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Characterization of the Production of Biogenic Amines and Gamma-Aminobutyric Acid in the Soybean Pastes Fermented by *Aspergillus oryzae* and *Lactobacillus brevis* S

Nam Yeun Kim[1] and Geun Eog Ji[1,2]*

[1]*Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University, Seoul 151-742, Republic of Korea*
[2]*Research Institute, Bifido Co., Ltd., Hongchun 250-804, Republic of Korea*

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**Corresponding author*
Phone: +82-2-880-8749;
Fax: +82-2-884-0305;
E-mail: geji@snu.ac.kr

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The production of gamma-aminobutyric acid (GABA) using GABA-producing lactic acid bacteria (LAB) has been considered to be an attractive strategy. However, some LAB may produce biogenic amines (BA), which may be of concern from the safety viewpoint. The aim of the present study was to characterize the production of GABA and BA in the soybean pastes fermented by *Aspergillus oryzae* (*A. oryzae*) FMB S46471 and GABA-producing *Lactobacillus brevis* (*L. brevis*) GABA 100. After a ripening period of 90 days, the levels of BA (putrescine, cadaverine, histamine, and tyramine) and GABA in the fermented soybean were assessed by high-performance liquid chromatography. The soybean pastes fermented by *A. oryzae* and *L. brevis* showed a range of 7,130–11,592 mg/kg for GABA, 178–305 mg/kg for tyramine, 139–163 mg/kg for putrescine, 7.4–10.8 mg/kg for histamine, and 7.1–7.9 mg/kg for cadaverine, whereas the soybean pastes fermented by *A. oryzae* only showed a range of 30–1,671 mg/kg for GABA, 0.8–189 mg/kg for tyramine, 1.3–85 mg/kg for putrescine, up to 3.6 mg/kg for histamine, and 0.2–2.4 mg/kg for cadaverine. The results showed that the production of GABA was accompanied by the increase in the production of BA, even though the production levels of histamine and cadaverine were very low. This is the first study to simultaneously characterize the production of BA and GABA in GABA-enriched fermented soybean pastes, and warrants further study to minimize the production of BA while optimizing the production of GABA.

Keywords: Biogenic amines, gamma-aminobutyric acid, *Lactobacillus brevis*, fermentation, soybeans

Introduction

The production of gamma-aminobutyric acid (GABA) in fermented foods has been considered to be desirable. The administration of GABA regulates pain and anxiety, and reduces lipid levels in serum [15, 22]. Furthermore, the consumption of GABA-rich foods inhibits cancer cell proliferation [23] and improves memory and learning abilities [22]. The Korean Food and Drug Administration has approved a daily consumption for GABA of 20 mg in functional foods for the regulation of blood pressure [16]. GABA production using lactic acid bacteria (LAB) has been developed in a number of studies [3, 11, 19, 21, 24]. LAB are generally considered to be non-toxic and nonpathogenic.

L. brevis GABA 100 has shown the capability of GABA production in previous studies [11, 12] and is used to develop GABA-rich soybean paste [14].

Biogenic amines (BA) are organic, basic, nitrogenous compounds, mainly formed through decarboxylation of amino acids [8, 17]. BA are undesirable because they exert toxicological effects on human health, such as hypertension, headache, diarrhea, rash, and excess inflammation [8]. The responsible enzymes, amino acid decarboxylases, are widely present not only in the spoilage microorganisms but also in the various LAB strains [4]. In fact, some LAB showed the production of BA during fermentation [20, 25]. For these

reasons, major BA (putrescine, cadaverine, histamine, and tyramine) from the developed soybean pastes were determined in the present study.

In this study, GABA-rich soybean pastes (more than 1% GABA) containing a wide range of salt content were developed by using *Aspergillus oryzae* (*A. oryzae*) FMB S46471 and GABA-producing *L. brevis* GABA 100 as starter strains, and the contents of the major BA as well as GABA were determined.

