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Antioxidant Activity Improvement of Soybean Meal by Microbial Fermentation

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Abstract: The improvement of antioxidant activity of soybean meal (SBM), an important protein source for monogastric animals, was studied by solid state fermentation. Fermented soybean meal (FSBM) was produced using pure culture of each *Bacillus subtilis* strain MR10 and TK8, *B. natto* and *Rhizopus oligosporus* that are accepted as GRAS microorganisms. It was found that the antioxidant activity in term of scavenging effect and reducing power was increased significantly at $p<0.05$. FSBM produced by *Bacillus* sp. and *R. oligosporus* showed percentage scavenging activity at the level of 70-99 and 49%, respectively, while only 25% of scavenging activity was found in SBM. Furthermore, the reducing power value (A_{700}) of every FSBM and SBM was between 0.827-1.031 and 0.299, respectively.

Key words: Antioxidant activity, scavenging activity, reducing power, fermented soybean meal, *Bacillus* sp., *Rhizopus oligosporus*

INTRODUCTION

Soybean meal (SBM) is a high quality and the most important source of dietary protein for monogastric animals, especially swine and poultry. It is a by-product obtained after oil extraction process of soybean including drying, dehulling, flaking, expanding, extraction and meal drying. SBM contains 47-51% DM of protein, while the other important nutritional compositions are carbohydrates (35% DM), fat (1.2-3.1% DM) and variety of minerals. However, during oil extraction by solvent, some phytochemicals may be removed from soybean as well, for example vitamin E (α-tocopherol), lecithin and isoflavones. About isoflavovones, there was a report confirmed that it was significantly lost during soaking and heat treatment (Xie and Hettiarachchy, 2001). These compounds function as natural antioxidants preventing many diseases caused by reactive free radicals for human and animals (Lee *et al*., 2005; Tavva *et al*., 2006; Yang *et al*., 2000; Yamamoto *et al*., 2003). Actually, almost organisms including human and animals are well protected against free radical damage by endogenous enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherol and glutathione but when this mechanism become imbalance by many factors such as aging or deterioration of physiological functions, pathological stress may occur (Yang *et al*., 2000). For animals, pathological stress may be found when they are fed in high stress condition or exposed to some chemicals. Supplementation of antioxidants or enhancement of antioxidant in food for human and animals may be used to reduce oxidative stress in body caused by free radicals. Synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate

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(PG) and tertiary butyl hydroquinone (TBHQ) are widely used because of their effectiveness and cheaper than natural antioxidants. However, their safety and toxicity of synthetic type must be concerned before use. Natural antioxidants are then very interesting source in food and feed application.

Fermented foods represent on average one-third of total food consumption (Nout and Kiers, 2005), especially fermented soybean that are widespread found in many part of the world as a local food for example Tua-nao (Thailand), Natto (Japan), Tempe (Indonesia) and Kinema (India). Those products have been reported on higher in antioxidant activity via microbial fermentation (Nout and Kiers, 2005; Hattori *et al*., 1995; Yang *et al*., 2000; Pyo *et al*., 2005). There are many reports about diversity of microorganisms found in fermented foods, especially in fermented soybean and related products for example *Bacillus* sp., lactic acid bacteria, *Rhizopus oligosporus*, *Mucor* sp., *Actinomucor* sp. and yeasts (Sarkar *et al*., 2002; Omafuvbe *et al*., 2002; Han *et al*., 2001; Han *et al*., 2003; Hesseltine, 1998). However, *Bacillus* sp., especially *B. subtilis* is the most important producing bacteria found in many fermented soybean products (Hesseltine, 1998). Moreover, *R. oligosporus* is another microbe that is the main producing mold found in tempe. Both *B. subtilis* and *R. oligosporus* have been accepted as GRAS (Generally Regarded as Safe) strain (Fererira *et al*., 2005; Lin *et al*., 2006). Not only growing on soybean as sole substrate but those can grow on SBM as well. These microbes were then expected as antioxidant activity enhancer for SBM as the found in fermented soybean. Therefore, in this research, we focused on the improvement of antioxidant activity of SBM for animal feed application using microbial fermentation. Fermented soybean meal (FSBM) production by the selected microorganisms was then studied and the microorganisms used were *B. subtilis*, *B. natto* and *R. oligosporus* isolated from Tua-nao, natto and tempe, respectively. The antioxidant activity in term of scavenging effect and reducing power of SBM and various FSBM will be reported in this study.

