NUTRITIVE VALUE BETWEEN FERMENTED AND GERMINATED SOYBEAN: γ-AMINOBUTYRIC ACID, AMINO ACIDS CONTENT AND ANTIOXIDANT PROPERTIES

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ABSTRACT. *In this study, soybean was subjected to both fermentation and germination process that was exposed either in an aerobic or anaerobic condition or combination of both. The γ-aminobutyric acid (GABA), amino acids content and antioxidants properties of both fermented and germinated soybean were analyzed. In all cases, the fermented soybean inoculated with Rhizopus 5351 strain were noted to have high content of GABA, essential amino acids and better antioxidant activities than germinated soybean. It was observed that fermented soybean subjected to both combination of aerobic and anaerobic incubation treatment (FSB3) had the highest content of GABA with the abundant of beneficial free and essential amino acids content, which was 0.328, 3.212 and 1.104 g/100g dry weight, respectively. In addition, sample FSB3 also showed the highest content of total phenolic compound (22.56 mg gallic acid equivalent/g extract) and antioxidant activity with the lowest IC_{50} value of 20 mg extract/mL among other soybean treatment. Within germination process, anaerobic germinated soybean at 72 h appeared to have better yield of GABA and amino acids content with higher antioxidant activities than other germinated soybean under aerobic condition. This sample was observed to contain higher amount of total phenolic compound and ferric reducing antioxidant power with lower value of IC_{50}. This study showed that the fermented soybean inoculated with Rhizopus 5351 strain is able to produce better nutritive value when compared to germinated soybean either in aerobic or anaerobic condition.*

KEYWORDS. Antioxidant; Fermentation; γ-amino butyric acid (GABA); Germination; Soybean

INTRODUCTION

Globally, there is an increase interest in nutritional and health functional foods. Many consumers are looking for the potential healthy foods that are able to bring pharmaceutical effects on human body. Soybean, world's most important legume in term of production and trade**,** has been the main choice of food in many countries mainly because of the higher protein content compared to other legumes. However, raw soybean has been reported to have low nutritive value due to low amount of essential amino acids and the presence of antinutritional factors. Therefore, many attempts have been done to improve its nutritive value of this legume. In general, both germination and fermentation process are the common practices to improve its nutritional profile by eliminate or reduce the antinutritional and indigestible factors in legumes (Bau *et al.*, 1997; Watanabe *et al*., 2008; El-Adawy, 2002; Park & Oh, 2007; Jannoey *et al*., 2010). The process of soybean protein breaking down either through germination or fermentation process has contributed to the changes in amino acid distribution and other nutritive value. During germination of beans, trypsin inhibitor activity,

tannin and phytic acid was drastically reduced as reported by previous few germination studies (Khalil & Mansour, 1995; El-Adaway, 2002). On the other hand, fermentation has been well recognized as a process to enhance the nutritional composition and metabolic regulatory functions of various foods. The employment of various culture of microorganism in beans has substantially enhanced the γ-aminobutyric acid (GABA), flavonoid content, free amino acids and other bioactivities constituent. It is well known that fermentation on soybean brings changes in texture, aroma, flavor of the product and improves the nutritional quality. Some features as lipids, oligosaccharides and protein profile, several vitamins like vitamin B_{12} are improved after fermentation (Liem *et al*., 1977; Bisping *et al*., 1993, Ran *et al.*, 2007).

