

Production of GABA (gamma amino butyric acid) by Lactic Acid Bacteria

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Abstract

Gamma-amino butyric acid (GABA) is a kind of pharmacological and biological component and its application is wide and useful in Korea specially, becoming aging society in the near future. GABA is request special dose for the purposed biological effect but the production of concentrated GABA is very difficult due to low concentration of glutamic acid existed in the fermentation broth. To increase GABA concentrate using fermentation technology, high content of glutamic acid is required. For this reason, various strains which have the glutamic acid decarboxylase (GAD) and can convert glutamic acid to GABA, were isolated from various fermented foods. Most of GABA producing strains are lactic acid bacteria isolated from kimchi, especially added monosodium glutamate (MSG) as a taste enhancer. Optimizing the formulation of culture media and the culture condition, GABA conversion yield and amounts were increased. Finally GABA concentration of fermentation broth in batch or fed batch fermentation reached 660 mM or 1000 mM, respectively. Furthermore formulation of culture media for GABA production developed commercially. Many studies about GABA-rich product have been continued, so GABA-rich kimchi, cheese, yogurt, black raspberry juice and tomato juices has been also developed. In Korea many biological effects of GABA are evaluated recently and GABA will be expected to be used in multipurpose.

Key words: lactic acid bacteria, fermentation, GABA, kimchi, high concentration

Introduction

Meat consumption per capita is increasing depending on the increase of the national income. According to the Korea Rural Economic Institute, annual meat consumption per capita in 2011 was 40.4 kg (average consumption per capita was 110.6 g/day) and it has increased to 4.2% (1.6 kg) compared with that of 2010, 38.7 kg. Compared with 32 kg consumption in 2005, consumption amount is increased about 26.2% (KREI, Agricultural and livestock industry products statistics, 2011). The increase of meat consumption is changing to factory-style and corporate-style in domestic livestock industry. The factory-style livestock industry means adapting livestock's ecology to the industrial scale. So, it affects the increase of scale of livestock husbandry and density. In early 2000s, the numbers of livestock increased to three times for the chicken and five times for the pig compared with the early 1980s. Consequentially, poultry farmers raising more than 30,000

chickens increased to 48.9% in 2007, whereas 0.2% in 1990, and ones raising less than 5,000 chickens decreased to 3.4% from 97.2%. This overcrowding and unnatural breeding environment gives livestock causing a lot of stress and creates a vicious cycle of weakening immune system and antibiotic overdose (Moon, 1998; Song *et al.*, 1998).

On the other hand, the European Union banned the antimicrobial growth-promoter for formula feed since 2006 responding to the worldwide needs of safety and high-quality livestock products. Korea also banned the use of antimicrobial growth-promoter from July 2012 (Ministry for Food, Agriculture, Forestry and Fisheries. Announcement No. 2010-3; The Agriculture, Fisheries and Livestock's News. 2011). The development of various natural feed additives that can be replaced by it is completed or in progress (Kim, 2010; Lim *et al.*, 2011).

In accordance with increasing domestic consumers' tendency to prefer eco-friendly livestock products, consumers' interest on food safety is also increasing and the development of feed additives to promote livestock's function as a way of producing eco-friendly livestock products (Lee *et al.*, 2008; Lee, 2009). The researches and developments for functional feed additives are indispensable for both consumers and producers for the future of the livestock

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industry.

The stress arising from the overcrowding of the factory-style livestock industry as well as the sanitary problems causes infectious diseases or diarrhea by reducing livestock's immunity and finally reduces productivity and coefficient of reproduction. It is possible to rely on the facilities in order to minimize various stresses that occur when livestock breeding, but it has the cost problem. Therefore, developing a substance being able to reduce stress and using it for livestock breeding can respond effectively to the factory-style livestock industry. Besides, various environmental and nutritional stresses arising from the modern livestock industry necessarily lead to the reduced livestock growth and feed availability and result in economic losses of farmers, so the study of anti-stress functional feed additives is essential (Jung *et al.*, 2010; Magoto *et al.*, 2004).

γ -Aminobutyric acid (GABA) is the non-protein amino acid that is widely distributed in nature, and generated irreversibly through the α -decarboxylation of L-glutamic acid in a reaction catalyzed by glutamate decarboxylase (GAD). GAD and GABA have been found from bacteria to higher organisms widely. GABA is well known as inhibitory neurotransmitter. The concentration of glutamate as excitable neurotransmitter and GABA is controlled by GAD (Ueno, 2000).

GABA promotes the metabolism of brain cells by increasing the oxygen supply and activating cerebral blood flow and inhibits the secretion of the vasopressin (antidiuretic hormone) by acting on the vasomotor center of medulla oblongata. Furthermore, GABA regulates growth hormone secretion, drops the blood pressure by expanding the blood vessels, and has the diuretic, anti-depressive, anti-oxidant effects, effective pain relief, as well as a medicine for the stroke treatment, especially (Hao and Schmit, 1993; Kono and Himeno, 2000; Leventhal *et al.*, 2003; Shelp *et al.*, 1999). In recent years, its sleep-inducing effect was proved by the animal experiment which establishes the production of serotonin and melatonin which act as sleep inducer, is facilitated significantly in the GABA-containing milk feeding group (Kim *et al.*, 2010). The interest of GABA as a functional food ingredients has been heightened due to the recent proof of various biological functions of GABA and it is reported that GABA-reinforced soy, tea, red yeast rice and chlorella drop the blood pressure in the experiment in Spontaneously Hypertensive Rats (Abe *et al.*, 1995; Bae, 2008; Hayakawa *et al.*, 2004; Nakamura *et al.*, 2000; Tsuiji *et al.*, 1992).

