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Antioxidant Activity and Total Phenolic Content of Dried Fermented-Soybean Products Fermented with *Bacillus subtilis a*nd Lab: Potential for Functional Food Application.

Arachaporn Chonkeeree, Monrodee Chaowarat and Sumonwan Chumchuere[+]

Department of Biotechnology Technology, Faculty of Technology, Mahasarakham University, Kantarawichai, Mahasarakham, Thailand

Abstract. Dried fermented soybean products were prepared with *Bacillus subtilis* TISTR 001and LAB *(Lactobacilus fermentum* TISTR 055, *Lactobacillus plantarum* TISTR 920 and *Lactobacillus casei* subsp. *rhamnosus* TISTR No.108). These organisms are commonly used as starters in the fermentation of many traditional, oriental food products. The total phenolic content and antioxidative activities of the water extract and ethanol extract of these dried products were compared with specific reference to 1,1-diphenyl-2-picrylhydrazine (DPPH) radicals scavenging effects and Fe^{2+} chelating ability. Total phenolic content increased in soybean after 72 h fermentation. Fermentation, also displayed enhanced antioxidative activates in comparison with the non-fermented soybean (control). Among the samples tested, that ethanol extract of dried soybean product fermented with *B. subtilis* exhibited the highest levels of DPPH-free radicals scavenging activity (66.91%) and Fe^{2+}-chelating ability (0.11 mmol Fe (II) / g extract). Also, the highest total phenolic content (14.59 mg GAE/g extract) was found in ethanol extract of dried fermented soybeans prepared with *B.subtilis*. These results show the potential of dried fermented-soybean products for developing a healthy food supplement with soybean fermented by *B. subtilis* and LAB.

Keywords: soybean, *Bacillus subtilis*, LAB, antioxidant activity, total phenolic content, dried soybean products, functional food

1. Introduction

Antioxidants are believed to play an important role in the body defense system against oxygen-free radicals and other reactive oxygen species, which are plays a significant pathological role in human disease [1]. Natural antioxidants in plants are related to three major groups: carotenoids, vitamins and phenolics [2]. Phenolic compounds are plant-derived antioxidants that possess metal chelating capabilities and radical scavenging properties [3]. Soybean and soybean products containing phenolic compounds have been shown to possess antioxidant ability. Various researchers have observed that the intake of antioxidants containing soy food was associated with a reduced cardiovascular risk resulting from lower blood pressure and homocysteine [4]. Therefore, it is proposed that intake of food-derived antioxidants in our daily diet may reduce oxidative damage and exert a corresponding beneficial effect on health [5]. Furthermore, several reports have found that many fermented soybean foods exhibit high contents of antioxidative agents than unfermented soybeans [6]. Such data have been reported and documented in various kinds of fermented soybeans such as Japanese natto, Korean chungkookjang, Indian kinema and Thai Thua Nao. However, consumer groups are concerned about the distribution and easiness on selection of health products [7].

The objectives of this study are (1) to determine the nutritional values of dried fermented soybean products (2) to determine these antioxidant effects by measuring total phenolic content (TPC), free-radical scavenging activity of 1,1-diphenyl-2-picrylhydrazine (DPPH) and Fe^{2+}- chelating ability (FRAP) of dried fermented-soybean products

[+] Corresponding author. Tel.: + (089-7116018); fax: +(043-754086).
E-mail address: mon_msu@yahoo.com

prepared with *Bacillus subtilis* and LAB.

2. Materials and methods

2.1. Test microorganisms and soybean

In the present study, *B. subtilis* TISTR 001, *L. fermentum* TISTR 055, *L. plantarum* TISTR 920 and *L. rhamnosus* TISTR 108 obtained from the Thailand Institute of Science and Technological Research TISTR were used as the test microorganisms. *B. subtilis* was activated in nutrient broth (NB broth) and incubated at 37 °C, 150 rpm for 24 h. To prepare inoculum of starter culture for solid state fermentation, the activated culture was inoculated into nutrient broth and incubated at 37 ℃, 150 rpm for 18h (ca 7 log cfu/ml). The cell suspension was concentrated and used as the inoculum for the fermentation of soybeans. While *L. fermentum*, *L. plantarum* and *L. rhamnosus* were activated in MRS broth and incubated at 37 ℃ (%5 CO_2) for 24h. Each activated culture was inoculated into MRS broth and incubated in at 37 ℃ (%5 CO_2) for 20 h (ca 8 log cfu/ml). The cell suspension was concentrated and used as the inoculate for the fermentation of soybeans. Soybean [*Glycine max* (L.) Merrill] was obtained from the local market.