One of its bioactive compounds that increased after the germination or fermentation process is GABA, a non-protein constituent amino acid with molecular formula, $C_4H_9NO_2$ (Aoki *et al*., 2003; Park & Oh, 2007; Jannoey *et al.*, 2010). GABA has numerous physiological functions and has been reported to be a neurotransmitter inhibitor (Möhler, 2011), an anti-diabetic (Adeghate & Ponery 2002) agent, anti-inflammatory agent (Park *et al*., 2009) and anti-hypertension agent (Nakamura *et al*., 2000; Aoki *et al*., 2003) as well as being capable of inhibiting the proliferation of cancer cells (Ortega, 2003; Oh & Oh, 2004). Other health benefits of fermented soybean arising from antioxidant activity of phenolic compounds are also improved during fermentation (McCue & Shetty, 2004; Watanabe *et al*., 2008; Babu *et al*., 2009). Consequently, the development of functional food enriched with GABA and antioxidant property is increasingly in demand. The production of GABA-enriched soybean was developed based on the theory that GABA accumulates in the stress condition. When exposed to stress, the GABA transaminase enzyme was blocked by the accumulation of succinic semialdehyde in the Krebs cycle metabolic pathway, thus it is enhancing the GABA yield.

Therefore, the aim of the present work was to investigate the effect of different germination and fermentation treatment on the GABA, amino acids distribution and antioxidant property of soybean compared to raw beans. The anaerobic stress was applied in both germination and fermentation process on soybean with the objective to accumulate GABA in conjunction with Krebs cycle and anaerobic stress. In this investigation, GABA enriched soybean via fermentation process was conducted either in an aerobic condition or combination of aerobic and then successively anaerobic fermentation using the *Rhizopus* 5351 strain obtained from MARDI's Centre of Functional Food Cultures (CFFC) collection.

MATERIALS AND METHODS

Materials

The *Rhizopus monosporus* 5351 strain was obtained from MARDI's Culture Functional Food Centre (CFFC). Raw soybean was bought from local supermarket (imported from Canada). The amino acids mix standards (2.5 mM of amino acids & 1.25 mM of cysteine) were bought from Waters, including histidine (His), serine (Ser), arginine (Arg), glycine (gly), aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), lysine (Lys), tyrosine (Tyr), methionine (Met), valine (Val), isoleucine (Ile), leucine (leu) and phenylalanine (Phe). The γ-aminobutyric acid (GABA) was purchased from Sigma-Aldrich. All the solvents used either analytical or HPLC grade as described in details in the methods used.

Solid state fermentation of soybean

Soybean was soaked overnight at room temperature (28°C) in distilled water at a ratio of 1: 3 (w/v) for 18 h. The dehulled soybean were washed few times and were boiled in water (1: 6 w/v) for 60 min, drained and cooled to room temperature. The cooked soybean was fan dried for an hour to the moisture content of 50 - 55% before being inoculated with MARDI's *Rhizopus* 5351 strain at the level of 2 g/kg soybean, packed in perforated polyethylene bags (thickness: 15 – 20 mm, perforation distance: 1 cm apart, 100 g cooked soybean per pack). The inoculated soybean was subjected to three different fermentation treatments: a) FSB1 – the inoculated soybean was incubated aerobically for 30 h at 30°C; b) FSB2 – the inoculated soybean was incubated aerobically for 48 h at 30°C; c) FSB3 - the inoculated soybean was initially incubated aerobically at 30°C for 30 h and then successively subjected to anaerobic fermentation (anaerobic jar) for 20 h. Finally, the fermented soybean was dried at 70°C for 2 days to obtain the moisture content of 4 – 5% before grinding to fine powder.

Germination Process of soybean

A 100 g of soybean was weighed and washed a few times to remove any dirt. The cleaned soybean was soaked with 500 mL distilled water overnight. The soaking soybean was subjected to aerobic and anaerobic germination separately and incubated at 24, 48 and 72 h. The anaerobically germination process was done in an anaerobic jar and incubated in the dark condition at room temperature. Then, the germinated soybean was dried at 70°C for 2 days before grinding to fine powder.

Extraction of water soluble free amino acids and GABA

The water soluble free amino acids and GABA was extracted from germinated, fermented and the control soybean using distilled water. A total of 1 g fine treated soybean or raw soybean powder was mixed with 20 mL distilled water. The mixture was vigorously shaken at 300 rpm (30°C) for 30 min, followed by centrifugation at 10,000 rpm for 5 min. The supernatant obtained was stored overnight at -40°C prior to being freeze dried to obtain a concentrated bioactive extract for GABA and the amino acids profile analysis.