GABA is available in many fruits and vegetables but the concentration is low in nature of which range is from 0.03 to 2.00 $\mu\text{mol/g}$ -wet weights (Fougère *et al.*, 1991; Rhodes *et al.*, 1986), which is known to be difficult to have biological activity. However, it is reported tea contains sufficient amount of GABA to hypertensive patient, showing the effect in lowering blood pressure without any side effects. It means various functional foods can be developed in case of available concentration of GABA added.

Various researches to increase GABA contents are in progress, and the method of increasing the amount of GABA in green tea under anaerobic conditions is one of the examples (Chang *et al.*, 1992; Sawai *et al.*, 2001; Tsuchida and Murai, 1987; Wang *et al.*, 2006). GABA production is stimulated through the action of decarboxylase in embryos by water immersing (Saikusa *et al.*, 1994), increasing GABA content using high-pressure and germination of brown rice (Miwako *et al.*, 1999), producing GABA containing yogurt (Park and Oh, 2007). Most of these methods produce GABA from glutamic acid by promoting the reaction of decarboxylase existing in the natural products. Therefore, the amount of GABA is proportional to the amount of glutamic acid existing in the various materials and addition of glutamic acid should be necessary in order to obtain a high concentration of GABA.

The study for the production of high concentrations of GABA using microorganisms is progressing in order to overcome these limitations. Recently, it is reported that lactic acid bacteria isolated from variety of fermented foods such as kimchi and salted seafood produces GABA using glutamic acid as a substrate. PharmaFood company (Japan) produced GABA by conversion of monosodium glutamate using lactic acid bacteria isolated from kimchi and sells as a functional food ingredient and it has reported to produce 6.3 mM GABA by inoculating *Lactobacillus brevis* IFO 12005 isolated from kimchi to Soju jigemi made from rice (Ueno *et al.*, 1997; Yokoyama *et al.*, 2002), 302 mM of GABA by adding pyridoxal phosphated as a coenzyme of GAD by *Lb. paracasei* NFRI 7415 isolated from Japanese traditional fermented food funasushi (Komatsuzaki *et al.*, 2005). Recently, Yang *et al.* (2008) has reported to produce 78 mM of GABA using *Streptococcus salivarius* in China, and domestic researchers are developing the isolation of strains producing GABA and have reported to produce 19 mM, 251 mM of GABA respectively by isolation of GABA-producing strains such as *Lb. brevis* OPK 3, *Lb. buchneri* from traditional fermented foods such as kimchi (Cho *et al.*, 2007; Park and Oh, 2006).

The research for the commercial production of GABA is also developing and 660 mM of GABA produced using the 5 ton fermenter with *Lb. sakei* B2-16, isolated from Kimchi (Kook *et al.*, 2010), and fed-batch process by immobilized *Lb. brevis* GABA 057 strains using alginate bead has changed 12% of MSG into GABA for 48 h (Choi *et al.*, 2006). It succeeded to produce 1005.8 mM of GABA from 3.1 M MSG by *Lb. brevis* NCL912 isolated from Paocai in China (Li *et al.*, 2010).

Intake of effective concentration of GABA, which is not available with the naturally enhanced GABA containing food, is available due to the increase the GABA productivity by the isolation of various strains and the study of fermentation. As a result, GABA began applying for components of the foods and cosmetics and recently functional feed additives depending on the cost of production was lowered, so it is necessary to arrange the research associated with the production of GABA by the fermentation at this point.

Subject

Isolation of strains

In Korea, most of GABA is produced by lactic acid bacteria isolated from kimchi. The traditional fermented foods such as salted seafood and sauces have been used occasionally, but it now appears that most strains, which have high conversion yield of GABA or have been studied on the production by continuous fermentation and efficacy evaluation, are lactic acid bacteria from kimchi.

GABA-producing strain isolated from aged natural cheese is used to increase GABA content in milk fermented products in Japan (Nomura *et al.*, 1998) and various strains with GABA-producing capacity isolated from dairy foods are used as starters for milk fermentation in Korea (Park and Oh, 2006), but it has been reported that their conversion yield is lower than conversion yield of lactic acid bacteria isolated from kimchi for the production of GABA. In recent years, the high-GABA-productive yeast was isolated from the vicinity of the ocean (the Pacific Ocean) by Japanese researchers (Masuda *et al.*, 2008). It has the advantage of increasing the GABA content of fermented bean foods by applying GABA-producing strain to yeast fermentation with beans as the main ingredient, but yet the conversion rate of glutamic acid is lower than that of lactic acid bacteria that are used in domestic for industry.

On the other hand, most of strains applied in various studies on the production of GABA are lactic acid bacte-

ria isolated from kimchi, and have the advantage of verification for safety. Because there may be no problem to use for foods as raw materials by strains isolated from fermented foods which have been eaten traditionally.

In the case of health supplements or foods containing GABA by lactic acid bacteria fermentation, the additional verification procedure of safety is needed, but the safety of general foods can be recognized in case of GABA-containing fermentation broth by the GABA producing lactic acid bacteria.