MATERIALS AND METHODS

Microorganisms

B. subtilis MR10 and *B. subtilis* TK8 were isolated from Tua-nao sample in Chiang Mai province, Thailand. They were identified to specie level using the method according to Norris *et al*. (1981). While *B. natto* and *R. oligosporus* were kindly obtained from Microbiology Section, Faculty of Science, Chiang Mai University, Thailand. *Bacillus* sp. and *R. oligosporus* were maintained in nutrient agar and potato dextrose agar, respectively. The experiment was conducted at Department of Biology, Chiang Mai University, Thailand during 2006.

FSBM Production and Sample Preparation

Ten gram of SBM was mixed with 20 mL distilled water, mixed well and sterile at 121°C 15 min. After cooling, in case of *Bacillus* sp., the 10^5 cfu of 8 h -old starter was transferred to solid medium while for *R. oligosporus*, the 10^5 spore was inoculated. After mixing well, these media were incubated at 37°C for 24 h. FSBM were subsequently freeze dried and ground to fine powder for the next step.

For antioxidant extraction, 5 g of powdered sample was mixed well with 70% (v/v) ethanol (Merck®) (prepared by deionized water), filtered through Whatman® paper No. 1. The filtrate was harvested and used as tested crude extract.

Scavenging Effect on ABTS Radical Cation

To generate $ABTS^{\bullet+}$, the protocol according to Re *et al*. (1999) was used. The 5 mL 14 mM ABTS (0.0385 g ABTS in 5 mL deionized water) and 5 mL potassium persulfate (0.0066 g potassium persulfate in 5 mL deionized water) were mixed together and stand in the dark for 12-16 h before use. To determine scavenging activity of FSBM extract, 10 μL of extract was added to 990 μL of $ABTS^{\bullet+}$ solution (adjusted the absorbance at 734 nm to 0.700±0.020 before used) and recorded the decreasing

of A_{734} every 1 min until stable. The standard antioxidants used in this study were α-tocopherol (Merck®), ascorbic acid (Fisher Chemicals ®, bultylated hydroxyanisole (BHA, Fluka).®For α-tocopherol and BHA, the solution were prepared in ethanol (Merck®) while ascorbic acid was prepared in 0.2 M sodium phosphate buffer pH 6.5 (Yang *et al*., 2000). The percent of scavenging activity at 1 min of reaction can be calculated by the formula:

$$\frac{A_{734}\text{ at 0 min} - A_{734}\text{ at 1min}}{A_{734}\text{ at 0 min}}$$

Reducing Power of FSBM Extract

The reducing power of FSBM extracts was determined according to the method of Oyaizu (1986). FSBM extract (2.5 mL) was mixed with sodium phosphate buffer (2.5 mL) and 1% (w/v) potassium ferricyanide (2.5 mL) and subsequently incubated at 50°C 20 min. After that 2.5 mL of 10%(w/v) trichloroacetic acid was added and centrifuged at 3000 rpm 10 min. The 5 mL supernatant was added with 5 mL deionized water and 1 mL of 0.1%(w/v) $FeCl_2$. After exact 2 min of reaction time, the absorbance at 700 nm was measured. A higher in A_{700} indicates a higher in reducing power.

Statistical Analysis

Data obtained were analyzed statistically by determining standard errors of the mean and analysis of variance using software STATISTIX version 8.

RESULTS AND DISCUSSION

Scavenging Effect on ABTS Radical Cation and Reducing Power

Scavenging effect on $ABTS^{\bullet+}$ by several crude extract of FSBM and SBM was determined; it was found that all extracts showed scavenging effect on this free radical as shown in Fig. 1. The absorbance at 734 nm was decreased rapidly at the first minute of reaction and slightly decreased until stable. The FSBM extracts showed higher in scavenging rate on $ABTS^{\bullet+}$. Among these extracts, FSBM produced by *B. subtilis* TK8 and mixed *Bacillus* sp. completely removed free radical since 5 and 2 min after reaction time, respectively, while almost complete free radical removal was found from those of

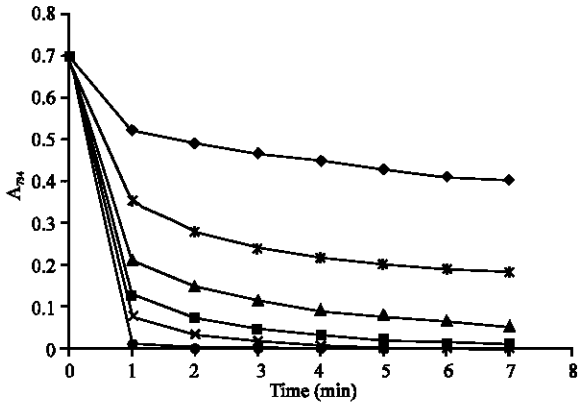

Fig. 1: Scavenging effect profile of methanolic extract of SBM and FSBM; SBM (♦), *R. oligosporus* (*), *B. natto* (▲), *B. subtilis* MR10 (■), *B. subtilis* TK8 (×) and mixed *Bacillus* sp (●)

