

Antioxidant Properties and Total Phenolics of *Thua Nao* (a Thai Fermented Soybean) as Affected by *Bacillus*-fermentation

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Abstract

The present study aims to investigate antioxidant activity and phenolic contents of Thai *thua nao*. Methanolic extracts of cooked non-fermented soybeans (CNF) and *thua nao*—fermented soybeans prepared by naturally occurring bacteria (TNMX) and *Bacillus subtilis* TN51 (TNB51) were prepared and used to determine antioxidant activities by β -carotene-linoleic acid system and DPPH-radical scavenging assay. Total phenolic contents of *thua nao* samples (both TNMX and TNB51) were significantly higher than those of the CNF samples (with an increase around 21 and 35%, respectively). A correlation between larger amount of total phenolics and higher antioxidant activity as determined by the β -carotene-linoleic acid model was observed in these extracts. All these results indicate that production process and bacterial fermentation are responsible for improvement of total phenolics and antioxidant activity. The potential use of *B. subtilis* TN51 appears to be a suitable fermenting strain to promote antioxidant property in cooked soybean.

Keywords: Antioxidant activity; *Bacillus subtilis*; Fermented soybean; Phenolics; *Thua nao*

Introduction

Thua nao, indigenous to Thailand, is an alkaline-fermented food condiment made from soybeans. It is essential in several local recipes as flavouring agents and has long been used as an important protein source in the Northern region of Thailand. The consumption of soy-fermented foods has been shown to promote several beneficial effects on human health; these include a good source of protein and soy-isoflavones which are well known for their pharmacological and antioxidant properties [1,2]. Although known as an important source of nutritive protein, these fermented soybeans have been described to possess biological activities such as anticancer [3], antidiabetic [4], antimicrobial [5], and antioxidant activities [6]. Such activities can be described by the ability of antioxidants present in soy-fermented foods to scavenge free radicals responsible for oxidative damage of biomolecules (i.e., lipids, nucleic acids, and proteins). Recently, more and more researchers have paid a great attention to natural antioxidants of fermented soybeans [1,2,6]. Most of these antioxidants are poly phenolic compounds functioning as metal chelators, reducing agents, and singlet oxygen quenchers. Of these, isoflavones (i.e., genistein and daidzein) are found at high concentration and they are known to act against some diseases such as cancer [7] and atherosclerosis [8].

Several reports have found that many fermented soybean foods exhibit high contents of anti-oxidative agents than unfermented soybeans [9]. Such data have been reported and documented in various kinds of fermented soybeans such as Japanese *natto* [10], Korean *chungkookjang* [11], and Indian *kinema* [12]. However, antioxidative effects of Thai *thua nao* remain unknown although one previous report has demonstrated that aglycone amounts could be enhanced in *thua nao* when a pure starter culture of *Bacillus* species was used [13]. This present study was therefore undertaken aiming to examine antioxidative property of *thua nao* using β -carotene-linoleic acid system and DPPH-radical scavenging assay.

Materials and Methods

Bacterial strain and culture conditions

Bacillus subtilis TN51 previously isolated from commercial *thua*

nao was used as pure starter culture due to its high proteolytic activity [14]. The bacteria were routinely cultured on nutrient agar and, for stock culture, the 20% glycerol bacterial culture was prepared and kept at -20°C. For inoculum preparation, the bacteria were grown in nutrient broth at 37°C for 24 h. The cells were then harvested by centrifugation (14000 rpm, 10 min, 4°C), resuspended in sterile distilled water and adjusted to 10⁴ CFU/mL using Haemocytometer slides. This cell suspension was ready to serve as inoculum for soybean fermentation.

