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# γ-Aminobutyric Acid (GABA) Production and Angiotensin-I Converting Enzyme (ACE) Inhibitory Activity of Fermented Soybean Containing Sea Tangle by the Co-Culture of *Lactobacillus brevis* with *Aspergillus oryzae*

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To enhance the γ-aminobutyric acid (GABA) content, the optimized fermentation of soybean with added sea tangle extract was evaluated at 30°C and pH 5.0. The medium was first inoculated with *Aspergillus oryzae* strain FMB S46471 and fermented for 3 days, followed by the subsequent inoculation with *Lactobacillus brevis* GABA 100. After fermentation for 7 days, the fermented soybean showed approximately 1.9 g/kg GABA and exhibited higher ACE inhibitory activity than the traditional soybean product. Furthermore, several peptides in the fraction containing the highest ACE inhibitory activity were identified. The novel fermented soybean enriched with GABA and ACE inhibitory components has great pharmaceutical and functional food values.

**Keywords:** γ-Aminobutyric Acid, *Lactobacillus brevis*, *Aspergillus oryzae*, angiotensin-I converting enzyme inhibition, soybean, sea tangle

γ-Aminobutyric acid (GABA), which is known as a neurotransmitter in the central nervous system, has various physiological functions in animals and humans, including antihypertensive activity [3, 4]. Moreover, angiontensin-I converting enzyme (ACE)-inhibitory activity can be also related to enzymatic proteolysis and can regulate the pressure of blood by binding with ACE [15]. The aim of this study was to develop a novel functional fermented soybean with both high GABA and ACE inhibition characteristics. To increase the GABA content and ACE inhibitory activity in the fermented soybeans, optimization of the soybean fermentation process was conducted for several fermentation conditions. Sea tangle (*Saccharina japonica*), known as a seaweed that has plentiful glutamic acid, was used to provide a natural source of glutamate [12]. ACE inhibitory activities were analyzed at each stage, from the fermentation to the digestion of the fermented soybeans, and the molecular masses and amino acid sequences of the peptides that were present in the ACE inhibitory fraction were identified.

## Optimization of GABA Production in Fermented Soybean

Basal steamed soybeans were prepared as follows. Soybeans crushed by a blender were added to sea tangle extract (STE). STE was prepared by autoclaving the mixture with sea tangle powder and distilled water, and filtered through filter paper. Next, soybeans containing STE were sterilized by autoclaving at 121°C for 15 min. After fermentation, the amino acids and GABA were assessed by TLC and HPLC [6, 8]. Three microorganisms were used for the fermentation; *A. oryzae* FMB S46471 and *B. subtilis natto*, which were derived from *meju*, and *L. brevis* GABA 100, which was isolated from *kimchi*, as previously reported [7, 9]. *A. oryzae* FMB S46471 was inoculated at $5 \times 10^4$ spores/ml at the beginning of the fermentation, whereas *B. subtilis natto* and *L. brevis* GABA 100 were inoculated at the 1% (v/v, $10^8$ CFU/ml) level at the beginning of fermentation, or after 3 days of fermentation. GABA production was observed when *L. brevis* GABA 100

was cultured. The fermented soybean cultured with *B. subtilis natto* or co-cultured with *B. subtilis natto* consumed the glutamate; thus, GABA was minimally produced even in the presence of *L. brevis* GABA 100 (data not shown). These results were similar to the usual fermentation process with *A. oryzae* and *B. subtilis* [5].

