content, DPPH free radical scavenging ability and ABTS free scavenging ability of antioxidant activities in methanolic extractions were observed in natto fermented by various B. subtilis strains.

Conclusion

The isoflavone aglycone concentration in fermented natto was affected by various B. subtilis strains after fermentation processing, which caused different DPPH free radical-scavenging activities and ABTS radical-scavenging abilities. The antioxidant activities in methanolic extracts from cooked soybeans fermented by BS205 and BSCO were significantly higher (P<0.05) than those fermented by BS14718, suggesting a role for fermentation in enhancing antioxidant activities at appropriate concentrations of isoflavone aglycone during fermentation periods.

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