Preparation of *thua nao*

Protein-rich soybeans variety TG145 obtained from the Field Crop Research Center, Institute of Agriculture, Chiang Mai, Thailand, were washed and soaked in tap water for 16 h at ambient temperature (~25°C). Before fermentation, different methods were used to prepare cooked non-fermented soybeans (CNF). Soaked soybeans were cooked by boiling for 4 h (CNF1) and left to ferment by naturally occurring microbes (TNMX). In contrast, for *Bacillus*-inoculum fermentation, soaked soybeans were sterilised at 121°C for 40 min (CNF2), and inoculated with 10⁴ CFU (per g of sterilised soybeans) of *B. subtilis* TN51 starter culture (TNB51). Fermentation process was then allowed to proceed at 42°C for 72 h. At the end of the fermentation period, all CNF and fermented soybean samples were smashed, freeze-dried by a lyophiliser (Labconco, USA), and stored in airtight bottles at -18°C until analysis.

Preparation of methanol extracts of *thua nao*

Based on the procedure of Lee et al. [15], the ground powder of

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the lyophilised sample (30 g) was extracted with 300 mL of 80% (v/v) methanol for 16 h at room temperature with continuous shaking. The extracts were filtered through Whatman No.1 paper, concentrated under vacuum at 40°C and freeze-dried. The lyophilized extracts were stored at 4°C and, before use; the extracts were dissolved in methanol.

Assay of total phenolics

Total phenolics were analysed using the protocol of Lin et al. [16]. The methanolic extract solution (0.1 mL) was added to a mixture of 1.9 mL of deionised water and 1 mL of Folin-Ciocalteu phenol reagent. After 8 min incubation at room temperature, 5 mL of 20% (w/v) sodium carbonate were added and this mixture was then heated in a boiling water bath (~95°C) for 1 min. The absorbance was measured at 750 nm by a spectrophotometer and the results expressed in mg of gallic acid per gram extract. Gallic acid (0 – 1 mg/ml) was used in this study for the standard calibration curve.

β-carotene-linoleate model assay

The antioxidant activity of sample extracts was determined using the β-carotene linoleic acid model system [17]. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 mL of chloroform. Two milliliters of this solution were transferred into a 100 mL round-bottom flask. After the chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 mL of aerated distilled water were added to the flask with vigorous shaking. This emulsion (4.8 mL) was aliquoted into 0.2 mL of 10mg/mL sample extract. For control, 80% methanol was used in the reaction instead of the sample extracts. The mixture was then shaken and stored at 50°C for 2 h. The absorbance of the samples was measured at 470 nm at the beginning (0 min) and at the end of the experiment (120 min). Antioxidant activity was then calculated using the following equation: % Antioxidant activity = 100 x [1 - {(A_{so} - A_{se})/(A_{co} - A_{ce})}] where A_{so} and A_{se} were absorbance of the sample at 0 and 120 min, and A_{co} and A_{ce} were absorbance of the control at 0 and 120 min [17].

Determination of DPPH radical-scavenging activity

Antioxidant activity of the extracts was determined using the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method [18]. One millilitre of methanolic lyophilized extracts (1 – 20 mg/mL) was added to 2.0 mL of 75 mM methanolic solution of DPPH. The mixture was shaken and allowed to stand in the dark at room temperature for 5 min. The decrease in absorbance at 517 nm was then measured against methanol. The inhibitory percentage of DPPH was calculated according to the equation as follows: % scavenging activity = [1 - (As/Ac)] x 100; where As and Ac were the absorbance values at 517 nm of the DPPH solutions with sample and without sample (control), respectively [18]. The percentage of scavenging activity

obtained was subsequently plotted against the sample concentration. The half maximal inhibitory concentration (IC₅₀) was then calculated from the equation analyzed from the logarithmic regression between sample concentration and inhibition rate. In addition, the efficient concentration representing amount of antioxidant required to decrease the initial DPPH concentration by 50% (EC₅₀) was calculated from the following formula: EC₅₀ = IC₅₀/ [DPPH] in mg/mL. The antiradical power (ARP) describing the effectiveness of antioxidant and radical scavenging capacity was also determined as follows: ARP = 1/(EC₅₀ x 100) [19].