Various combinations of the soybean, sea tangle, and water were composed according to Table 1. Groups G1 to G9 comprised the various compositions of soybean and sea tangle in 5 g quantities; Groups G10 and G11 were compared with G1, and then the optimal fermentative conditions for GABA production were selected based on TLC analysis. As a result, GABA was produced in greater quantities by increasing the soybean content compared with sea tangle (Fig. 1A). The fermented soybean added with sea tangle extract showed lower levels of GABA than that added with sea tangle powder. The sea tangle component present in the powder but excluded in the extract may have contributed to the elevation of GABA production. For instance, group G5 (composed only with STP) contained GABA after the fermentation, whereas in group G9 (composed only with STE), the pattern of GABA was not detected. In Fig. 1B, the pattern of glutamate in groups G10 and G11 by the third day declined compared with the beginning of the fermentation period. In other words, the glutamate from the sea tangle was not used to produce GABA during fermentation by the third day, which was partially consumed by the microbes. Furthermore, the effects of temperature (25°C, 30°C, and 37°C), pH (4.0 to 6.0), inoculum age, and several carbohydrates were investigated. The optimal fermentative conditions were found to be 30°C, pH 5.0, with no added carbohydrates based on the TLC (data not shown). *A. oryzae* FMB S46471, which was inoculated at the beginning of fermentation, might have extended the period of protein hydrolysis. *L. brevis* GABA 100, which has GABA-producing activity, showed the highest GABA content when inoculated on the third day. Moreover, as shown in Fig. 1C, adding the sea tangle to the soybean had a beneficial function in regulating the pH of the soybean medium during fermentation. Groups G10 and G11 were able to maintain a pH of approximately 6.0 or below, whereas the pH of G1 increased. In this study, GAD of *L. brevis* played an important role in converting glutamate to GABA. Therefore, the sea tangle was able to lower and maintain the pH to the nearly optimal condition for GAD activity and GABA production.

The selected fermentation conditions based on TLC analysis were those found in group G1 (as a control group), and then G10 and G11, which contained the added sea tangle and the highest soybean composition. The fermented soybean–containing STP cultured under optimal conditions produced 1.9 g/kg of GABA.

**Table 1.** The various medium compositions with different ratios of soybean, sea tangle, and water, used for the fermentative production of GABA.

| Group | Composition of the steamed soybean | | | | | Change of glutamate and GABA during fermentation[a] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | SB (g) | STP (g) | DW (ml) | STE (g/ml) | | Glutamate (mg/kg) | | GABA (mg/kg) | |
| | | | | ST (g) | DW (ml) | 0 day | 7 days | 0 day | 7 days |
| G1 | 5.0 | - | 50 | - | - | 46.20 | 1,058.57 | 16.31 | 1,503.14 |
| G2 | 3.75 | 1.25 | 50 | - | - | | | | |
| G3 | 2.5 | 2.5 | 50 | - | - | | | | |
| G4 | 1.25 | 3.75 | 50 | - | - | | | | |
| G5 | - | 5.0 | 50 | - | - | | | | |
| G6 | 3.75 | - | - | 1.25 | 50 | | | | |
| G7 | 2.5 | - | - | 2.5 | 50 | | | | |
| G8 | 1.25 | - | - | 3.75 | 50 | | | | |
| G9 | - | - | - | 5.0 | 50 | | | | |
| G10 | 5.0 | - | - | 2.5 | 50 | 1,384.20 | 936.11 | 64.75 | 1,894.12 |
| G11 | 5.0 | 2.5 | 50 | - | - | 1,413.64 | 389.22 | 50.55 | 1,919.72 |

SB, soybean; STP, dried sea tangle powder; STE, sea tangle extract; DW, distilled water.

STE was prepared by autoclaving a mixture of sea tangle powder and distilled water, and was filtered through filter paper.

[a]Groups G1, G10, and G11 were selected to measure glutamate and GABA, both of which were assessed by HPLC. Other groups were not evaluated, because they showed lower GABA than G1, G10, and G11 analyzed by the TLC method (data not shown).