Materials and Methods

Fungal Strains and Cultures

The characterization of *A. oryzae* FMB S46471 and *L. brevis* GABA 100 were previously reported and obtained from the Food Microbiology Laboratory at the Department and Food and Nutrition at Seoul National University, Korea [11, 13]. *A. oryzae* FMB S46471 was grown on potato dextrose agar (Becton, Dickinson and Company, MD, USA) at 30°C under aerobic conditions prior to the inoculation into the steamed soybeans. *L. brevis* GABA 100 was cultured in Lactobacilli MRS broth (Becton, Dickinson and Company) with 0.05% (w/v) L-cysteine-hydrochloride anhydrous (Sigma-Aldrich Co., LLC., USA) at 30°C under anaerobic conditions prior to the inoculation.

Preparation of Soybean Pastes

For the production of GABA in fermented soybean pastes, the following processes were carried out [14]. Briefly, soybeans (3.5 kg) steamed at 121°C for 15 min were prepared by Andong Nonghyup (Andong, Korea), by using a commercialized manufacturing process. Briefly, a spore suspension of *A. oryzae* FMB S46471 was inoculated (10^6 cells/g) in the steamed soybeans. The soybeans were incubated under aerobic conditions at 30°C for 12 days. Then, 10^8 cells (CFU/ml) of *L. brevis* GABA100 (5% (v/v)) were added to the soybeans and adjusted to pH 5.0 [12] with 1 M citric acid (Junsei Chemical Co., Ltd., Tokyo, Japan) and further fermented for the following 5 days at 30°C under anaerobic conditions. These 17-days fermented soybean pastes were salted (Local Market, Seoul, Korea) to produce G0, G1.5, G3, G6, G9, G12, G15, and G18 containing 0%, 1.5%, 3%, 6%, 9%, 12%, 15%, and 18% of salt, respectively. To produce G-18, soybeans fermented with *A. oryzae* FMB S46471 under aerobic conditions at 30°C for 12 days, followed by pH adjustment to pH 5.0, were further fermented without *L. brevis* GABA 100 for the following 5 days at 30°C under anaerobic conditions and then salted to contain 18% salt. To produce CA, soybeans inoculated with *A. oryzae* FMB S46471 were fermented for 17 days at 30°C under aerobic conditions and then salted to contain 18% salt. To produce CT, commercially available soybean pastes (*meju*) (local market, Seoul, Korea) made by traditional natural fermentation were purchased at a local supermarket and then salted to contain 18% salt. All of these soybean pastes described above were further ripened in a cold room (4–6°C) for 90 days. For another control, commercially available soybean pastes (*doenjang*) (CC) (local market, Seoul, Korea), which were being sold after completion of fermentation and ripening, were purchased at a local supermarket and analyzed.

Viable Cell Counts of LAB and *A. oryzae*

Viable cell numbers of LAB in the fermented soybean samples were determined using MRS agar plates containing 55 g/l MRS broth (Becton, Dickinson and Company) with 0.05% (w/v) L-cysteine-hydrochloride anhydrous (Sigma-Aldrich Co.), 20 g/l agar powder (Becton, Dickinson and Company), and 1.2 g/l bromocresol purple (Sigma-Aldrich Co.). The sample was diluted to $1/10^6$–$1/10^9$. The diluted sample (100 µl) was inoculated onto MRS agar plates and the plates were incubated at 30°C for 2 days under anaerobic conditions. Isolation of fungal species from fermented soybean samples was determined using DRBC (Dichloran Rose Bengal Chloramphenicol) (Sigma-Aldrich Co.) agar plates with 0.01% (w/v) Chloramphenicol Selective Supplement (Sigma-Aldrich Co.). The sample was diluted to $1/10^7$. The diluted sample (100 µl) was inoculated onto DRBC agar plates and the plates were incubated at 30°C for 5 days under aerobic conditions. Then, the plates including the *A. oryzae* strain were morphologically selected and counted by staining with lacto phenol blue (Sigma-Aldrich Co.). The mean values and the standard deviation were calculated by duplicate independent trials.