Statistical analysis

Data were expressed as means ± standard deviation of triplicate observations. The data were also subjected to analysis of variance (ANOVA), *t*-test, and Duncan's multiple range test (DMRT). Differences between means at 5% (*P*<0.05) were regarded as significant.

Results and Discussion

Extraction yield

Earlier reports on phenolic contents and antioxidant activity of plants have been based on solvent extraction. For soy-fermented food, methanol was found to be the most suitable solvent used for extracting antioxidant components [20]. In addition, the methanolic extract was also reported to exhibit higher antioxidant activity than that of other extracts in several model systems [21]. This present study thus employed this solvent and extraction yield of the CNF and *thua nao* samples is shown in Table 1. When using methanol, antioxidants could be extracted and accounted for 12.22 to 17.97% of lyophilized soybean tested samples (Table 1). There was no significant difference (*P*>0.05) in the extraction yield between the CNF1 and CNF2 samples. It should be noted, however that there was a significant increase (*P*<0.05) of the extraction yield of both *thua nao* samples (TNMX and TNB51). This implied that there was a bioconversion of some materials in soybeans after bacterial fermentation and the use of methanol was able to extract more soluble compounds from fermented soybeans.

Total phenolics content

Total phenolic content, expressed as mg of gallic acid equivalents (GAEs) per g of extract, was also shown in Table 1. There was no significant difference (*P*>0.05) in amounts of phenolic compounds between the CNF samples which were 27.67 and 29.03 mg GAEs/g extract. However, the increase of phenolic contents was observed in *thua nao* extracts ranging from 21-35% as compared with the original concentrations of the CNF samples. The amount of phenolic compounds in the TNMX extracts was highest (37.29 mg/g), and followed by the TNB51 extracts (35.18 mg/g). Such an increase of total phenolic contents in soybeans after fermentation as shown in this

Sample	Yield of extract (%)	Total phenolics (mg GAE/g ext.)	Phenolics/Yield of extract (%)
Natural fermentation			
CNF1	12.22 ± 0.30b	27.67 ± 1.41c	2.77 ± 0.14c
TNMX	17.97 ± 0.85a	37.29 ± 1.00a	3.73 ± 0.10a
Pure culture fermentation			
CNF2	12.63 ± 1.93b	29.03 ± 0.14c	2.90 ± 0.01c
TNB51	16.53 ± 0.29a	35.18 ± 1.03b	3.52 ± 0.10b

*Data are means ± S.D. of triplicate measurements. Means in the same column with different letters were significantly different (*P*<0.05).

**CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *thua nao* fermented by naturally occurring bacteria and *B. subtilis* TN51.

Table 1: Yield (%) and total phenolics (mg GAE/g extract) of methanolic extracts of cooked non-fermented soybeans (CNF) and *thua nao*.

Sample	AOX (%)	Free radical scavenging activity		
		IC ₅₀	EC ₅₀	ARP
Natural fermentation				
CNF1	54.55 ± 1.16d	3.37 ± 0.05b	113.93 ± 1.82b	0.88 ± 0.01b
TNMX	65.65 ± 0.39a	5.18 ± 0.43a	175.28 ± 14.54a	0.57 ± 0.05c
Pure culture fermentation				
CNF2	58.49 ± 2.30c	2.70 ± 0.01c	91.30 ± 0.40c	1.10 ± 0.00a
TNB51	62.12 ± 0.95b	3.27 ± 0.01bc	110.74 ± 0.41bc	0.90 ± 0.01b

*Data are means ± S.D. of triplicate measurements. Means in the same column with different letters were significantly different ($P < 0.05$).

**CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *thua nao* fermented by naturally occurring bacteria and *B. subtilis* TN51.

***AOX = Antioxidant activity determined by the β -carotene linoleic acid system at 10 mg/mL of dried sample extracts; IC₅₀ = Half maximal inhibitory concentration (mg/mL of sample extract); EC₅₀ = Efficiency concentration (mg/mg DPPH); ARP = antiradical power.