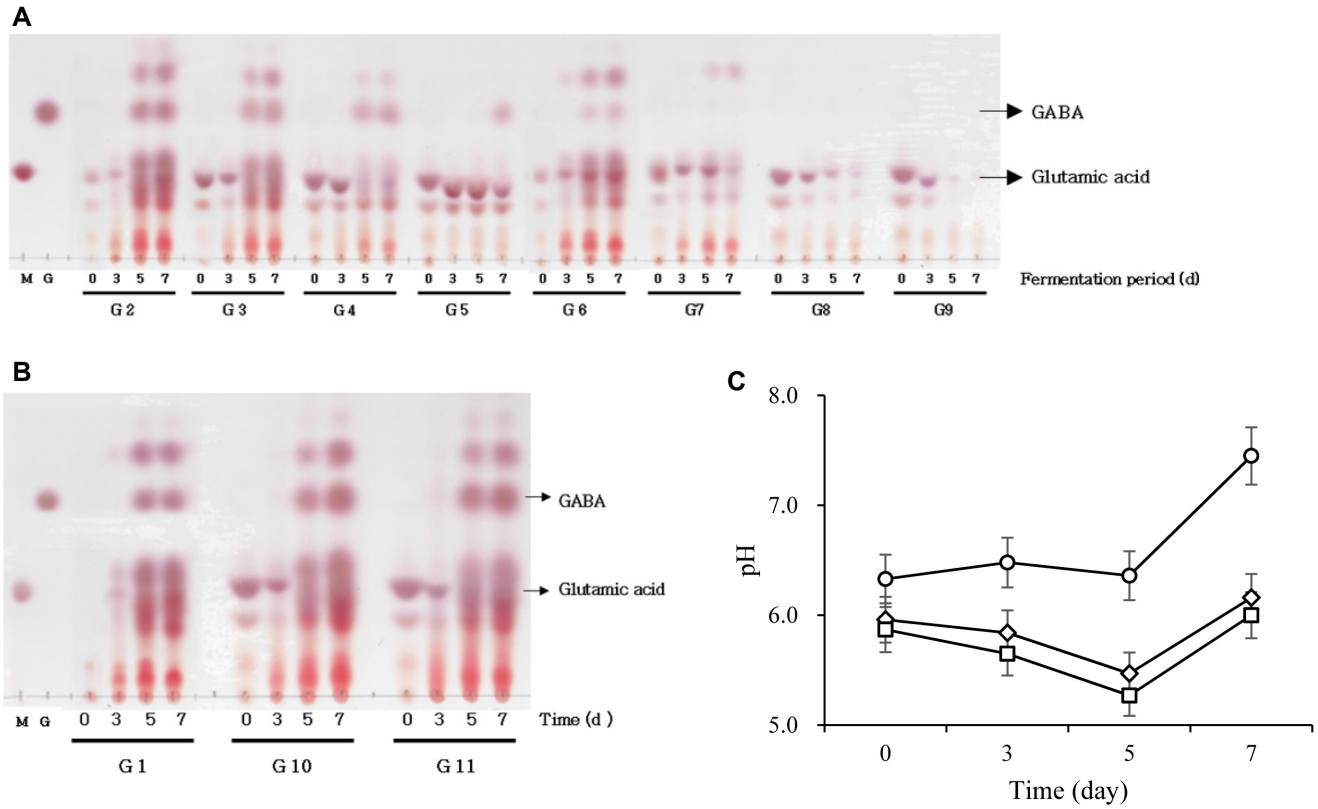


**Fig. 1.** Effects of various media compositions on the production of GABA.
The pattern of amino acid production depending on medium composition (**A** and **B**) and pH (**C**) (circles, G1; diamonds, G10; squares, G11). M, MSG; G, GABA.

## Measurement of the ACE Inhibitory Activity of Fermented Soybean

The ACE-inhibition effect of fermented soybeans was determined *in vitro* using a previously described method [10]. As shown in Fig. 2A, ACE inhibitory activities were increased in the soybeans fermented by *A. oryzae* FMB S46471 compared with those fermented by *L. brevis* GABA 100 or *B. subtilis natto*. Only group A0G3 showed a high level of GABA and ACE inhibition, which was fermented with *A. oryzae* FMB S46471 at the beginning of fermentation and then inoculated with *L. brevis* GABA 100 at 3 days after fermentation. Thus, subsequent experiments were conducted by using the inoculum combination of A0G3 to increase GABA production and ACE inhibitory activity simultaneously.

When *L. brevis* was inoculated into the soybean, the pH typically decreased to approximately 4.3 within 24 h, whereas *A. oryzae* and *B. subtilis* increased the pH by hydrolyzing the soybean protein. The change of pH until the third day and the pattern of amino acid production in group A0G0 were similar to that of G0 (data not shown). Production of amino acids and GABA was affected by a pH below 5.0, which was able to inactivate or decrease the activities of enzymes, such as protease and GAD. Release of amino acids by *A. oryzae* was observed at the third day after inoculation, and the pH failed to increase, as presented in Fig. 1C. This means that *A. oryzae* needed 3 days to grow and produce proteases after inoculation. Thus, the ACE inhibitory activity of *A. oryzae* could be decreased by the lower pH when *L. brevis* was inoculated at the beginning of fermentation.