Determination of GABA content and amino acids profile using ultra performance liquid chromatography (UPLC)

The GABA and amino acids profile of treated soybean or raw soybean was separated using AccQ-Tag^{TM} Ultra column (2.1 mm x 100 mm, 1.7 µm) at the flow rate of 0.7 mL/min with column temperature controlled at 55°C under the UV spectra of 260 nm. A total of 10 µL of water extract from germinated, fermented and the control soybean was derivatized with 70 µL of AccQ-Tag^{TM} Ultra borate buffer and mix vigorously. Then, 20 µL of AccQ^{TM} Fluor reagent was added and vortex for a while before heating at 55°C for 10 min, followed by injecting 1 µL into the UPLC system as described in the UPLC amino acid analysis application solution. The gradient elution consisting of AccQ-Tag^{TM} Ultra Eluent A and AccQ-Tag^{TM} Ultra Eluent B. Gradient elution was conducted as follows: from 0 to 0.54 min, maintained at 99.9% A; from 0.54 to 5.74 min, linear gradient from 99.9 to 90.9% A; from 5.74 to 7.74 min, linear gradient from 90.9 to 78.8% A; from 7.74 to 8.50 min, linear gradient from 78.8 to 40.4% A and then hold for 0.3 min at 40.4% A; from 8.80 to 8.90 min, linear gradients from 40.4 to 99.9% A and then maintained at 99.9% for another 2.1 min. Quantification was made using calibration curves obtained by injecting known amounts of amino acids standard and GABA as external standards with known retention times. The total essential amino acids were calculated based on the sum of phenylalanine, threonine, methionine, leucine, isoleucine, lysine and valine. All analyses were performed in triplicate.

Water soluble extract for antioxidant profile assay

A total of 20 g treated soybean or raw soybean was extracted by boiling with 200 mL distilled water at 100°C for 10 min. The residue samples were then extracted with another 100 mL boiled distilled water as described earlier. The combined water extracts were filtered and freeze-dried. The freeze dried samples will be subjected to three different antioxidant assays as described below.

Determination of total phenolic content

The total phenolic content of water extract of treated soybean or raw soybean was determined according to Marina *et al*., (2009) with some modifications. Aliquots of the 1 mL water extract (2 mg/mL) were taken in a test tube and mixed with 5 mL Folin-Ciocalteu reagent (1:1 with water) and allow to stand at room temperature for 5 min. A total of 4 mL of sodium carbonate solution (7.5%, w/v) were added sequentially and vortex the reaction mixture. The tubes were placed in the dark for 2 h and the absorbance was recorded at 765 nm against the reagent blank. The amount of total phenolic content was calculated as gallic acid equivalents (mg GAE/g extract) from a gallic acid calibration curve.

Determination of free radical scavenging activity

The free radical scavenging activity of treated soybean or control was determined using the 1,1-diphenyl-2-picryl-hydrazil (DPPH) according to Thaipong *et al.*, (2006) with minor modifications. The beans were extracted by boiling with distilled water at 100°C for 10 min. The DPPH stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored at -20°C. The working solution was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 ± 0.02 units at 517 nm using spectrophotometer. A total of 2850 µL of freshly prepared methanolic DPPH solution was added to the test tube that containing 150 µL of the water extract sample and then shaken vigorously. The decolourizing process was recorded after left stand for 30 min in the dark condition. The absorbance (Abs) was measured at 517 nm and compared with a blank control. IC_{50} (Inhibitory concentration of 50%) was determined from the DPPH calibration curve, denoted the concentration of sample required to scavenge the 50% of the DPPH free radicals. The scavenging ability was calculated as follows:

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

Determination of ferric reducing antioxidant power (FRAP)

The FRAP assay was done according to method of Thaipong *et al.* (2006). The stock solutions including 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-*s*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before use. A total of 150 µL of water extract (5 mg/mL) was allowed to react with 2850 µL of FRAP solution and shaken vigorously before being left to stand for 30 min in the dark. The reading of the colored product (ferrous tripyridyltriazine complex) was taken at 593 nm. The ascorbic acid was used as a standard and was linear in the range in between 20 to 200 ppm. The result was expressed as ascorbic acid equivalent (mg AAE)/ g extract.