Table 2: Antioxidant activities of methanolic extracts of cooked non-fermented soybeans (CNF) and *thua nao*.

study is in agreement with the findings of other researchers [12,16,22]. These studies also suggest that β -glucosidase, which produced from fermenting bacteria plays a key role in increasing phenolic contents of the products. Different scenario was reported however by Shon et al. [17] in which there was no change in total phenolics contents between *chungkukjang* and cooked non-fermented soybeans.

Antioxidant activity

In this study, antioxidant property was assessed by two different assays: β -carotene-linoleate system and DPPH free radical scavenging. For β -carotene-linoleate system, free radicals yielded from linoleic acid oxidation attack unsaturated β -carotene molecules. As a result, the existence of different antioxidants can prevent the degree of β -carotene bleaching by neutralising the linoleate-free radicals [23]. As shown in Table 2, the antioxidant activity (AOX) of the *thua nao* extracts was higher than that of the CNF samples. It should be noted interestingly that the AOX of the TNMX sample was highest, followed by the TNB51, CNF2 and CNF1 samples. In general, the AOX values are very high ranging from 54.55-65.65%. The marked increase in the AOX values of the *thua nao* extracts is of great interest representing more than 62%. These values, albeit used at low concentration of the extracts (10 mg/mL), are much higher than those of *kinema* (44%) described by Moktan et al. [12]. It is understandable that many factors such as soybean variety, cooking process, and fermenting starter culture(s) could affect these results [12,17].

Alternatively, the DPPH free radical scavenging, one of the widely used methods in antioxidative assay was also used in this study. The DPPH radical is stable and can easily accept an electron or hydrogen radical into a stable molecule. This technique can be used to evaluate the antioxidant activity of specific compounds or extracts by measuring the change of absorbance at 517 nm in a short time. Table 2 shows the antioxidant activities evaluated in terms of inhibitory concentration (IC₅₀), efficiency concentration (EC₅₀), and anti-radical power (ARP). The lower the IC₅₀ and EC₅₀, the higher the antioxidant capacity. In contrast, the ARP was calculated from the EC₅₀; the larger the ARP, the more efficient the antioxidant activity. Based on the DPPH assay, it was found that the CNF samples exhibited higher antioxidative activity than the *thua nao* samples. Considered from the ARP values, the antioxidant activity of these extracts tested are as follows: CNF2 > TNB51 > CNF1 > TNMX.

It has previously been reported that the antioxidant activity is well correlated with the phenolic contents [24]. In the present study, it is worth noting that such a relationship has also been observed but only for the antioxidant activity determined by β -carotene bleaching model. The DPPH assay gave a different result in which the CNF samples

showed higher antioxidant activity although they contained less phenolic contents. One possible explanation is due to high contents of aglycone compounds in fermented soybeans. Aglycones (i.e., daidzein and genistein) are described as inactive substrates in radical scavenging assay [25].

In addition to phenolic content, antioxidant activity can be resulted from synergistic effects of other antioxidant components such as oligoproteins, free amino acids and melanoidins as previously described in soy-fermented foods [26,27]. Peptides and free amino acids are also known as source of the anti-free radicals and anti-linoleic acid oxidation in Chinese *douchi* [28]. In our present study, the ratio between total polyphenolics and total extractable substances in all soybean samples ranged from 2.77 to 3.73% (Table 1) which implies that there exists a significant amount of other antioxidants apart from phenolic compounds.

Conclusion

Our study clearly showed that the methanolic extracts of cooked non-fermented soybeans and their fermented counterparts (*thua nao*) exhibited antioxidant and free radical scavenging properties. It was also found that there was a strong relationship between total phenolic contents and antioxidant activity (as determined by the β -carotene-linoleate method). Moreover, the use of *B. subtilis* TN51 appears to enhance phenolic amounts and increase antioxidant activity although data of free radical scavenging assay were inconsistent. From our results, it can be concluded that production process and bacterial fermentation play the key role in improving these properties. Further study on (bio) chemical characteristics of these antioxidative components as well as their metabolic biotransformation remains to be investigated.

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