In group A0G3, the pH ranged from 5.5 to 6.5 for 3 days, which was when *A. oryzae* was growing during the fermentation. Then, *L. brevis* lowered the pH to 5.0–6.0, at which time GAD efficiently converted glutamate into GABA. Likewise, this pH range might not affect the inhibition of the growth and the protease activity of *A. oryzae*.

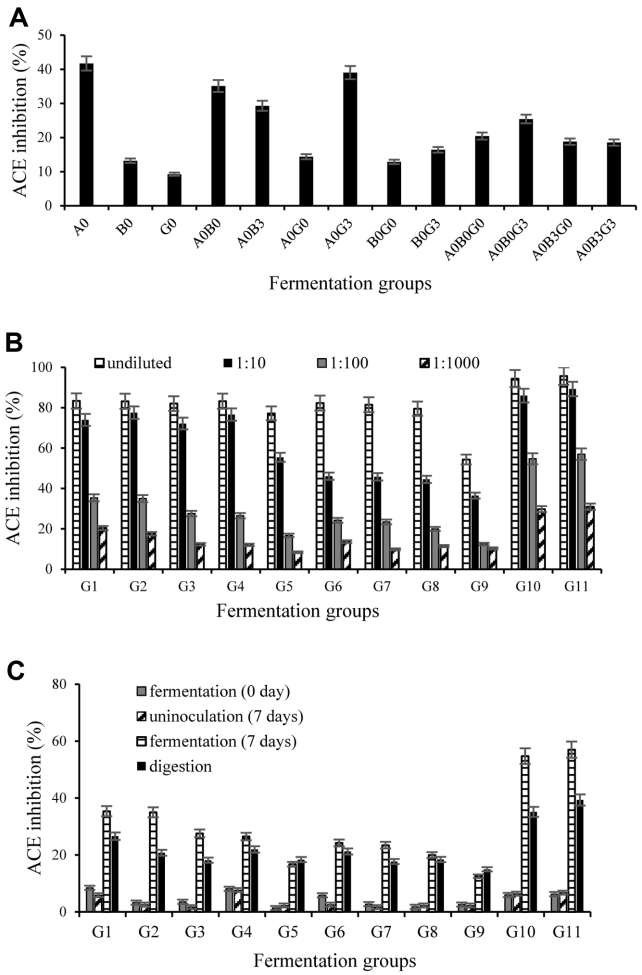


**Fig. 2.** ACE inhibitory activities of fermented soybeans depending on the inoculation combination (**A**), the addition of sea tangle (**B**), and the stage of fermentation and digestion (**C**).

(**A**) A, *A. oryzae* FMB S46471; G, *L. brevis* GABA 100; B, *B. subtilis natto*. The inoculation time of each microorganism is written on the right of the microorganism abbreviation in the fermented groups. (**B**), (**C**) Refer to Table 1 for the details of each fermentation group; data are expressed as averages in triplicate.

In the *A. oryzae* FMB S46471 and *L. brevis* GABA 100 co-cultured fermented soybean, the combination of soybean and sea tangle in the fermented soybeans also affected ACE inhibition, and the results showed that the ACE inhibitory activities were positively proportional to the level of GABA production. The diluted groups of G10 and G11 (at the ratio of 1:100 (v/v)) were above 50% (Fig. 2B). In comparison, the diluted Korean traditional fermented soybeans (at the ratio of 1:100 (v/v)) showed 22–31% ACE inhibitory activity in this study. The addition of STE (groups G6–G9) was less effective on the ACE inhibitory activity than STP addition (groups G2–G5). ACE inhibition was correlated with a high degree of hydrolysis [2], and the amount of GABA was influenced by the amount of glutamate produced from protein sources. The *in vitro* ACE inhibitory activity of the fermented soybean (at a dilution ratio of 1:100) was evaluated by its *in vitro* treatment with various digestive enzymes (pepsin, trypsin, and α-chymotrypsin) and the subsequent estimation of the ACE inhibition activities.