Various kinds of kimchi were used as the sample for isolation of lactic acid bacteria, and most researchers have used the commercial brand kimchi, traditional market kimchi, restaurants kimchi and homemade kimchi for the sample, because kimchi is made of a various ingredient depending on the purpose or region. And in order to isolate the superior lactic acid bacteria which convert glutamic acid to GABA, kimchi with various amount of MSG are compared, and then, one with good producer of GABA is chosen. For the first time, it is known that lactic acid bacteria used in PharmaFood company in Japan for the industrial production of GABA was isolated from kimchi and GABA-containing mung bean ferment (product name: Phyto-GABA) which is exporting to China for cosmetic raw material is known as the product using lactic acid bacteria isolated from kimchi of a domestic venture company (Park *et al.*, 2005).

Isolation of GABA producer

First, lactic acid bacteria were isolated from kimchi, after then, strain having the producing ability of GABA was selected, and vice versa. The isolation of lactic acid bacteria from kimchi samples used the plate count medium with BCP (bromocresol purple) as a selective media, that is, adjusted to pH 6.8 ± 0.2 after dissolving peptone 5.0 g, yeast extract 2.5 g, dextrose 1.0 g, tween 80 1.0 g, L-cystein 0.1 g, bromocresol purple 0.05 g, agar 15.0 g in 1,000 mL of distilled water according to the Food Standards Codex (Korean Food Standards Codex, 2011).

Samples for isolation of lactic acid bacteria are spread onto the plate count medium with BCP, typically prepared kimchi from various regions is grinded with soup using a sterile homogenizer and the suitable number of single colony is obtained after dilution at a rate of about $1/10^6 \sim 1/10^8$. Generally, sterile saline solution or BPW (Buffered peptone water) is used for dilution in accordance with the Food Standards Codex. After cultivation for 72 h at 35 to 37°C, lactic acid bacteria colonies with yellowish color are selected.

Table 1. Comparison of culture conditions and GABA productivity

Source of isolation/ manufacturer	Microorganism	Major component of medium	Fermentation conditions	Final concentration of GABA	Reference
Bifido Co., Ltd.	<i>Lb. brevis</i> GABA 057	1% glucose, 1% yeast extract 0.5% poly peptone, 0.2% sodium acetate 12% MSG	pH 4.2, 34°C, 48 h, Cell immobilization	66.1 g/L	Choi <i>et al.</i> , 2006
Cheese	<i>Lb. buchneri</i> OPM-1 <i>Lactobacillus</i> sp. OPM-2	MRS broth, 1% MSG	30°C, 48 h	4.4 g/L 5.6 g/L	Park and Oh., 2006
kimchi	<i>Lb. brevis</i> AML15	MRS broth, 5% MSG	pH 5.0, 37°C, 48 h	10.7 g/L	Shin <i>et al.</i> , 2007
kimchi	<i>Lb. brevis</i> BFC-110	2% maltose, 3% yeast extract. 5% MSG	30°C, 100 rpm, 72 h	26.7 g/L	Kim, 2009
kimchi	<i>Lb. buchneri</i> MS	MRS broth, 5% MSG	pH 5.0 (initial), 30°C, 36 h	25.8 g/L	Cho <i>et al.</i> , 2007
kimchi	<i>Lb. sakei</i> B2-16	10% Rice Germ extract 4% sucrose, 1% yeast extract 7% MSG	30°C, 48 h	39.7 g/L	Kook <i>et al.</i> , 2009
kimchi	<i>Lb. sakei</i> B2-16	10% Rice Bran extract 4% sucrose, 1% yeast extract 12% MSG	pH 6.0 (initial), 30°C, 50 rpm, 48 h	68.1 g/L	Kook <i>et al.</i> , 2010
kimchi	<i>Lactobacillus</i> sp. OPK2-59	MRS broth, 1% MSG	30°C, 48 h	1.58 g/L	Bae <i>et al.</i> , 2009
Pacific ocean	<i>Hachinohe</i> No.6 (yeast)	2% glucose, 2% peptone 1% yeast extract, 3% NaCl 1% MSG	25°C, 120 rpm, 65 h	1.2 g/L	Masuda <i>et al.</i> , 2008
Paocai	<i>Lb. brevis</i> NCL912	5% glucose, 3% Soya peptone 9.3 % MSG 50 g/L glucose, Soya peptone 30.25 g/L 7.5 % MSG : 0 h 28 % MSG : 12 h 22 % MSG : 24 h	pH 5.0, 30-35°C Batch fermentation pH 5.0, 32°C, 36 h Fed batch fermentation	35.6 g/L 103.5 g/L	Li <i>et al.</i> , 2010
Sea water	<i>Pseudomonas</i> sp. Strain G-17	Sea water mineral Putrescine, Alanine, Glucose-nitrate 0.187% MSG	pH 7.3-7.6, 25°C, 60 rpm, 24 h	0.134 g/L	Mountfort and Pybus, 1992
Soy sauce	<i>Lactobacillus</i> sp. Strain E1	10% soy sauce, 0.3% yeast extract 1% glucose, 3.3% NaCl (to give a final conc. 5%) 50 mM MSG	pH5, 30°C, 72 h	1.03 g/L	Higuchi <i>et al.</i> , 1997

MRS medium with MSG as a source of glutamic acid is used in order to select the strains with GABA-producing capability among the isolates. GABA-producing capability of strains can be determined by analyzing the GABA content in culture broth after 3-4 d of culture with 1% level of MSG in the medium. Generally, in case of matured kimchi with MSG as a sub material, the GABA-producing strains were found and the most of them are lactic acid bacteria. There is no basis which one is more efficient, selecting lactic acid bacteria or GABA-producing bacteria but it can be a time-saving way to search preferentially strains of superior GABA-producing capability in appropriately fermented kimchi suitable for eating.