Analysis of GABA

Samples were ground by using a cell strainer (BD Biosciences, NC, USA) and filtered through a 0.2 µm syringe filter (PALL Life Sciences, MI, USA). The quantitative determination of GABA was performed at the National Instrumentation Center for Environmental Management (NICEM), Seoul National University (Seoul, Korea). The samples were diluted with distilled water and filtered through a 0.2 µm syringe filter (PALL Life Sciences). Primary and secondary amino acids were automatically derivatized into fluorescent substances within the autosampler using *o*-phthalaldehyde (Agilent Technologies, USA) and 9-fluorenyl methyl chloroformate (Agilent Technologies), respectively. Then, the separation of the different amino acid derivatives was performed using an INNO C-18 column (150 mm × 4.6 mm, 5 µm; Youngjin Biochrom Co., Ltd., Korea). Standard amino acids were purchased from Agilent Technologies. The mobile phase was a mixture of 10 mM Na_2HPO_4 (Sigma-Aldrich Co.) and 10 mM $Na_2B_4O_7$ (Sigma-Aldrich Co.) and water-acetonitrile-methanol (10:45:45 (v/v/v)) (Honeywell Burdick & Jackson Inc., NJ, USA), which was pumped at a constant flow rate of 1.5 ml/min. The quantitative determination of GABA was performed using a fluorescence detector (excitation: 340 nm; emission: 450 nm). The analysis was performed using the Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc., USA). The mean values and the standard deviation were calculated by duplicate independent trials.

Analysis of BA

The extraction procedure was carried out as described in a

previous report with some modifications [7]. Briefly, a 5 g sample was weighed and vortexed with 25 ml of 0.1N HCl (Sigma-Aldrich Co.) for 5 min. After the resulting homogenate was centrifuged at 4,000 ×*g* for 15 min (4°C) (2236R high-speed centrifuge; Labogene Aps, Denmark), the aqueous layer was collected and the residue was re-extracted as described above. Combined extracts were filtered through Whatman No. 4 filter paper (Whatman Int'l., Ltd., UK). Then, 1 ml of the extract or standard amine solution was spiked with 0.1 ml of internal standard (1,7-diaminoheptane, 100 ug/ml) and mixed in a glass tube with 0.5 ml of saturated sodium carbonate (Sigma-Aldrich Co.) and 0.8 ml of 1% dansyl chloride (Sigma-Aldrich Co.) in acetone (Sigma-Aldrich Co.). After thoroughly mixing, the test tube was incubated in dark water bath (WBC 1510A; Jeio Tech. Co., Ltd., Korea) at 45°C for 60 min. Subsequently, 0.5 ml of 10% proline (Sigma-Aldrich Co.) and 5 ml ether (Sigma-Aldrich Co.) were added to each sample for removing residual dansyl chloride. The supernatant was suspended and evaporated (Scanvac Speed Vacuum Concentrator; Labogene Aps) at 20°C until dry. The dry residue was diluted with 1 ml of acetonitrile (Sigma Chemical Co.). The reconstituted sample and standard were filtered through a 0.2 μm syringe filter for HPLC analysis. The HPLC analysis for tyramine, histamine, putrescine, and cadaverine was performed at NICEM, Seoul National University. HPLC determinations were performed using an INNO C-18 column (150 mm × 4.6 mm, 5 μm; Youngjin Biochrom Co.). The gradient elution system consisting of water (solvent A) and acetonitrile (solvent B) was started at 60% B, increased *via* linear gradient to 100% B at 20 min and held for 5 min, and then returned to the initial composition within 5 min. The total run time of analysis was 30 min with a flow rate of 0.8 ml/min. The column temperature was kept at 30°C. A volume of 10 μl was injected into the HPLC system. Components were tentatively identified by comparison of their retention time with those of authentic standards under identical analysis conditions at 250 nm. The analysis was performed using the Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc.). The mean values and the standard deviation were calculated by duplicate independent trials.

Results

Viable Cell Counts of LAB and Fungus from the Fermented Soybean

The soybean samples fermented with *L. brevis* GABA 100 showed a range of 5.7–6.9 log CFU/g for viable cell numbers of LAB (Table 1). The fungal morphology of *A. oryzae* was shown in the plates from G0, G1.5, G3, G6, G9, G12, G15, G18, G-18, and CA, which were inoculated with *A. oryzae* FMB S46471 (Table 1).

Determination of GABA in the Fermented Soybean

The content of GABA in the 10 types of soybean pastes that were fermented and ripened as described in the Materials and Methods is shown in Table 2. The GABA contents of G0, G1.5, G3, G6, G9, G12, G15, and G18 ranged 7.1–11.6 g/kg , whereas that of G-18 was about 1.7 g/kg (Table 2). The production of GABA decreased as the salt content of the soybean pastes was increased (Table 2, Fig. S1). CA, CT, and CC showed low contents of GABA (0.03, 0.47, and 0.17 g/kg, respectively) (Table 2, Fig. S1).