Table 1: Scavenging activity of methanolic extract of SBM and FSBMs at 1 min of reaction

Samples	Percentage scavenging activity±SD
Soybean meal	25.17±1.07[e]
B. subtilis MR10	82.17±1.29[b]
B. subtilis TK8	91.84±4.81[a]
B. natto	70.00±0.27[c]
Mixed *Bacillus*	98.97±0.35[a]
R. oligosporus	49.46±0.25[d]

Each value represents mean±SD (n = 3) and different letter (s) are significantly different at $p<0.05$

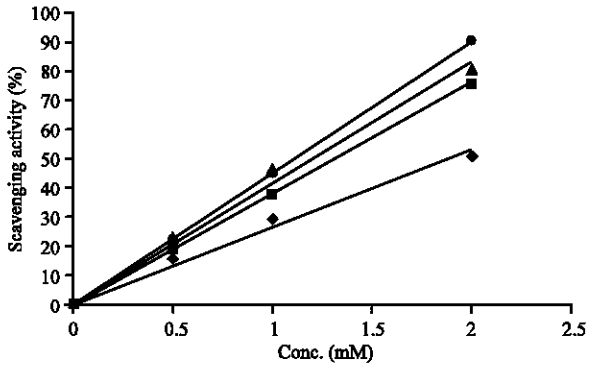

Fig. 2: Scavenging effect of standard antioxidants; α-tocopherol (♦), ascorbic acid (●), BHA (▲) and Trolox (■), at various concentration and 1 min of reaction time

B. subtilis MR10 and *B. natto*. When percentage scavenging activity of the extracts at 1 min of reaction time were calculated, the results showed that FSBM extract of mixed *Bacillus* sp. and TK8 significantly showed the highest percentage scavenging activity at 98.97 and 91.84%, respectively and the lowest value was found in SBM extract (25.17%) at $p<0.05$ (Table 1). While FSBM extract of *B. subtilis* MR10, *B. natto* and *R. oligosporus* removed free radical at 82.17, 70 and 49.45%, respectively. Therefore, we found that there was approximately 2-4 times higher in percentage cavenging activity of SBM when microbial fermentation was applied, especially, by the action of *Bacillus* sp.

The scavenging effect of some standard antioxidants was also investigated to compare with SBM and FSBM extract. It was found that at 1 min of reaction time, FSBM extract of *B. subtilis* TK8 and mixed *Bacillus* still exhibited higher in scavenging effect than those of every standard antioxidant at concentration 2 mM. On the other hand, those of SBM extract gave the lower activity than every standard antioxidant. The percentage scavenging activity of α-tocopherol, ascorbic acid, BHA and trolox at 2 mM and 1 min of reaction time were 51.09, 89.75, 80 and 75%, respectively (Fig. 2).

From the results above, we found the fermentation of soybean meal could improve the ability in $ABTS^{\bullet+}$ removal and we also found the fermentation by bacteria could enhance scavenging activity more satisfy than the one by mold. The difference of growth rate between bacteria and mold might be the reason for this result. Among these *Bacillus* sp. used in this study, there was a difference in scavenging effect obtained. We found that *B. subtilis* TK8 gave the best activity when compared to *B. subtilis* MR10 and *B. natto*. *B. subtilis* TK8 and MR10 were isolated from Tua-nao, a traditional fermented soybean of Thailand. *B. subtilis* TK8 is bacteria that capable of higher in cellulase production than *B. subtilis* MR10 and *B. natto* (data and detail not shown). Cell wall of soybean might be hydrolyzed by cellulase activity, therefore better secretion of active compounds from soybean cell was obtained and resulting in better scavenging activity. The active compounds found in soybean seed against free radicals are α-tocopherol, isoflovones and their glycosides such as malonyl glycitin, genistin, daidzin, daidzein, genistein and glycitin (Lee *et al*., 2005; Pyo *et al*., 2005; Yamamoto *et al*.,

Table 2: Reducing power of methanolic extract of SBM and FSBMs

Samples	A_{700}±SD
Soybean meal	0.299±0.003^{c}
B. subtilis MR10	0.910±0.006^{ab}
B. subtilis TK8	0.827±0.125^{b}
B. natto	0.848±0.017^{b}
Mixed *Bacillus*	1.031±0.010^{a}
R. oligosporus	0.835±0.150^{b}
α-tocopherol	1.575±0.138
Ascorbic acid	2.296±0.039
BHA	2.219±0.080

Each value represents mean±SD (n = 3) and different letter (s) are significantly different at $p<0.005$