2.2. Fermentation of soybean

The whole beans were first washed and then soaked in distilled water that was three times their weights at room temperature overnight. After decanting the water, the soaked soybeans were steam cooked in an autoclave at 121 ℃ for 15 min and then dried in a hot air oven at 65 °C for 2 h before being used in the fermentation process. After cooling, the steamed beans (100 g) were inoculated with each test microorganism strains by equally adding 10 % (w/w) concentrated cell of *B. subtilis*, *L. fermentum*, *L. plantarum* and *L. rhamnosus* and then fermented at 37 °C for 72 h according to modified methods described by [9].

2.3. Proximate analysis

The recommended methods of the Association of Official Analytical chemists (AOAC, 2000) were used for the determination of moisture content, ash content, fat, and protein content. Total carbohydrate content was calculated by difference.

2.4. Preparation of extracts

After the end of fermentation process, the fermented bean was dried in Vacuum Dryer at 40 ℃ for 18 h. The soybean koji was mashed using mortar and pestle. The mashed samples were extracted by shaking at room temperature for 24 h with distilled water and 80% methanol (1:10, w/v). After filtering through Whatman No. 1 filter paper (Whatman, Maidstone, UK), the extract was vacuum concentrated and dried in hot air oven at 55^{o}C overnight. The samples were analyzed for total phenolic content and antioxidative activity according to modified methods described by [10]

2.5. Determination of the total phenolic content

The total phenolic content of the samples was determined based on the modified method described by [11]. An aliquot of extract (0.5 ml) was added to1.0 ml Folin - Ciocalteau phenol reagent (Sigmae Aldrich Co.,St. Louis, MO,USA) and 1.5 ml of distilled water. After mixing and allowed to react for 30 min, 2.0 mL of 20% Na_2CO_3 was added. The mixer was incubated at room temperature for 60 min comparatively to gallic acid standard. The absorbance was then measured at 765 nm using a spectrophotometer (Shimadzu ,UV-160 A., Tokyo, Japan). Total phenolic content were expressed as mg gallic acid equivalents (GAE) /g of extract.

2.6. Determination of antioxidative activity

The DPPH free radical scavenging activity of the extract was determined modified method described by [12] with minor modification. Briefly, 0.6 mM alcohol solution of DPPH (Sigma - Aldrich Co.) (1.0 ml) was added to 2.0 ml of the test samples. After a 30 min incubation period in darkness at room temperature, the absorbance at 517 nm of reaction mixer was determined. The inhibitory percentage of DPPH was calculated according to the following equation:

$$\text{Scavenging effect (\%)} = [1- (Abs_{control} - Abs_{sample})/ Abs_{control}] \times 100$$

Where, $Abs_{control}$ is the absorbance of the control DPPH^{o} solution without extracts and Abs_{sample} is the absorbance of the sample.

FRAP assay was performed according to a method described by [13]. Briefly, the bean extract (150 µl) was mixed with 2.85 ml of FRAP solution and kept in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured at 593 nm. The results were expressed as mmol of Fe (II)/g extract.

2.7. Statistical analysis

The experimental results were expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one way analysis of variance (ANOVA) and differences between samples were determined by Duncan's Multiple Range test using the Statistical Analysis System program (SPSS version 16.0). Differences between means were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Proximate analysis

Table 1 showed the percentage of moisture content, ash content, protein, fat and carbohydrate content of the dried seed soybean, dried non-fermented-soybean and dried fermented-soybean products .The moisture content ranged from 7.41% in dried non-fermented-soybean and 10.81% in dried fermented-soybean prepared with *L. fermentum.* Ash content was approximately about 0.04-0.05% in both non-fermented-soybean (control) and dried fermented-soybean products.

