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Production of γ-amino Butyric Acid by Lactic Acid Bacteria in Skim Milk

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Lactic acid bacteria were isolated from a variety of fermented seafoods and sea creatures from the East Sea Rim, Korea and were screened for γ-amino butyric acid-producing (GABA) activity. Through a 16S rRNA sequence analysis, the bacteria of interest, which were GABA-positive on the thin-layer chromatography analysis, were recognized as three isolates of *Lactobacillus* (*Lb.*) *brevis* and one isolate of *Lactococcus* (*Lc.*) *lactis*. *Lb. brevis* FSFL0004 and FSFL0005 were isolated from fermented anglerfish and *Lb. brevis* FSFL0036 was derived from salted cutlass fish. The *Lc. lactis* strain FGL0007 was isolated from the gut of a brown sole flounder. According to HPLC analysis, the GABA contents produced by FSFL0004, FSFL0005, FSFL0036 and FGL0007 were equivalent to 10,754.37 μg/ml, 13,082.79 μg/ml, 12,290.19 μg/ml, and 45.07 μg/ml respectively in 1% monosodium glutamate-supplemented methionyl-tRNA synthetase (MRS) broth. The four strains were inoculated in skim milk with 1% monosodium glutamate to commercialize the strains as starter cultures for GABA-enriched dairy products, and TLC results displayed the production of γ-amino butyric acid by all four strains in the adaptation media. *Lc. lactis* FGL0007 demonstrated the greatest GABA production (431.42 μg/ml) by HPLC analysis. The GABA production by lactic acid bacteria strains in the skim milk demonstrated in the present study may be helpful for the production of GABA-enriched dairy products.

Key words : GABA, lactic acid bacteria, *Lactobacillus brevis*, *Lactococcus lactis*, γ-Amino butyric acid

Introduction

Gamma amino butyric acid (GABA, γ-Amino butyric acid), a four-carbon amino acid is a notable inhibitory neurotransmitter in the central nervous system with an extended list of physiological functions in humans [5]. It is produced by irreversible α-decarboxylation of glutamic acid catalyzed by the enzyme glutamate decarboxylase [19]. Physiological benefits include hypotensive, hypoglycemic and anti-cancer activities [2, 16, 23]. Sleeplessness and depression that might occur during the menopausal or pre-senile period can also be alleviated [2, 11].

The amino acid hampers overly excitatory impulses of the brain hence called an inhibitory neurotransmitter. It is also described as the 'brain's natural calming agent' and researchers have even documented its ability to defend against neurotoxicant-induced cell death [7, 14]. Conversely, glutamate is a neurotransmitter associated with excitatory processes in the brain and altered levels of both glutamate and GABA in the central nervous system have been correlated with neurological and psychological disorders including Alzheimer's disease, schizophrenia and epilepsy and studies show that it is essential to measure not only the amount but also the variation of amino acid neurotransmitter concentration in different areas of the brain to distinguish between drug efficiency and drug abuse [6, 8, 21, 22]. Owing to a great number of physiological benefits, GABA-enriched functional food has become a vibrant field of research [4, 15]. Microorganisms have also been attributed to the production of the amino acid, especially lactic acid bacteria (LAB) and have been cultivated as starter cultures in raw material or fermentative media [13]. Here we report the methods and materials to utilize marine-derived LAB as candidate strains in fermented skim milk for industrial purposes.

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Materials and Methods

Sample collections and bacterial strains

The fermented seafood and brown sole flounder intestines were purchased at Jukdo fish market, Pohang, Korea. All samples were kept in sterile poly bags with ice-box, and

transported to the laboratory. All samples were stored at 4-8°C until use. Bacterial strains were isolated from the fermented anglerfish, cutlass fish and the gut of brown sole flounder using de Man, Rogosa and Sharpe (MRS) medium (BD, Becton, Dickinson and company, Sparks, MD, USA). The isolated LAB strains were stored in 10% glycerol at -80°C before use. For the growth of the strains, stock solutions were streaked onto MRS agar plates with an inoculating loop and incubated at 30°C for 24 hr. A single colony was selected after growth for each strain, inoculated in MRS broth and incubated under the same conditions. The culture broth was used in further experiments.

Identification of isolated LAB strains

Stock solutions were streaked onto MRS agar plates and incubated at 30°C for 24 hr. Culture agar plates were sent to Solgent Company (Dajeon, Korea) for 16S rRNA sequence analysis. Basic Local Alignment Search Tool (BLAST) was used to analyze obtained 16S rRNA sequences and a species with the greatest percentage of sequence identity was selected.