RESULTS AND DISCUSSION

GABA and amino acids content

Changes in GABA and amino acids content of control soybean, germinated or fermented soybean were investigated and the results are presented in Figure 1. In comparison with germinated soybean, the GABA, free and essential amino acids of treated soybean increased drastically in fermented soybean inoculated with *Rhizopus* 5351 strain especially for the sample FSB3. This inoculated soybean that was initially incubated aerobically at 30°C for 30 h and then successively subjected to anaerobic fermentation for 20 h, exhibited the highest content of GABA with higher beneficial free and essential amino acids content, which was 0.328, 3.212 and 1.104 g/100 g dry weight, respectively. Similarly, this finding was also reported by Jannoey *et al.*, (2010), whereby the fermented rice showed higher GABA concentration than the germinated rice. Although fermented soybean under aerobic incubation also showed an increase in GABA and free amino acids content than raw soybean, however, prolong fermented soybean under aerobically incubation period was noted to have lower yield of GABA as evidently shown in sample FSB1 (30 h) and FSB2 (48 h), which were 0.284 g and 0.154 g/100 g dry weight, respectively. This phenomenon showed that anaerobic incubation was necessary based on the theory that GABA accumulates under stress conditions (Brouché & Fromm, 2004). However, the production of free and essential amino acids was noted to increase with the aerobic fermentation period. Nevertheless, combination of both aerobic and anaerobic fermentation in sample FSB3 showed a drastic increment of free and essential amino acids content than sample FSB2.

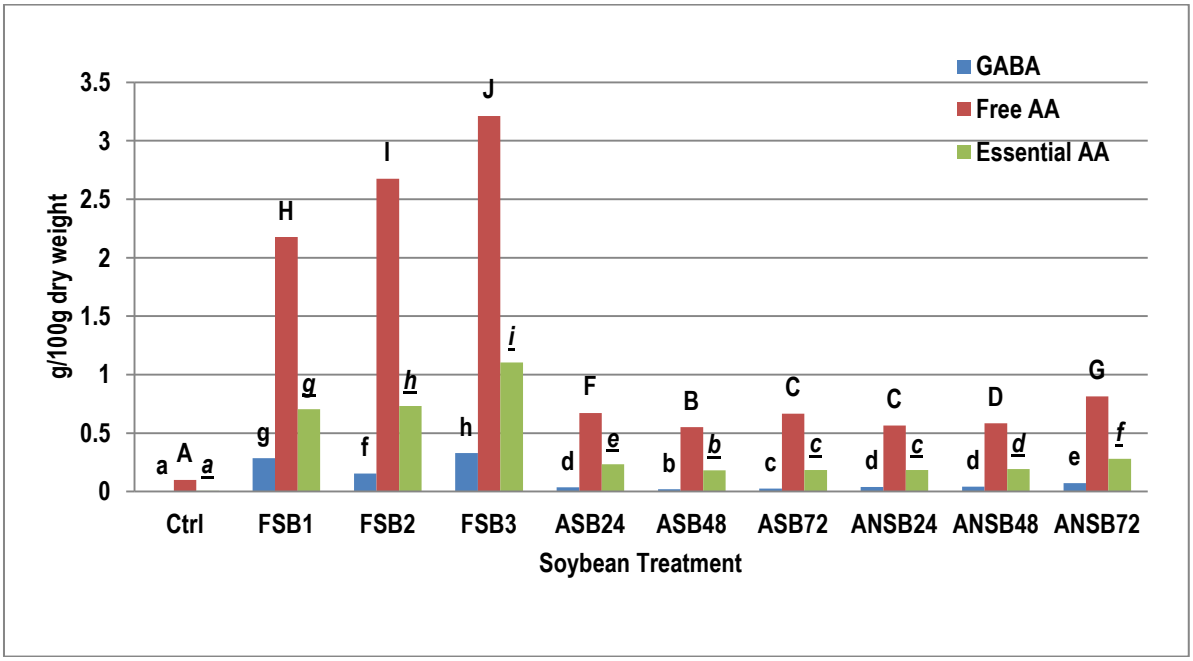

Figure 1: The GABA, free and essential amino acids content of germinated, fermented and the control soybean under different incubation period.