Protein was found in high levels in dried fermented-soybean products which is significantly higher ($p < 0.05$) than that non-fermented soybean (control). Also, the dried products of dried fermented-soybean prepared with LAB are significantly higher ($p < 0.05$) than that fermented with *B. subtilis*. The protein content varied between 23.49% in dried non-fermented soybean and 40.94% in fermented soybean prepared with *L.plantarum*. The increasing in protein content may be due to the ability of the microorganisms to digest proteins and change to small size peptide as well as functional properties such as anti-cancer and various clinical compounds)[14]. Obviously report, fermentation of legumes and cereals can increase in the amino acid contents [15]. On the other hand, the fat content in dried products of fermented soybean prepared with *B. subtilis* are significantly higher ($p < 0.05$) than that fermented with LAB and ranged from 4.18% in dried non-fermented soybean and 6.47% in dried fermented soybean prepared with *B. subtilis.*

Particularly carbohydrate content in the non-fermented soybean was significantly higher ($p < 0.05$) than those fermented-soybean products. The reduction in carbohydrate content was expressed because of the soybeans are hydrolyzed by β-glucosidase produced by microorganisms [16, 17]. The carbohydrate content ranged from 64.86% in dried non-fermented soybean and 43.29% in dried fermented soybean prepared with *L.fermentum* and with *L.plantarum* respectively.

Table 1: Proximate analysis of the dried soybean and soybean products

dried soybean products	Proximate analysis				
	moisture	ash	protein	fat	carbohydrate
Seed soybean	7.49 ±0.09 ^{c}	0.05 ±0.00 ^{ab}	23.34 ±0.37 ^{c}	4.46 ±0.04 ^{c}	64.66 ±0.51 ^{a}
Control non fermented	7.41 ±0.22 ^{c}	0.04 ±0.00 ^{b}	23.49 ±0.41 ^{c}	4.18 ±0.18 ^{c}	64.86 ±0.81 ^{a}
fermented with					
B.subtilis	10.36 ±0.03 ^{b}	0.05 ±0.01 ^{a}	36.27 ±0.13 ^{b}	6.47 ±0.50 ^{a}	46.84 ±0.61 ^{b}
L.fermentum	10.81 ±0.10 ^{a}	0.04 ±0.00 ^{b}	40.35 ±0.08 ^{a}	5.50 ±0.48 ^{b}	43.29 ±0.50 ^{c}
L.plantarum	10.35 ±0.03 ^{b}	0.04 ±0.00 ^{b}	40.94 ±0.04 ^{a}	5.37 ±0.30 ^{b}	43.29 ±0.31 ^{c}
L.rhamnosus	10.36 ±0.17 ^{b}	0.05 ±0.00 ^{ab}	40.48 ±0.50 ^{a}	5.58 ±0.03 ^{b}	43.52 ±0.30 ^{c}

Values are presented as means ± SD (n=3), and the means in the same column with different lowercase letters (a,b) were significantly different by Duncan's multiple range test $p<0.05$

3.2. Contents of total phenolics

It has been suggested that the phenolic content of plant materials is correlated with their antioxidant activity [18]. In plant, phenolics are usually found in conjugated forms through hydroxyl groups with sugar and glycosides in plant materials. As shown in Fig. 1, the total phenolic content of extract of the various soybean products ranged between 3.11 and 14.59 mg gallic acid/g extract. It was found that total phenolic content of extract of dried fermented soybean was higher than that of the non-fermented soybean extract. In

present study, the increased total phenolic content in soybean after fermentation is consistent with findings reported by other investigator [19].

Additionally, it was also found that the total phenolic content of the ethanol extracts of dried fermented soybean are significantly higher ($p < 0.05$) than the distilled water extracts. The total phenolic content of extract of the various dried fermented-soybean products ranged between 6.07 and 14.59 mg gallic acid/g extract depending on the starter microorganism. For example, the water extract of dried fermented soybeans prepared with *B.subtilis* showed the highest total phenolic content of 8.29 mg gallic acid/g extract, while the highest phenolic content of 14.59 mg gallic/g extract was found in ethanol extract with the respective extract of fermented soybeans that fermented with *B.subtilis*.