In all tested samples of fermented soybean with various combinations of soybean and sea tangle, ACE was increased by fermentation, but it tended to be decreased by digestive enzymes. This suggests that ACE inhibitors generated from fermented soybeans might be hydrolyzed by gastrointestinal digestive enzymes, such as pepsin, trypsin, and α-chymotrypsin. From the results of these comparisons, the fermentation of soybeans was shown to be a beneficial process that improved bioactivity, such as GABA production and ACE inhibitors, although fermented soybean hydrolysate decreased ACE inhibition compared with prior digestion. Several studies reported that fermented soybeans, such as soy milk and Korean soybean paste, lowered the blood pressure in *in vivo* models, although they were fermented under slightly different conditions [13, 14].

## Purification and Identification of Peptides in the High ACE Inhibitory Fraction

The fermented soybean G10 hydrolysate (FSH) was fractionated using UF membrane filters. The ACE inhibitory activity of the fraction below the 3 kDa MW cut-off showed the lowest $IC_{50}$ (11.69 μg/ml). Then, the identification of peptides in the ACE inhibitory fraction having the highest activity was assessed by nano-LC-ESI-MS/MS. The MS/MS spectra of the peptide fractions below 3kDa indicated nine novel peptides derived from soybeans that had not been previously reported (Table 2).

A peptide, WAMLGALGCVFPELLARNGVKFGEASWFK, contains hydrophobic-rich amino acids at the N-terminus and Trp and Phe at the C-terminus as a potent inhibitor, which may easily bind on the structure of ACE [1]. In addition, the peptides VFDGELQEGR and LQESVIVEISKK were mentioned in a patent indicating that several polypeptides or polypeptide fragments that included these sequences could be isolated from soybean oil body–associated proteins; they may be useful for the treatment or prevention of cardiovascular disease because of their suppression of cholesterol uptake by Caco-2 cells in a dose-

**Table 2.** Identified peptides in fermented soybean hydrolysate (FSH [a]) fractionated below 3 kDa.

| Sequences | Charge | m/z (Da) | Position | NCBI Accession No and source protein |
|---|---|---|---|---|
| LQESVIVEISK | 2 | 622.8587 | 309-319 | gi\|9967357 Alpha subunit of beta-conglycinin, partial [Glycine max] |
| EEGQQQGEQR | 2 | 594.7692 | 299-308 | |
| GSEEEQDER | 2 | 539.7177 | 74-82 | gi\|9967361 Alpha' subunit of beta-conglycinin, partial [Glycine max] |
| LQESVIVEISK | 2 | 686.9056 | 325-335 | |
| LQESVIVEISKK | 2 | 686.9056 | 325-336 | |
| DNPNWTSDTR | 2 | 603.2662 | 788-796 | gi\|126406 Lipoxygenase-3 (Seed linoleate 9S-lipoxygenase-3) [Glycine max] |
| QLEALIETLSK | 2 | 622.8587 | 218-228 | gi\|571468558 Uncharacterized protein LOC102659859 [Glycine max] |
| WAMLGALGCVFPEL LARNGVKFGEASWFK | 4 | 800.1650 | 84-112 | gi\|115779 Chloroplastic, Chlorophyll a-b binding protein (LHCII type I CAB), precursor [Glycine max] |
| VNGNLVSPAHVLANAE VVEIITYNALSSKSAFQRHK | 3 | 1293.0221 | 601-646 | gi\|356570978 Uncharacterized protein LOC100814134 [Glycine max] |
| VFDGELQEGR | 2 | 575.2829 | 382-391 | gi\|15988119 Chain C, Crystal Structure Of Soybean Proglycinin A1ab1b Homotrimer [Glycine max] |

[a]Fermented soybean hydrolysate was treated with pepsin, trypsin, and α-chymotrypsin. Pepsin was used at a ratio of 1:100 (w/w) (enzyme:substrate), and pepsin hydrolysis was proceeded at pH 2.0 for 5 h. Trypsin and α-chymotrypsin were each used at a ratio of 1:200 (w/w) (enzyme:substrate). Trypsin and α-chymotrypsin were added into previous pepsin hydrolysate, and hydrolysis was proceeded at pH 7.5–8.0 for 5 h.

dependent manner [11]. Therefore, the fermented soybean samples manufactured in this study are expected to regulate blood pressure based on the enriched GABA content and the increased ACE inhibitory activity.

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