Thin Layer Chromatography (TLC) analysis of GABA

TLC was performed to screen isolates for their GABA producing ability. The developing solvent consisted of n-butanol:acetic acid:distilled H_2O [3:2:1(v/v/v)]. Sample solutions were prepared as follows: 1% inoculum, cultivated in MRS broth for 24 hr, was transferred to MRS broth with 5% sodium L(+)-glutamate monohydrate (MSG, Daejung Chemicals and Metals Co., Ltd, Siheung, Korea). 3 mM pyridoxal phosphate monohydrate (Wako Pure Chemical Industries, Ltd. Osaka, Japan) was added to the sample up to 0.01g/dl and the final mixture was incubated at 30°C for 3-4 days then centrifuged at 1,200 rpm at 4°C for 10 min to obtain the supernatant for GABA identification. 1 µl aliquot of the supernatant was spotted onto TLC silica gel glass plates (Merck™ Millipore, Darmstadt, Germany) and immersed in the developing solvent for 120 min. Standard GABA (Sigma Aldrich, St. Louis, USA) solution was prepared as final concentration of 1% w/v in distilled H_2O. The developed TLC plate was briefly air-dried and sprayed with a ninhydrin solution (0.2% w/v-distilled H_2O, Sigma Aldrich) as a color reagent. Heat was applied to the plate with a dryer until GABA spots became visible for observation.

High Performance Liquid Chromatography (HPLC) analysis of GABA

To determine the amount of GABA produced by each strain, HPLC was performed as described below: The mobile phase A solvent was prepared as pH 6.1 with a mixing solution of 140 mM NaHAc, 0.15% TEA, 0.03% EDTA, 6% CH_3CN and the mobile phase B solvent was 60% CH_3CN and 0.015% EDTA. A linear gradient of solvent B (0-100%) was carried out for the analysis of compounds and run for 30 min. The flow rate was fixed at 1.0 ml/min and Waters Nova-Pak® C18 4 µm (3.9×300 mm, Waters Corporation, Seoul, Korea) was used for separation of compounds at the temperature 46°C with a variable wavelength detector (254 nm). Sample solutions were prepared by centrifugation of culture broth that had been cultivated in MRS media with 5% sodium L (+)-glutamate monohydrate at 3,000 rpm at 4°C for 15 min and the supernatant was filtered with a 0.45 µm membrane filer to remove impurities. The supernatant was dehydrated, derivatized with the solvent MeOH:H_2O:TEA:PITC (7:1:1:1) and incubated at room temperature for 30 min. Sample solutions were dried after incubation and eluted with the mobile phase A solvent and filtered with a 0.45 µm membrane filer to remove impurities. The samples were centrifuged and the supernatant was analyzed for GABA content. HPLC was performed at Korea Basic Science Institute, Dajeon, Korea.

Skim milk fermentation

GABA producing LAB strains were cultivated in MRS broth and 1% inoculum was transferred to an adaptation medium containing 10% skim milk (BD, Becton, Dickinson and company, Sparks, MD, USA), 0.5% yeast extract (BD, Becton, Dickinson and company, Sparks, MD, USA), 2% D-(+)-glucose and 1% MSG after 24 hr incubation at 30°C. 3 mM pyridoxal phosphate monohydrate was added to the sample up to 0.01g/dl and the final mixture was fermented for 36-48 hr at 30°C. The supernatant was prepared by centrifugation at 13,000 rpm for 10 min at 4°C to perform TLC as described in the above to confirm GABA production of candidate strains in skim milk.

Casein hydrolysis test

Stock solutions were streaked onto MRS agar plates and incubated at 30°C for 24 hr. A single colony was selected and streaked in a straight line with an inoculating loop onto tryptic soy agar plates with 20% skim milk and incubated

Table 1. Identification of isolated LAB strains

Strains	Identification	Identity %	Source of isolation	Accession No.
Lb. brevis FSFL0004	*Lb. brevis* strain NOS 7317	99%	Fermented anglerfish	MF966557
Lb. brevis FSFL0005	*Lb. brevis* strain b1	99%	Fermented anglerfish	FJ227308
Lb. brevis FSFL0036	*Lb. brevis* strain KCCM200080	99%	Salted cutlass fish	MF992224
Lc. lactis FGL0007	*Lc. lactis* subsp. *lactis* strain NWAFU3002	99%	Flounder gut	MG551103

at 30°C for 16-24 hr. If a zone of clearing was observed on the agar medium surrounding the bacteria, the distance between the outer edge of the clear zone and the outer edge of the bacteria was measured.