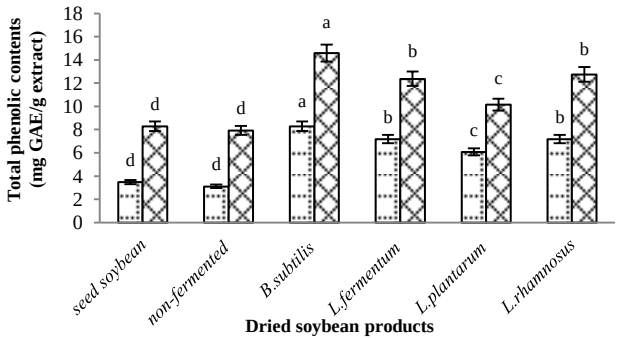

Fig.1 : Total phenolic content (mg GAE /g extract) of the extracts of dried soybean and soybean products fermented with *B.subtilis* and LAB. Values are presented as means ± SD (n=3). Means (bar values) with different letters are significantly different by Duncan's multiple range test $P<0.05$ (▨ water extract ▩ ethanol extract)

3.3. Antioxidant activity

Since DPPH, having a proton free radical, DPPH free radical-scavenging assay was used to determine the proton scavenging activity of the various extracts of dried soybean and soybean products[20]. Additionally, the chelating ability toward Fe^{2+} ion was investigated for antioxidant activity in present study.

Table 2: Free radical scavenging effect (DPPH) and Fe^{2+} chelating ability of the extracts of dried soybean and and soybean products

dried soybean products	antioxidant activity			
	DPPH scavenging effect (%)		Fe^{2+} ion Chelating ability (mmol Fe (II) / g extract)	
	water extract	ethanol extract	water extract	ethanol extract
seed soybeans control	0.75±49.21 ^{b}	1.83±45.94 ^{c}	0.060.00± ^{d}	0.00±0.07 ^{d}
non-fermented fermented with	1.92±48.00 ^{b}	1.11±50.42 ^{b}	0.060.00± ^{d}	0.00±0.07 ^{d}
B. subtilis	0.21±60.61^{a}	2.54±66.91^{a}	0.00±0.09 ^{a}	0.00±0.11 ^{a}
L. fermentum	1.31±38.91 ^{c}	0.55±40.85 ^{d}	0.00±0.08 ^{b}	0.00±0.10 ^{b}
L. plantarum	2.38±33.45 ^{d}	38.872.22 ^{de}	0.07 0.00±^{c}	0.090.00± ^{c}
L. rhamnosus	1.37±35.88^{cd}	1.38±35.88 ^{e}	0.080.00± ^{b}	0.100.00± ^{b}

Value are presented as means ± SD (n=3) and the means in the same column with different lowercase letters (a,b) were significantly different by Duncan's

Table 1 shows the antioxidant activities of water and ethanol extracts of dried fermented-soybean products evaluated in terms of DPPH radical-scavenging activities, and Fe^{2+} ion chelating activity (FRAP). It was found that the antioxidant activity of all ethanol extracts of dried fermented soybean are significantly higher ($p<0.05$) than the water extracts. Moreover, the antioxidant activity of the extracts depended on the starter organisms employed for fermentation. The present results, similar to obviously findings that reported the antioxidant activity of fermented soybean and fermented of black beans with various microorganism extracted with ethanol were higher than extraction with water [21, 22]

Based on the DPPH assay, it was found that the ethanol extract of dried non fermented and fermented samples showed the scavenging effect ranging between 33.45 and 66.91%. Both ethanol and water extract of dried soybean samples prepared with LAB exhibited lower scavenging effect and Fe^{2+} ion chelating activity than samples fermented with *B. subtilis*. The highest level of antioxidant activity based on DPPH radical-scavenging activities and

Fe^{2+} ion chelating activity of the ethanol extract of the dried fermented-soybean samples prepared with *B. subtilis* were 66.91% and 0.11 mmol Fe (II) / g extract, respectively. The results were a correlation between total phenol content and antioxidant activity of the soybean extracts. The higher total phenolic content of the soybean extracts, the higher scavenging effect and Fe^{2+} ion chelating activity were estimated.

4. Conclusion

Our study demonstrated that dried fermented soybean products prepared with *B. subtilis* and lactic acid bacteria (LAB) exhibited the high level in nutritional values. The ethanol extracts of dried fermented soybeans enhanced DPPH radical-scavenging effect, Fe^{2+}-chelating ability and higher total phenolic content than non-fermented soybeans. It is clear that the enhanced effect on antioxidative activity varied with the starter microorganism. On the other hand, our results have shown the potential for developing a healthy food supplement, or dietary adjunct, with soybean fermented by the *B. subtilis* and LAB.

5. Acknowledgement

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6. References

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