Depends on the fact that GABA-producing strain having the resistance to high concentrations of MSG primarily, isolating GABA-producing bacteria which have tolerance to high concentration of GABA. Generally, the MSG resistant strains are isolated by adding 5% (w/v) of MSG in MRS medium and it is reported that the resistant strains of high concentrations of MSG are isolated by increasing to 10% of MSG content (Kim, 2009).

Qualitative and quantitative analysis of GABA

TLC (Thin Layer Chromatography) and RP-HPLC (Reverse Phase High Performance Chromatography) are used in GABA qualitative and quantitative analysis, respectively.

The qualitative analysis using TLC is assumed in order to confirm GABA-producing capability of a large number of bacteria in a short period of time. Typical developing solvent is n-Butanol-Acetic acid-H₂O [5:3:2 or 4:1:1 (v/v/v)], and it is modified to match the experimenters (Holdiness, 1983). Developed TLC plate is dried after applying the ninhydrin solution (0.2%, w/v-ethanol) as a color reagent. The spot of GABA and MSG can be easily confirmed by GABA standard solution and MSG solution because GABA and MSG are detected with the red spot when it is dried at around 60°C after spraying the ninhydrin solution (Fig. 1). The amount of loading sample varies by researchers and can be determined experimentally. A clear GABA spot can be obtained with 1 µL of the sample removed solids fraction from the fermentation broth containing GABA by centrifugation.

Quantitative analysis of GABA uses RP-HPLC and many different protocols have been established as well as various TLC developing solvent by the researchers. First, Kook *et al.* (2009) modified the analysis protocols reported by Ibolya and Vasanits (1999), Tcherkas *et al.* (2001) for the quantitative analysis of GABA in the culture broth. After centrifugation of the sample for 10 min at 8,000

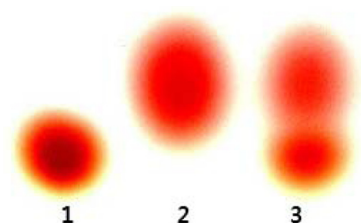


Fig. 1. TLC profile of monosodium glutamate (MSG) and gamma aminobutyric acid (GABA) during *Lactobacillus brevis* B3-18 fermentation. 1. MSG standard; 2. GABA standard; 3. Cultural supernatant of *Lactobacillus brevis* B3-18.

rpm, the supernatant were filtered with 0.45 µm cellulose acetate membrane filter (Advantec, MFS. Inc., Japan) and diluted to the appropriate concentration. The analysis is carried out using RP-HPLC after derivatization process using *o*-phthalaldehyde (OPA). The OPA solution (pH 9.3) is made by mixing 1.0 mL of a methanolic OPA, 250 µL of borate buffer (pH 9.9) and 25 µL of 2-mercaptoethanol. Methanolic OPA is made by dissolving 2.56 g of OPA in 50 µL of methanol. For borate buffer, 0.2 M potassium was put in after mixing 0.2 M boric acid and 0.2 M sodium hydroxide by 50:50 (v/v). 20 µL of derivatized sample was injected into the column. RP-HPLC system is composed of Waters 2487 Dual λ Absorbance detector, Waters 1525 Binary HPLC pump, Waters 717 plus Autosampler, U.S.A and XTerra column (Waters, RP18 5 m × 4.6 mm × 150 mm, U.S.A) for HPLC column. 0.05 M sodium acetate (pH 7.2) is mobile phase A, and the mixture (pH 7.2) of 0.1 M sodium acetate, acetonitrile (HPLC grade) and methanol (HPLC grade) by 46:44:10 (v/v) is mobile phase B. Mobile phase were controlled by gradient conditions. The flow rate was fixed at 1 mL/min; GABA was detected by 358 nm of UV detector (Fig. 2).

Kim (2009) modified the method of Chen *et al.* (1997) to the analysis of GABA. After centrifugation of the culture broth for 15 min at 3,000 rpm, the supernatant were filtered with 0.45 µm CA membrane filter, the filtered sample were diluted to 0.2 M sodium bicarbonate solution (pH 9.8) by 1:9, after then, the derivatization reagent, Dansyl chloride, mixed to the sample till 8.0 g/L. It is analyzed, after incubation for 1 h at 30°C with blocking light. The mobile phase A is the mixed solvent with tetrahydrofuran-methanol-50 mM pH 6.2 sodium acetate (5:75:420, v:v:v) and the mobile phase B is methanol, and the gradient condition is A:B = 100:0 (0 min), 60:40 (10 min), 0:100 (10 min). The flow rate was fixed at 1 mL/min, the column is OptimaPak C18 (4.6×250 cm) and its temperature is 40°C. GABA was detected by 280 nm of