(Abbreviations: ASB24, ASB48, ASB72 = Aerobic germination of soybean at 24, 48 and 72 h, respectively; ANSB24, ANSB48, ANSB72 = Anaerobic germination of soybean at 24, 48 and 72 h, respectively; FSB 1 = Aerobic fermentation of soybean at 30 h; FSB2 = Aerobic fermentation of soybean at 48 h; FSB3 = Aerobic fermentation of soybean at 30 h, followed by anaerobic fermentation at 20 h; Free AA = sum of His, Ser, Arg, Gly, Asp, Glu, Thr, Ala, GABA, Pro, Lys, Tyr, Met, Val, Ile, Phe, Tryp content; Essential AA = sum of His, Thr, Lys, Met, Val, Ile, Leu, Phe, Tryp content. Means with each bar with different superscripts are significantly ($P < 0.05$) different).

Overall, the germination process was found to be less effective in the production of GABA and free amino acids when compared to fermentation treatment on soybean. All

germinated soybean either incubated aerobically or anaerobically has exhibited lower yield of GABA and free amino acids content. However, anaerobic germinated soybean at 72 h appeared to have better yield of GABA, free and essential amino acids content than other germinated soybean samples, which was 0.073 g, 0.813 g and 0.279 g/100 g dry weight, respectively. This germinated soybean gave higher GABA concentration than other anaerobically germinated barley grain as reported earlier, which contained only 0.0143 g GABA/100 g (Chung *et al.*, 2009).

Total phenolic content and antioxidant profile

The total phenolic content and antioxidant profile of germinated, fermented and the control soybean were compared and the results are summarized in Table 1. Both germination and fermentation process have caused an increase in the content of total phenolic and antioxidant capacity compared to control soybean. The same findings were also reported by a few scientific studies (Watanabe *et al*., 2007; Hiran *et al.*, 2011; Wu *et al*., 2011). Fermentation process was known to improve the antioxidant profile of legumes. The antioxidant activity as estimated by 1,1,-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays indicated that fermented soybean can quench the superoxide free radical better than germinated soybean and possibly scavenge the hydrogen peroxide generated in the reaction mix. It was found that phenolic compounds and antioxidant capability of fermented soybean under the combination of aerobic and anaerobic treatment were 4-5 folds greater than the control. The antioxidant activities of fermented soybean might be attributed by various groups, namely free amino acids, peptides and phenolic compounds as claimed by Watanabe *et al.* (2007). During fermentation process, nutritional quality of fermented soybean increased because some enzymes such as amylases, xylanases and proteases derived from the grain and microbes contribute to the modification of grain composition (Katina *et al.*, 2007). Among all fermented soybean treatment, soybean inoculated with MARDI's *Rhizopus* 5351 strain under aerobic fermentation for 30 h and then successively anaerobic fermentation for 20 h at 30°C gave the highest yield of total phenolic content (22.56 mg gallic acid equivalent/g extract) and FRAP value (11.27 mg ascorbic acid equivalent/g extract) with the lowest of IC_{50} value (20 mg extract/mL). This result indicated that the additional process of anaerobic incubation has increased the nutritional quality of fermented soybean than raw soybean or aerobically incubated fermented soybean.

Table 1. Antioxidant profile of germinated, fermented and the control soybean under different incubation condition[a].