Determination of BA in the Fermented Soybean

The BA contents in the soybean pastes are shown in Table 2. The BA levels in GABA-rich soybean pastes (G0, G1.5, G3, G6, G9, G12, G15, and G18) showed a range of 178.2–304.7 mg/kg for tyramine, 139.6–163.2 mg/kg for putrescine, 7.1–7.9 mg/kg for cadaverine, and 7.4–10.8 mg/kg for histamine. The contents of BA decreased as the salt contents of the soybean pastes were increased (Table 2, Fig. S2). The contents of tyramine, putrescine, and cadaverine in G-18 were 188.9, 84.8, and 2.4 mg/kg, respectively (Table 2, Fig. S2). The highest production of BA was of tyramine, followed by putrescine, and the lowest were cadaverine and histamine. CA, CT, and CC showed low levels of BA (Table 2, Fig. S2).

Discussion

Soybean is rich in amino acids. Glutamic acid is produced by the hydrolytic action of *A. oryzae* during the fermentation of soybean paste, which can be used as a substrate for the production of GABA by GABA-producing LAB. The GABA production in the fermented soybean paste before maturation was 6.5 g/kg (data not shown). The GABA contents in soybean paste matured for 90 days were increased about 2-folds, which was 11.3 g/kg at 0% salinity.

Table 1. Viable cell counts of LAB and *A. oryzae* in the experimental fermented soybean pastes.

	Sample											
Index	G0	G1.5	G3	G6	G9	G12	G15	G18	G-18	CA	CT	CC
LAB (log CFU/g)	6.9 ± 0.1	6.6 ± 0.2	6.4 ± 0.2	6.3 ± 0.5	6.2 ± 0.2	5.9 ± 0.1	6.0 ± 0.2	5.7 ± 0.4	5.4 ± 0.1	4.5 ± 0.5	5.7 ± 0.4	N.D.
A. oryzae (10^4 cells/g)	3.7 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.7 ± 0.2	3.8 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	4.0 ± 0.0	3.9 ± 0.1	4.2 ± 0.6	-	-

LAB, lactic acid bacteria; N.D., not detected at 2 log CFU/g; *A. oryzae*, *Aspergillus oryzae*; -, *A. oryzae*'s morphology not detected using DRBC (Dichloran Rose Bengal Chloramphenicol) agar plate.

Table 2. Determination of BA and GABA in the experimental fermented soybean pastes. (mg/kg, wet weight)

		Sample											
		G0	G1.5	G3	G6	G9	G12	G15	G18	G-18	CA	CT	CC
BA	Tyramine	287.1 ± 1.1	304.7 ± 0.8	258.2 ± 2.9	242.8 ± 0.9	217.3 ± 0.5	205.0 ± 1.3	201.6 ± 0.8	178.2 ± 0.9	188.9 ± 0.1	0.8 ± 0.1	32.1 ± 1.6	3.4 ± 0.4
	Histamine	7.4 ± 0.1	8.3 ± 0.6	8.8 ± 0.2	9.1 ± 0.3	10.2 ± 0.5	9.1 ± 0.3	9.7 ± 0.7	10.8 ± 0.0	N.D.	N.D.	N.D.	3.6 ± 0.3
	Putrescine	163.2 ± 0.7	159.3 ± 0.8	151.6 ± 0.9	145.2 ± 0.9	149.2 ± 0.8	147.3 ± 0.5	144.3 ± 0.6	139.6 ± 0.8	84.8 ± 0.7	1.3 ± 0.1	2.7 ± 0.3	2.6 ± 0.2
	Cadaverine	7.7 ± 0.1	7.9 ± 0.1	7.1 ± 0.4	7.6 ± 0.2	7.4 ± 0.4	7.5 ± 0.4	7.4 ± 0.5	7.5 ± 0.1	2.4 ± 0.1	0.2 ± 0.1	0.6 ± 0.2	1.1 ± 0.1
AA	GABA	11,315 ± 275	10,914 ± 135	10,622 ± 435	11,592 ± 262	9,418 ± 96	8,656 ± 135	7,815 ± 149	7,130 ± 155	1,671 ± 72	30 ± 6	473 ± 22	165 ± 15

BA, biogenic amines; AA, amino acids; GABA, gamma-aminobutyric acid; N.D., not detected.