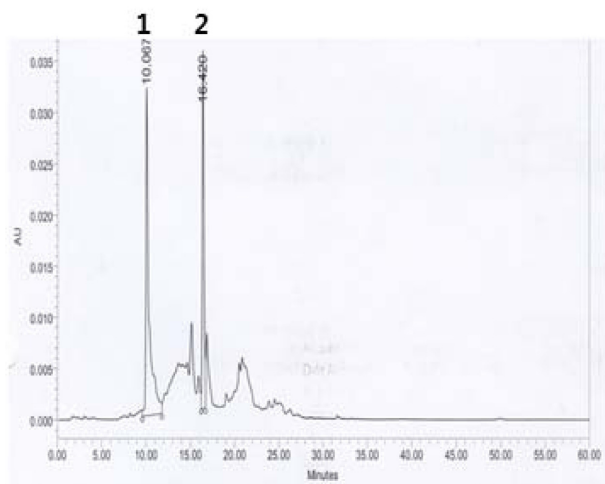


Fig. 2. RP-HPLC profile of monosodium glutamate (MSG) and gamma aminobutyric acid (GABA) during *Lactobacillus brevis* B3-18 fermentation. Analysis conditions: mobile phase A. 0.05 M Sodium Acetate; mobile phase B. 0.1M Sodium Acetate: Acetonitrile:Methanol (46:44:10, v/v/v); Column. Xterra (C18, Waters); 1. MSG; 2. GABA

UV detector.

And, Oh and Oh (2003) modified the method of Baum *et al.* (1996). After adding 200 μ L of sample to 800 μ L of methanol-chloroform-water (12:5:3), GABA-containing supernatant is obtained by centrifugation for 15 min at 4°C, 13,000 g. The centrifugation is conducted again to remove impurities from the supernatant and then, it is freeze-dried and used after dissolving in water before the analysis. The sample dissolved in water is filtered by 0.45 μ m membrane and derivatized to 6-aminoquiolyl-N-hydroxysuccinimidyl carbonate (AQC) (Cohen and Michaud, 1993), followed analysis by amino acid analysis system (2690, Waters, USA).

There is the way to quantify the content of GABA by measuring the activity of the GABAse, not using RP-HPLC but spectrophotometer (Sethi, 1993), however, it is not common in Korea. As described above, researchers apply quantitative analysis methods using RP-HPLC by some variants. Now, GABA is not listed in food ingredients or food additives and the name is not free to use, therefore there is no standardized assay. From now on, food ingredients or food additives listing and standard analysis settings will be the important task to researchers.

Production of GABA by fermentation

Medium composition

It is basic technique to culture GABA-producing bacte-

ria in the optimal medium where lactic acid bacteria can proliferate actively in order to produce GABA. Generally, GABA-producing lactic acid bacteria were cultured in MRS medium at the first stage when the study on production of GABA was started, after that time, the study to examine the composition of the medium to increase the productivity of GABA was actively conducted (Choi *et al.*, 2006; Kook *et al.*, 2009; Kook *et al.*, 2010; Oh, 2006; Seok *et al.*, 2008).

Also, the optimization of medium composition for increasing the productivity of GABA and experiments to configure low-cost medium for the industrial production of GABA were carried out together (Kim, 2009; Kook *et al.*, 2009; Kook *et al.*, 2010). Generally, the optimal medium composition can vary depending on the strain of GABA producing lactic acid bacteria. Most of the strains isolated for the production of GABA in Korea were *Lb. brevis* isolated from kimchi (Kang, 2002) and *Lb. sakei*, so the research on two species of lactic acid bacteria for the production of GABA has been conducted in Korea.

Therefore, the composition of the medium for the growth of GABA producing lactic acid bacteria was studied initially, and carbon source, nitrogen source and trace elements for the growth of *Lb. brevis* and *Lb. sakei* are selected through experiments in general. As a Carbon sources, dextrose, sucrose, maltose, fructose, lactose, xylose, galactose and arabinose are considered, and yeast extract, soytone, peptone, beef extract and tryptone are as nitrogen sources, which are examined as the medium for the growth of strains and the production of GABA. Magnesium sulfate, sodium acetate, manganese sulfate, ferric sulfate, calcium chloride and poly-solvate are trace elements and they are added to the medium as the mineral. The composition of medium for GABA production would not differ significantly from the medium for proliferation of lactic acid bacteria. Glucose, maltose and sucrose as carbon source were selected, and yeast extract as nitrogen source showed to be suitable and the impact of a small amount of added metal ions was weak. The composition of medium was determined at the level of less than 4% of carbon source and nitrogen source respectively and at the 0.01% level of metal ions. The medium composition can vary depending on the final product whether a high concentrations of GABA or a particular product containing GABA, and generally the medium composed of carbon source, nitrogen source and minerals adds optimal concentration of glutamic acid to match the strain's ability to produce GABA and finally is used to obtain the produced GABA (Table 2).

Table 2. Comparison of GABA enhanced products

Raw Materials	Microorganism	Major component of medium	Fermentation conditions	Final concentration of GABA	Reference
Black raspberry juice	<i>Lb. brevis</i> GABA 100	black raspberry juice 2% MSG	pH 5.5, 30°C, 12 d	265 mg/100 mL- black raspberry juice)	Kim <i>et al.</i> , 2009
Cheese	<i>Lactococcus lactis</i> ssp. <i>lactis</i> 01-7	Cheese	pH 4.86, 16°C, 2 wk	197.9 mg/100 g-cheese	Nomura <i>et al.</i> , 1998
kimchi	<i>Lactobacillus</i> sp. OPK2-59	0.1% MSG	0~1°C, 21 d	18 mg/100 g-kimchi	Seok <i>et al.</i> , 2008
Soybean	<i>Lb. acidophilus</i> , <i>Bifidobacteria lactica</i> , <i>S. thermophilus</i>	10% soybean	-	97.79 mg/100 g-soybean	Kim <i>et al.</i> , 2007
Tomato juice	<i>Lb. brevis</i> B3-20	Tomato juice Tomato juice 0.5% yeast extract 3% MSG	37°C, 72 h	0.169g/100 mL-juice 1.479g/100 mL- juice	Cho <i>et al.</i> , 2012
Whole milk	<i>Lb. crispatus</i> RMK567	12% skim milk MSG 0.1%	40°C, 18 h	74.56 mg/100 mL-milk	Oh, 2006
Yogurt	<i>Lactobacillus</i> sp. OPY-1	20% brown rice powder 18% whole milk 2% skim milk 5% MSG	37°C, 24 h	83 mg/100 mL yogurt	Park and Oh, 2005