Results

Screening of GABA producing LAB strains and identification

Total four GABA producing LAB strains were screened through TLC analysis among LAB strains isolated from fermented seafood and fish intestines. Strains FSFL0004, FSFL 0005, and FSFL0036 were most similar to *Lb. brevis*, and FGL0007 to *Lc. lactis* through 16S rRNA sequence analysis (Table 1). *Lb. brevis* FSFL0004, FSFL0005, and FSFL0036 showed exceptionally high GABA production compared to *Lc. lactis* FGL0007, and relatively lower GABA production was observed on TLC analysis by *Lc. lactis* FGL0007 compared to other three strains (Fig. 1).

Quantitative determination of GABA production in 1% MSG supplemented MRS broth by isolated LAB strains

The contents of GABA production by *Lb. brevis* FSFL0004, *Lb. brevis* FSFL0005, *Lb. brevis* FSFL0036 and *Lc. lactis* FGL 0007 were determined by HPLC analysis, and the amounts were equivalent to 10754.37 μg/ml, 13082.79 μg/ml, 12290.19 μg/ml and 45.07 μg/ml respectively (Table 2). GABA production by strains FSFL0004, FSFL0005, FSFL0036 and FGL0007 was identified as peaks that projected after 13.55 min, 13.53 min, 13.55 min and 13.66 min of retention time (RT) respectively (Fig. S1).

GABA production in skim milk by isolated LAB strains

TLC results showed that GABA was produced in skim milk by all four cultivated strains at slightly varying degrees, but *Lc. lactis* FGL0007 exhibited the highest GABA production (Fig. 2). Skim milk without MSG was also used as

Table 2. Quantitative analysis of γ-amino butyric acid (GABA) production by GABA-producing LAB strains in 5% sodium L(+)-glutamate monohydrate supplemented MRS broth measured by HPLC

Strains	Source of isolation	GABA (μg/ml)
Lb. brevis FSFL0004	Fermented anglerfish	10754.37
Lb. brevis FSFL0005	Fermented anglerfish	13082.79
Lb. brevis FSFL0036	Salted cutlass fish	12290.19
Lc. lactis FGL0007	Flounder gut	45.07

Abbreviations for genus are as follows: *Lactobacillus*, *Lb.*; *Lactococcus*, *Lc.*

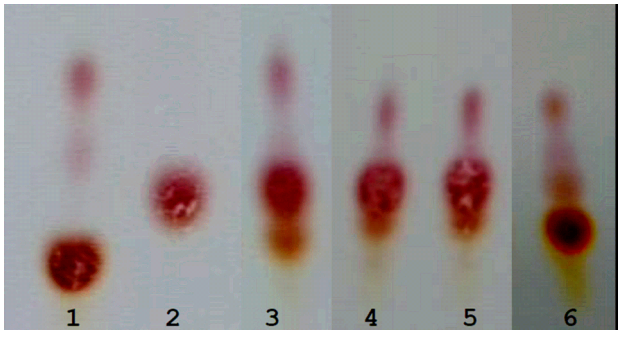

Fig. 1. TLC profile of γ-amino butyric acid production by marine-derived lactic acid bacteria strains. Lanes 1, Sodium L(+)-glutamate monohydrate; 2, GABA standard; 3, Cultural supernatant of *Lb. brevis* FSFL0036; 4, *Lb. brevis* FSFL0004; 5, *Lb. brevis* FSFL0005; 6, *Lc. lactis* FGL0007.

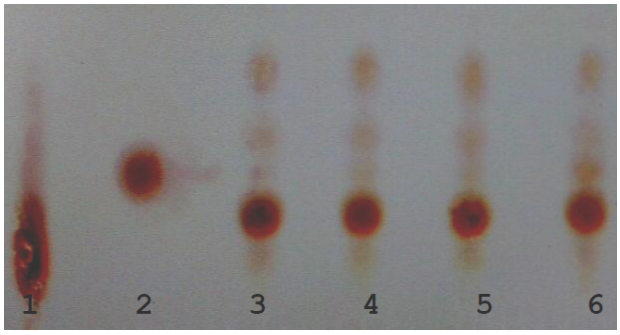

Fig. 2. TLC profile of monosodium glutamate (MSG) and gamma amino butyric acid (GABA) after skim milk fermentation. 1. Skim milk with 1% MSG; 2. GABA standard; 3. Supernatant of strain FSFL0004; 4. Strain FSFL0005; 5. Strain FSFL0036; 6. Strain FGL0007