2003). These active compounds can be found in many parts of soybean seed such as tocopherol that found mainly in cotyledon (92.8%), seed coat (5.4%) and axis (1.8%) (Yoshida *et al*., 2006), while 80-90% of total isoflavones were located in the cotyledon and the remainder in the hypocotyls (Hoeck *et al*., 2000). In case of *R. oligosporus* fermentation, there was a report on increasing of scavenging effect found in tempe caused by this mold (Nout and Kiers, 2005; Lin *et al*., 2006). That is consistent to our results, although our study was conducted in soybean meal not in whole seeds. The previous reports were studied on the scavenging effect of soybean and related products on several free radicals such as DPPH radical (Yang *et al*., 2000; Lee *et al*., 2005; Chen *et al*., 2005), superoxide anion radical and hydroxyl free radical (Yang *et al*., 2000; Yen and Hsieh, 1997) but our study showed that soybean meal and FSBMs extract could also eliminate ABTS radical.

Assay of reducing power was based on the reduction of Fe^{3+}/ferricyanide complex to the ferrous form in the presence of reductones (antioxidants) in the tested extracts (Oyaizu, 1986). In this study, the reducing power of the methanolic extracts of SBM and FSBMs was indicated as the higher in the formation of Perl's Prussian blue (A_{700}) and the results showed that microbial fermentation could improve the reducing power of soybean meal as found in the scavenging effect study. The reducing power of SBM extract was the lowest while the highest reducing power was found in the one of mixed *Bacillus* sp. at $p<0.005$. Among FSBM extracts, exception with FSBM extract of mixed *Bacillus* sp., no significant difference in reducing power of those FSBM extract was found at $p<0.005$ (Table 2). This result was consistent to their scavenging activity that microbial fermentation enhanced reducing power for SBM as approximately 2-3 times higher was found. In case of *R. oligosporus* fermentation, we found its reducing power was in the same level as found in extract of koji produced by this mold (Lin *et al*., 2006). The reducing power of every FSBM might be due to their hydrogen-donating ability as described by Shimada *et al*. (1992) such as reductone formed during fermentation that could react with active free radicals to stabilize and terminate radical chain reaction (Yang *et al*., 2000).

On overview of antioxidant activity improvement of SBM using microbial fermentation, many mechanisms might be concerned as same as found in the soybean fermentation. Isoflavones and their glycosides content in SBM and FSBM were considered as a potent scavenging effect and reducing power substances (Lee *et al*., 2005). Nout and Kiers (2005) reported referring to McCue and Shetty (2003) that during the tempe fermentation, at least a partial cleavage or change in the glucosides take place associated with increased glucosidase and glucuronidase activity, releasing potent antioxidant substances by transformation of flovonoids. Moreover, the other antioxidant substances found in fermented soybean by *R. oligosporus* were HAA (Esaki *et al*., 1996) and factor 2 (6, 7, 4-trihydroxyisoflavone) (Nout and Kiers, 2005). Lin *et al*. (2006) also reported that phenolic compounds that is antioxidants and found in soybean can be increased after fermentation. It was suggested that the liberation of lipophillic aglycones of isoflavone glucosides such as daidzein and genestein by the catalytic action of β-glucosidase during fermentation resulted in the increased antioxidative activity of fermented soybean. Furthermore, there is another mechanism to explain the increasing of antioxidant activity of soybean by fermentation. It might be due to increasing of free amino acid caused by microbial protease activity, especially, tyrosine, methionine, histidine, lysine and tryptophan that are

generally accepted to be antioxidants (Wang and Mejia, 2005; Yen and Hsieh, 1997). Wang and Mejia (2005) also reported referring to Chen *et al*. (2005) that during protein hydrolysis, the soy protein structure will be altered and more active amino acid R group will be exposed. Therefore, soybean peptides can have higher antioxidant activity than intact protein. For example, after enzyme digestion of β-conglycinin and glycinin, the radical-scavenging activity was increased 3-5 times. Those mechanisms given above might be the reason to explain the improvement of SBM by microbial fermentation.

CONCLUSIONS

It could be concluded that the antioxidant activity of SBM, an important protein source for monogastric animals, was improved by the action of tested bacteria and mold that are accepted as GRAS. The scavenging effect on $ABTS^{\bullet+}$ of every FSBM was approximately 2-4 folds higher than SBM. FSBM fermented by *Bacillus* sp. exhibited higher scavenging effect than that by *Rhizopus oligosporus*. For reducing power, the results were in consistent to those of scavenging effect assay that FSBM gave better activity than SBM. In addition, there was no statistically significant difference between FSBM fermented by bacteria and mold. These obtained results suggested that FSBMs might be a potent product for animal feed industry to improve the animal performance.

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