The production of BA as well as GABA in the foods requires the availability of the precursor amino acids [25]. Specifically, fermented soybeans contain considerable amounts of various amino acids. The production of BA and GABA is mainly carried out by the microbial decarboxylases under the favorable conditions for their growth and decarboxylation activity [1, 5]. Hierro *et al*. [9] suggested that the environmental factors affecting the activity of decarboxylase are very important as well as the precursor availability. Considering that the soybean paste fermented by only *A. oryzae* FMB S46471 showed a considerably low amount of GABA and BA, the metabolic activity of *L. brevis* GABA 100 was mainly responsible for the production of GABA as well as the assessed BA, although the soybean paste that was not inoculated with *L. brevis* (G-18) showed the production of BA to some degree (Table 2).

It turned out that salt is an environmental factor to reduce the level of both GABA and BA. The partial inhibition of the growth of LAB by salt, as shown in Table 1, may explain this phenomenon. Meanwhile, as shown in the viable cell counts of LAB in G-18, CA, and CT, there was a considerable number of LAB compared with soybean samples inoculated with *L. brevis* GABA 100. To clarify this phenomenon, sequencing analysis of the target 16S rRNA gene was performed, showing *L. brevis* strain was a major bacterial population in the fermented soybeans with *L. brevis* GABA 100 (data not shown), whereas G-18, CA, and CT included mostly *L. paracasei*, *L. plantarum*, or *Pediococcus pentosaceus*. Those strains might be inoculated from the natural environment. In addition, *A. oryzae* was a major fungus population in the soybean pastes fermented with *A. oryzae* FMB S46471 after maturation for 90 days (Table 1). Soybean pastes with various salt contents were prepared in the present study to assess the level of salts at which the starter lactic acid bacteria inhibit the growth of spoilage bacteria. The results showed that the fermented soybean pastes successfully produced a range of 7,130–11,592 mg/kg for GABA, whereas the growth of *E. coli*/coliform was successfully inhibited at all of the salt ranges used in the present study (Table S1).

Upper limits of biogenic amines for human consumption have been suggested by several investigators as follows: histamine, 50–100 mg/kg; tyramine, 100–800 mg/kg; total biogenic amines, 1,000 mg/kg in foods [2, 4, 26]. Among the BA, histamine has been implicated as the causative agent in outbreaks of food poisoning where intoxication results from the ingestion of foods containing excessive amounts of histamine [26]. According to the surveillance report of the Korea Food and Drug Administration, the contents of BA in commercially available soybean pastes were detected in the average of 346.2 ± 274.8 mg/kg for histamine, 392.3 ± 288 mg/kg for tyramine, and 426.0 ± 428.6 mg/kg for putrescine [10]. Lee *et al*. [18] reported that commercially available soybean pastes contained 22.8 mg/kg cadaverine and 397 mg/kg putrescine on average [18]. The average contents of BA in those commercial soybean pastes were under the threshold limit of human consumption as defined by Food and Agriculture Organization/World Health Organization and European Food Safety Authority [4, 6]. Interestingly, as compared with commercially available soybean pastes, GABA-rich soybean pastes developed in the present study showed a very low level of histamine content (up to 10.8 mg/kg).

There are different types of fermented soybean pastes depending on manufacturing methods. The soybean paste fermented by serial fermentation using *A. oryzae* and

L. brevis has been newly developed in this study. Accordingly, the newly developed soybean paste was compared with other soybean pastes as references. CA designates a soybean paste fermented with only *A. oryzae* FMB S46471. CT is a naturally fermented soybean paste made by *meju* according to the traditional method, and CC is a fermented soybean paste purchased from a local market and was fermented by an *A. oryzae* starter culture.

This is the first study to evaluate the production of BA as well as GABA in the fermented food with enhanced functional benefits of GABA. This study will provide useful information on the development of GABA-enriched soybean pastes.

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