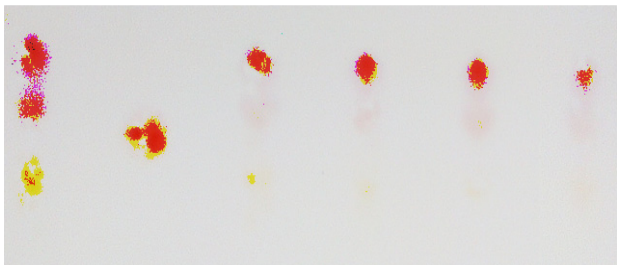

Fig. 3. TLC profile of skim milk fermentation without monosodium glutamate (MSG) 1. Skim milk with no MSG; 2. GABA standard; 3. Supernatant of strain FSFL0004; 4. Strain FSFL0005; 5. Strain FSFL0036; 6. Strain FGL0007

a medium to test whether the isolates were able to generate GABA without any additional MSG, but no GABA spots were apparent on the developed TLC plate (Fig. 3).

Quantitative determination of GABA production in 1% MSG supplemented skim milk by isolated LAB strains

The contents of GABA production by four GABA-producing LAB strains during skim milk fermentation were determined by HPLC analysis, and the amounts were equivalent to 132.22 μg/ml, 322.39 μg/ml, 334.60 μg/ml and 431.42 μg/ml respectively (Table 3). GABA production by strains FSFL0004, FSFL0005, FSFL0036 and FGL0007 was identified as peaks that projected after 13.68 min, 13.60 min, 13.75 min and 13.75 min respectively (Fig. S2).

Casein hydrolysis

Among four GABA-producing strains, only *Lc. lactis* FGL 0007 showed a positive outcome for protein assimilation and a clear zone with diameter of less than 5 mm surrounding the bacteria could be observed on the inoculated medium composed of casein and skim milk after a 24 hr incubation

Table 3. Quantitative analysis of γ-amino butyric acid production by GABA-producing LAB strains in 5% sodium L(+)-glutamate monohydrate supplemented skim milk measured by HPLC

Strains	Source of isolation	GABA (μg/ml)
Lb. brevis FSFL0004	Fermented anglerfish	132.22
Lb. brevis FSFL0005	Fermented anglerfish	322.39
Lb. brevis FSFL0036	Salted cutlass fish	334.60
Lc. lactis FGL0007	Flounder gut	431.42

Abbreviations for genus are as follows: *Lactobacillus*, *Lb.*; *Lactococcus*, *Lc.*

Table 4. Protein hydrolysis test of GABA-producing isolates

Strain no.	Casein hydrolysis*
Lb. brevis FSFL0004	-
Lb. brevis FSFL0005	-
Lb. brevis FSFL0036	-
Lc. lactis FGL0007	+

*Zone of clearing: + <5 mm, ++ ≥5 mm, +++ ≥10 mm
Abbreviations for genus are as follows: *Lactobacillus*, *Lb.*; *Lactococcus*, *Lc.*

period (Table 4). No clear zones were observed for any of the *Lb. brevis* strains.

Discussion

The employment of microorganisms, especially LAB, as starter cultures to manufacture GABA-enriched food is becoming increasingly popular as the commercial demand for naturally-occurring GABA is on the rise and former studies show an assortment of fermentative media used to cultivate bacteria and encourage production of the amino acid in the medium, including black raspberry juice, cheese, Kimchi, soybean, tomato juice, whole milk and yogurt [3, 9, 10, 13, 17, 18, 20]. Bacterial strains used in preparing GABA-enriched food have also been derived from various sources. Our goal was to screen for GABA-producing activity of isolated LAB from marine derived resources from the East Sea Rim of Korea such as marine organisms, including fish intestine, and fermented seafood. Other than a myriad of health benefits GABA-enriched food warrants, the process of adding preservatives can also be eliminated as LAB are known to extend shelf life by maintaining the original quality of the food over an extended period time [19].

The production of GABA is a result of the key activity of the enzyme Glutamic Acid Decarboxylase (GAD) and the activity of pyridoxal phosphate, a coenzyme that converts the substrate to glutamate or glutamic acid, yields the amino acid as an end product through an irreversible reaction of decarboxylation [12]. Former studies mention a remarkable fact that the production of GABA, arising from glutamate being altered, begins during the late logarithmic growth phase of the cultivated bacteria and the activity of GAD is maximized during the stationary phase [13]. Studies have shown that the pH plays a more predominant role during the conversion of GABA than fermentation conditions because the biosynthesis mechanism of GABA highly depends on the pH inside the cultivated bacteria and the acidic envi-

ronment of the culture medium [17, 19]. The pH of the medium is inevitably lowered due to the secretion of lactic acid as a byproduct during bacterial growth, but the pH within the cells of the growing bacteria remains consistent because when a molecule of hydrogen ion is utilized during the conversion of GABA from glutamate, it results in the increase of pH and contends with the decreasing pH and acidic environment of the fermentative medium [20].