Tomato paste (Cho *et al.*, 2012), yogurt (Park and Oh, 2005) and black raspberry juice (Kim *et al.*, 2009) are used for raw materials in order to obtain the fermented products enhanced GABA component (Table 3). At these processes, glutamic acid contained in the natural ingredients without artificial glutamic acid (MSG) may be converted to 100% of GABA or there is a case of adding a small amount of MSG with the aim of 100% conversion of added MSG. Also, the extract of various grains such as rice bran, rice germ and mungbean were added to a certain amount of carbon source and nitrogen for the production of high concentrations of GABA (Kook *et al.*, 2009).

Fermentation conditions

In general, the fermentation conditions for the growth of lactic acid bacteria have been equally applicable to the production of GABA. Most of lactic acid bacteria are facultative anaerobic, so additional aeration or strong agitation is not necessary. Regardless of the fermentation scale, the degree of agitation, that bacterial cells didn't subside during fermentation periods, is sufficient, and additional supply of air or oxygen is not necessary, either. The GABA is produced smoothly at a suitable temperature for the growth of strains having GABA-producing capability (Choi *et al.*, 2006; Kook *et al.*, 2009). However, the production of GABA is not always optimized in the fermentation condition optimized the growth of all strains. There was not difference in the production of GABA in the general pH range during fermentation period of lactic acid bacteria, but the productivity of GABA even fell down when the pH was adjusted to pH 4 at the lag phase of cell growth. The adjustment of pH during fermentation period has some sort of effect on the productivity of GABA (Kang, 2002; Kim, 2009). Thus, it is reported that the adjustment of pH is not requested for the productivity of GABA. It is determined that the pH is more important in the production of GABA than other fermentation conditions because the biosynthesis mechanism of GABA is closely related to the pH. The acidic pH conditions of culture broth, which is made by organic acid such as lactic acid produced during lactic acid fermentation, are necessary for GABA production by lactic acid bacteria, which convert glutamic acid into GABA. The biosynthesis mechanism of GABA is to maintain the pH within the cell constantly even though the environment around the cell becomes acidification, because glutamate consumes one molecule of H⁺ by decarboxylation and raises the pH within the cell.

So pH condition of culture broth is an important factor

Table 3. Raw materials and products of individual recognition (taken from : <http://www.foodnara.go.kr/hfoodi/>)

Ingredients / Product	Company	Authentication information	Addition amount
Theanine, etc. Compound extract	CJ Co., Ltd.	Recognized functionality as "May help improve memory, but yet the scientific evidence is insufficient" Functional level is "Other functions III" because it is confirmed that it can help animal improve memory on tests, but human studies data has not been submitted.	CJ theanine and Compound extract are made by mixing rice bran fermentation extract and L-theanine. Component indicators are GABA and L-theanine, and contents are more than 90% and more than 0.4% respectively and standardized.
GABA-containing powder derived from L-glutamic acid	Lotte shopping Co., Ltd.	"It can help people with high blood pressure" (Other functions II)	Daily intake: [GABA] 20 mg/d
HealthOne GABA omega3	Lotte Confectionery Co. Ltd. Health projects headquarters	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80.0-120.0 of [20 mg/4 capsules(2,200 mg)]
GABA 120	LG Life science Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120% of [20 mg/2 capsules(1,000 mg)]
GABA and Manganese	Nutri Biotech Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : 80-120% of 20 mg/1,000 mg
Chingkundang GABA	Nutri Biotech Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120% of 20 mg/1,000 mg
GABA and γ -Linlinol Acid	Nutri Biotech Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : 80-120% of 20 mg/1,400 mg
GABA Plus	Nutri Biotech Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120% of [20 mg/2 capsules(1,000 mg)]
GABATriple	LG Life science Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : 80-120% of 20 mg/1,400 mg
GABA and Jung	Nutri Biotech Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120% of [20 mg/1,400 mg]
GABA and omega3	Rexgene Biotech Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120 of [20 mg/1,150 mg]
GABA Premium	Cosmaxbio Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120% of 20 mg/1,000 mg
Energy GABA 129	Hanmi Commercial Co. Ltd.	[GABA-containing powder derived from L-glutamic acid] "It is helpful for people with high blood pressure. (Other functions II)	Gamma-amino butyric acid (GABA) : Labeled amount 80-120% of 20 mg/2,000 mg

in GABA production (Fig. 3). It showed that the point of being converted to GABA of glutamic acid was from the late logarithmic growth phase when the growth of strains progressed to some extent as previously reported, and which is consistent with the report that the activity of the key enzyme, GAD for converting to GABA becomes the best in the stationary phase (Hayakawa *et al.*, 1997). It can be seen that production of GABA is started at acidic pH conditions that lactic acid bacteria produce the organic acid during their growth period regardless of lactic acid bacteria strain. There is a little difference depending on the medium compositions and the wild strains, but the production of GABA is started usually from after 8 h and lasted up to 48 h. It can be seen that GABA production is sustained at a low level as long as the viable cell is maintained and glutamic acid is present.