Process	Treatment (mg GAE/g extract)		TPC (mg extract/mL)	IC_{50} (mg AAE/g extract)	FRAP
Control	-		4.59 ± 0.02	88.18 ± 0.64	2.92 ± 0.01
Aerobic Germination	ASB 24	24 h	10.39 ± 0.20	233.82 ± 8.00	4.00 ± 0.04
	ASB28	48 h	12.34 ± 0.18	367.94 ± 47.21	5.28 ± 0.03
	ASB72	72 h	13.35 ± 0.15	144.32 ± 3.24	5.99 ± 0.04
Anaerobic Germination	ANSB 24	24 h	5.53 ± 0.17	101.43 ± 1.20	2.85 ± 1.04
	ANSB48	48 h	11.24 ± 0.15	87.60 ± 2.58	6.27 ± 0.15
	ANSB72	72 h	16.53 ± 0.15	39.89 ± 2.39	9.83 ± 0.09
Aerobic Fermentation (by Rhizopus 5351 strain)	FSB1	30 h	19.75 ± 0.01	24.21 ± 0.36	11.43 ± 0.02
	FSB2	48 h	14.70 ± 0.10	20.66 ± 0.14	5.81 ± 0.01
Aerobic + Anaerobic Fermentation (by Rhizopus 5351 strain)	FSB3	30h + 20h	22.56 ± 0.31	20.00 ± 0.11	11.27 ± 0.03

[a]Each value in the table represents the mean ± standard deviation from triplicate analyses

Abbreviations: TPC = Total phenolic content; IC_{50} (Inhibitory concentration at 50%) = Concentration of sample required to scavenge the 50% of the DPPH free radicals; FRAP = Ferric reducing antioxidant power; GAE = Gallic acid equivalent; AAE = Ascorbic acid equivalent)

Phenolic compounds and antioxidant activities of fermented soybean were more positively correlated with the fermentation incubation period. The same phenomenon was also observed in the anaerobically germinated soybean. When compared to fermentation process, germination process was found to be less competitive in improving the nutritional quality of soybean in terms of total phenolic content and antioxidants capability. However, germinated soybean under anaerobic condition did improve the phenolic content and antioxidant profile than aerobically germinated soybean. Particularly, anaerobic germinated soybean after day 3 of germination showed higher antioxidant activity which correlates to higher total phenolic content than other germinated soybean samples and the control, with the total phenolic content of 16.53 mg gallic acid equivalent/g extract and IC_{50} value of 39.89 mg extract/mL.

In summary, the presence of GABA, various free amino acids and phenolic compounds in fermented soybean not only improve the nutritive value of food, but was also claimed to have other health advantages such as rapid absorption, antioxidant activity and anticancer. (McCue & Shetty 2004; Oh & Oh, 2004; Ran *et al*., 2007; Watanabe *et al*., 2007 and 2008; Babu *et al*., 2009). The anaerobic stress with the combination of a suitable *Rhizopus* 5351 strain as a GABA producer on soybean was an innovation of GABA functional food which was enriched with free amino acids and phenolic content and at the same time containing better antioxidant property. It was an efficient method to increase GABA yield in soybean with higher nutritional values.

CONCLUSION

As shown in this study, aerobic or anaerobic incubation either under germination or fermentation on soybean did affect the nutritional profile of soybean. All the processes have shown to improve the nutritional quality of soybean when compared to raw soybean. In general, fermented soybean had shown better nutritive value and antioxidant property than germinated soybean. The functional and nutritive value of fermented soybean was enhanced by applying microorganism with a high GABA producer (*Rhizopus* 5351 strain) with the combination of aerobic and anaerobic incubation to stimulate the GABA yield under stress condition. This fermented soybean exhibited the highest yield of GABA with the abundant of beneficial free and essential amino acids content. On top of that, this sample also showed the highest content of total phenolic compounds with the highest antioxidant activities when compared with other soybean sample. Moreover, the presence of these compounds in fermented soybean not only enhanced the taste of food but also have other nutritional advantages, providing alternative product that has a great potential to bring multiple health benefits if consumed constantly. In future work, few bioactivities assay will be conducted on this fermented soybean to assess its specific health benefits.

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