Skim milk was the adaptation medium used to culture the candidate strains to screen for the GABA-production in dairy product. By qualitative analysis using TLC, GABA spots were relatively minimal but clearly noticeable for all *Lb. brevis* strains as where the *Lc. lactis* FGL0007 that displayed fairly the highest GABA production in skim milk. It seems that this phenomenon lies in the fact that skim milk lacks an adequate amount of glutamate. Consequently, studies have revealed that usually 1% of MSG is added as an alternative source of glutamic acid in the medium. For that reason, researchers have openly suggested the utilization of bacterial strains that secrete ample amounts of protease or peptidase enzymes in order to hydrolyze protein and use it as an available source of free glutamate in the fermentative medium. As a result, a high level of L-glutamate may be theoretically liberated during fermentation and proteolysis [13].

TLC results show that the *Lc. lactis* FGL0007 yielded the greatest amount of GABA in the adaptation media that composed of 1% MSG and skim milk, owing to the fact that the particular strain may harbor protein-digesting enzymes and is able to hydrolyze protein in skim milk as demonstrated by the casein hydrolysis test. Conversely, negative results indicated that the *Lb. brevis* strains are not capable of protein hydrolysis. Moreover, upon observation of the fermented skim milk, the medium inoculated with the *Lc. lactis* FGL0007 was the only strain where the incubated medium appeared coagulated and slightly viscous, while the fermented skim milk that consisted of the *Lb. brevis* strains remained fairly watery [1]. Therefore, it can be suggested that the *Lc. lactis* FGL0007 readily uses the protein in the medium provided and was therefore capable of producing a greater amount of GABA over compared strains. However, experimental results show that neither was able to yield GABA at all in skim milk that did not consist of MSG. This poses a significant problem for potential mass production because MSG still has a poor social reputation as a chemical additive and use of the substance is shunned by most consumers despite its safety announced by FDA. In order to successfully commercialize the candidate strains as potential starter cultures in GABA-enriched food products, a different substance that could replaces MSG may be required. On the other hand, optimization of full turnover of MSG to GABA may be another solution to reduce MSG content in the final product. Moreover, optimal fermentation conditions of each particular strain could be tested to further increase the GABA output.

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초록 : 탈지방우유에서 가바생성 유산균 배양을 통한 가바생성 연구

차진영[1] · 김영록[1] · 백보람[2] · 박지헌[1] · 황철원[1] · 도형기[1]*
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동해안 지역 수산발효식품과 수산물로부터 다양한 종류의 유산균들을 분리하여 감마아미노낙산(GABA) 활성을 위해 분석을 하였다. 박층크로마토그래피(TLC)를 이용하여 GABA를 생성하는 4개의 균주를 확보하였으며, 16S rRNA sequencing 분석 결과를 통해 FSFL0004, FSFL0005, FSFL0036 균주는 *Lactobacillus* (*Lb.*) *brevis,* 그리고 FGL0007은 *Lactococcus* (*Lc.*) *lactis*에 가장 유사한 것으로 확인하였다. *Lb. brevis* FSFL0004와 FSFL0005는 발효된 아귀로부터 분리되었고, *Lb. brevis* FSFL0036는 갈치 젓갈로부터 분리되었으며, *Lc. lactis* FGL0007 균주는 참가자미의 내장으로부터 분리되었다. 고속액체크로마토그래피(HPLC)를 사용한 정량분석결과를 보면, FSFL0004, FSFL0005, FSFL0036과 FGL0007에서 각각 10,754.37 μg/ml, 13,082.79 μg/ml, 12,290.19 μg/ml, 45.07 μg/ml의 GABA가 생성되었다. GABA가 풍부한 낙농제품의 발효 종균으로서 상용화 실험을 위해 1% MSG를 포함한 탈지방우유에 4개의 균주를 각각 접종하였다. TLC 결과를 보면 4개의 균주 모두가 GABA 생성을 보였다. HPLC 분석 결과를 보면, 4균주 중 *Lc. lactis* FGL0007이 가장 높은 GABA 생성(431.42 μg/ml)을 보였다. 본 연구의 내용은 GABA가 함유된 유제품 개발의 기반이 될 수 있을 것으로 생각한다.