Therefore, GABA-producing strain should be fermented at the optimal temperature in order to maximize the productivity of GABA and at the non-adjusting pH is not required, however the pH environment should be acidified by the production of organic acids such as lactic acid during the culture of lactic acid bacteria. It is determined that the total activity (AU) and specific activity (AU/CFU-cell) of GAD are maximized in an environment such as optimal temperature and acidic pH etc., additionally, the substrate, MSG, added in the culture medium, is delivered into the cells by their own antiporter system and maximize the production of GABA.

GABA-producing capability

The concentration of GABA produced by the fermenta-

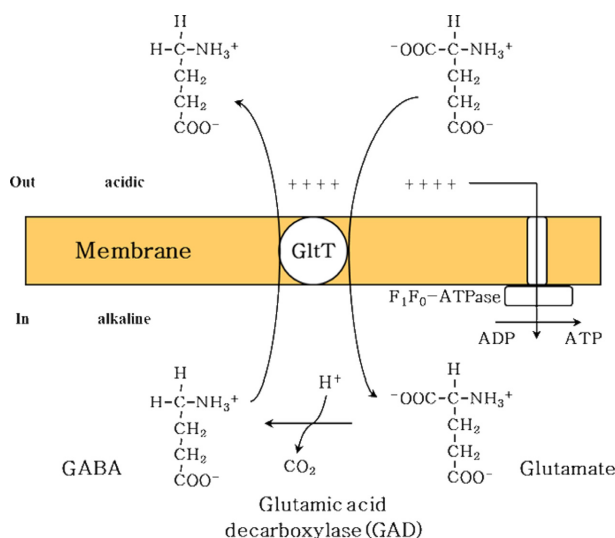


Fig. 3. Hypothetical scheme for acidic pH resistance mechanism and ATP production by glutamic acid decarboxylase in *Lactobacillus* strains (Kang, 2002).

tion shows the difference depending on the basic capability of screened strains and the optimization of fermentation condition. Each strain's GABA-producing capability is how much MSG can be converted into GABA during fermentation period and the conversion rate as well as the amount of added MSG is also an important factor. Especially, in case of GABA produced for food, the merchantability is much lower by domestic emotional issues to avoid MSG when it is not converted to GABA in the fermentation broth.

Theoretical production yield of GABA can be represented by how much glutamic acid added as MSG has been converted into GABA. The molecular weight of MSG is 169 and GABA is 103.1. When 8% (w/v) of MSG is added per 1 liter of medium, the GABA content in the medium is 4.88% (w/v) by calculated as $(103.1 \div 169) \times 8\%$, if added glutamic acid is converted to 100% of GABA. In general, most of MSG distributed as a food additive on the markets is mono hydrate type and the molecular weight is 187, so the molecular weight and the moisture content should be considered when calculating the conversion yield.

GABA-producing strain isolated from Japanese Soju produced 2 mM level of GABA without adding glutamic acid in the early time (Yokoyama *et al.*, 2002), according to the recent research in Korean, the fermentation broth containing over 600 mM (618 g/L) of GABA could be obtained by the batch fermentation at the optimal medium added 12% of MSG (mono hydrate), at this case of fermentation, GABA production yield was 100% (Kook *et al.*, 2010). In China, it was reported that 1,000 mM levels of GABA has been produced by fed-batch recently (Li *et al.*, 2010).

Accordingly, the production of GABA has been increased by screening of a new strain and optimization of fermentation conditions, and the various applications of purposes are possible.

Commercialization of GABA

Post-treatment of GABA-containing culture broth
GABA-containing culture broth is powered through the post-treatment process to make the material containing high concentrations of GABA. The post-treatment process removes water-insoluble fractions primarily, most of them were cells used in the fermentation, removed by filtration using membrane or continuous-type centrifugation. After remove the water-insoluble fractions, GABA containing broth is powdered through the spray drying add-

ing diluting material such as maltodextrin. Generally, GABA, carbon source and nitrogen source containing in the culture broth, are very hygroscopic, so it is impossible to be powered through the spray drying if the GABA content exceeds a certain concentration. Therefore, the highest level of GABA content that are produced by spray drying is approximately 20%

The crystallization process is necessary in order to increase the GABA content more than 20%. In this case, it's possible to increase the GABA concentration close to 100% but there would be no benefit for the fermentation process because it costs a lot and the discrimination with chemically synthesized GABA is eliminated by removing various substances produced by lactic acid bacteria during fermentation. In addition, the marketability of GABA-containing powder increased when extracts such as rice bran, rice germ, and mung bean used as a fermentation broth (Cho *et al.*, 2005).

Recently, various efficacies of GABA on the skin are revealed (Denda *et al.*, 2002; Han *et al.*, 2007) and GABA is developing as functional ingredients for the cosmetics. Using as food ingredients, GABA-containing culture broth can be used raw materials for cosmetics. However, it is necessary to remove the unique flavor of the fermentation broth and pass the decoloration in order to apply the fermentation broth to raw materials for cosmetics. The distillation method is commonly used for deodorization and the activated carbon for the decoloration mainly. In addition, preservatives that can prevent pollution and degeneration during distribution period can be added for the commercialization of liquid product.

Efficacies of GABA

The research for clinical efficacy of GABA began in earnest by identifying the effect on diseases such as insomnia, depression using defatted rice embryos enhanced the GABA content in Japan (Okada *et al.*, 2000). After that, the various functions have shown and studies for new applications of GABA are progressing.

Utilizing the research that driving exercise-induction of rats is suppressed when added GABA to feed because of its role of inhibitory neurotransmitter, that is, weight is increased by inhibiting the movement of the unintended energy consumption, a study for utilizing the efficacy by adding GABA to feed has been conducted and it might mean the possibility of GABA as a feed additives.

Also, Bae (2008) confirmed the efficacy of GABA related to the alcohol decomposition and improvement of liver function by confirming the fact that the liver function of

the mouse, which ate feed added GABA powder produced by lactic acid bacteria has improved.

This means that GABA can be used as a material with enough competitiveness in many domestic drinking populations. Also, Kim *et al.* (2010) conducted study on the sleep-inducing effects of GABA by obtaining the result about the significant increase of melatonin and serotonin in case of eating over a certain amount of GABA produced by lactic acid bacteria through animal test. It means that GABA can get the spotlight as the food ingredient to prevent insomnia increasing rapidly due to such a stress recently if the effectiveness is proven.

Others

"GABA" cannot be expressed in Korea yet because it is not registered in Korean Food Standards Codex and Korean Food Additives Codex. Accordingly, it is considered that GABA being distributed as a food material, is manufactured in "other food" and named "Fermented material" currently. Because the "registration" process is costly and time-consuming in Korea unlike Japan. It is because the study for GABA is continued in some small business such as ventures in most cases and the domestic market for GABA couldn't be expanded too much. Anyway, it is not possible to determine whether the registration of "GABA" was not tried by the small market or vice versa. However, several companies are try to certificate GABA as functional material utilizing a wide range of government support in recent years (Table 3).

A variety of physiological functions of GABA revealed so far can be both advantages and disadvantages at the same time in the commercialization of GABA, especially. Various experiments should be carried out to demonstrate the validity of function after selecting one which can highlight to consumers among the variety of physiological functions of GABA in order to certify as a functional material, all of which is difficult to have to invest a lot of money. However, it is necessary to utilize GABA which has various physiological functions. Its safety is already proved by fermented foods such as kimchi and various sauces which have been ingested traditionally. Especially, it is the material to be utilized to contribute to the national health in the domestic situation to enter the super-aging society unprecedentedly.

Also, the GABA added feed for companion livestock marketed in Japan recently and began to be supplied to the Korea through online purchase and started to apply to the factory-type livestock industry as functional ingredients.

As described above, it is known that GABA is synthe-

sized in eukaryotes, such as plants and the degree of synthesis are affected by environmental factors. The synthesis of GABA is considered to be compete the stress that comes from environments. Therefore, it is necessary to be ingested through food or functional food for human and feed for livestock because the GABA concentration becomes lower by stress and aging.

Conclusion

We reviewed the recent trends of study related to GABA produced by fermentation in Korea. The research of utilization of GABA as a functional ingredient started in Japan and has been progressing much in China recently.

A variety of products added GABA can also be found in US as well as Japan and those are sold as the type of mixture with vitamin and healthy foods for sleep induction unlike the Orient. However, consumers in US have used synthetic GABA without any burden due to their less reluctance for synthetic products. Accordingly, a various biologically active substances produced by microorganisms are not contained and have sold in low price with the content of more than 1000 mg for one drink. On the other hand, most of GABA products are produced by fermentation due to the reluctance on the synthetic products in Korea, China and Japan. It is considered that the continuous research for the production of GABA is needed in Korea because the GABA market is at the beginning unlike Japan. Especially, mungbean fermented broth containing GABA is exported to China as a cosmetics material, so it is expected that the GABA market in Korea will be expanded further if additional research on the skin-related efficacy of GABA is conducted.

It is determined that there are some subjects to discuss in the production of GABA by fermentation. At first, monosodium glutamate (MSG) is used as the source of glutamic acid supplied to the fermentation medium. MSG is good for the fermentation material for its solubility, reasonable price and edibility, but it has the problem that MSG may remain in culture broth if the conversion rate of MSG does not reach to 100% due to the vague reluctance on MSG surging the nation. Therefore, it is necessary to use substances other than MSG as a source of glutamic acid or find the method of adding glutamic acid in the medium after dissolving. Otherwise, the burden that should convert all of MSG during the fermentation period occurs. The second is that the sodium content of the final product increases by the residual sodium ions when MSG added during the medium is converted to the

GABA. It depends on the purpose, but the amount of sodium added to the product with GABA is small because the effective amount of GABA is also small at the level of tens to hundreds milligrams, however, the content of sodium which is one of the ingredients that should be avoided in the food as well as MSG has to be reduced. To do this, it is needed to use the method to remove the sodium ion in the culture broth after fermentation or develop a source of glutamic acid other than MSG.

It is determined that the fermentation optimization for production of GABA is not fulfilled yet despite some differences exist depend on the type of strains producing GABA. The study on the production of GABA by enzymatic conversion after separating GAD enzyme is conducted, but it can be not the fermentation product in the traditional sense. Therefore, in order to optimize the fermentation associated with the production of GABA, the pH adjustment at a specific time, production of high concentrations of GABA by cell recycle as well as the supply of medium and glutamic acid in the concept of continuous culture is ongoing in some sort. GABA being produced with a higher efficiency is going to be used as a material for the health of many people in aging modern times.

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