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ORIGINAL PAPER

Separation of gamma-aminobutyric acid from fermented broth

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Abstract Gamma-aminobutyric acid (GABA) is a non-proteinaceous amino acid that is widely distributed in nature and acts as the major inhibitory neurotransmitter in the mammalian brain. This study aimed to find a separation method for getting high-purity GABA from a fermented broth. Firstly, a fermented broth with a high content of GABA (reaching 997 ± 51 mM) was prepared by fermentation with *Lactobacillus brevis* NCL912. GABA purification was conducted by successive centrifugation, filtration, decoloration, desalination, ion-exchange chromatography (IEC), and crystallization. Inorganic salt (Na_2SO_4) was removed from the both by desalination with 70% ethanol solution. A ninhydrin test strip was designed for the real-time detection of GABA during IEC. The recovery rate for the whole purification process was about 50%. The purified product was characterized by thin-layer chromatography and HPLC, and its purity reached 98.66 ± 2.36%.

Keywords Gamma-aminobutyric acid · Separation · *Lactobacillus brevis* NCL912 · Fed-batch fermentation · Fermented broth

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Introduction

Gamma-aminobutyric acid (GABA) is a non-proteinaceous amino acid that is widely distributed in nature, from microorganisms to plants and animals [20, 30]. It acts as the major inhibitory neurotransmitter in the mammalian central nervous system, and has several other well-characterized physiological functions [1, 6, 7, 10, 18, 31]. The consumption of GABA-enriched foods has been reported to depress the elevation of systolic blood pressure in spontaneously hypertensive rats (SHRs) [7] and mildly hypertensive humans [9]. Doubtlessly, GABA has potential as a bioactive component in foods and pharmaceuticals [18, 23]. However, the direct addition of chemical GABA to food is regarded as unnatural and unsafe [12, 18, 26]. It is therefore necessary to find a natural and safe method to produce GABA. Recently, the bioproduction of GABA by lactic acid bacteria (LAB) has attracted growing attention [4, 14, 17, 27, 32, 33] due to the fact that LAB possess special physiological activities and are generally regarded as safe (GRAS).

The production of GABA by bioconversion involves two main processes, namely fermentation and separation of GABA. In previous studies, much attention has been paid to the microbial production of GABA, especially using some LAB strains [5, 14, 19, 29]. To our knowledge, however, few studies have been conducted on the purification of GABA from fermented broth, one exception being that Yokoyama et al. [33] prepared a GABA-containing solution with light color from alcohol distillery lees fermented by *L. brevis* IFO-12005, through centrifugation, flocculation, and decoloration. The separation of amino acids from fermented broth is rather difficult [3, 11] because various problems can be encountered. Firstly, the fermentation mother broth usually contains many different

components and impurities. Secondly, because amino acids are polar organic compounds, they cannot dissolve in typical organic solvents, so traditional solvent extraction and sublation methods cannot be used to separate them from the fermentation broth [2]. Thirdly, another challenge is to remove high concentrations of Na_2SO_4 from the fermented broth [19].

We previously screened a high GABA-producer *L. brevis* NCL912 [15] and developed a fed-batch fermentation process for the efficient synthesis of GABA [19]. However, more surplus glucose and L-glutamate remained in the broth when the fermentation was ended [19]. Therefore, the former process should be improved to ensure no glucose and glutamate residuals in the fermented broth so that the GABA could be easily separated. The present study was aimed at the preparation of fermentation broth containing a high content of GABA but no glucose and glutamate residuals. GABA purification was then conducted by successive application of routine technologies such as centrifugation, filtration, decoloration, desalination, ion-exchange chromatography (IEC), and crystallization. The purified GABA was characterized by thin-layer chromatography (TLC) and HPLC. Notably, a new efficient desalination technology using 70% ethanol was developed to remove Na_2SO_4, and a ninhydrin test strip (NTS) was designed for the real-time detection of GABA.

Materials and methods

Strain and medium

GABA-producing *L. brevis* NCL912 was isolated from paocai, a Chinese traditional fermented vegetable [15]. The seed medium was composed of (g/L): glucose, 50; soya peptone, 25; $MnSO_4 \cdot 4H_2O$, 0.01; L-glutamate, 150 mM; and Tween 80, 2 mL/L. The fermentation medium was composed of (g/L): glucose, 35; soya peptone, 25; L-glutamate, 400 mM; $MnSO_4 \cdot 4H_2O$, 0.01; and Tween 80, 2 mL/L. Nitrogen sources, glutamate, and the other compositions were autoclaved separately at 121°C for 20 min and mixed prior to use.

Cultivation

Lactobacillus brevis NCL912 was cultured in the seed medium at 32°C for 10 h till the absorbance value at 600 nm (A_{600}) was between 4.0 and 6.0 and then used for seed culture. The fed-batch fermentation was carried out in a 5-L fermentor (Labo-controller MDL-8C; B. E. Marubishi, Tokyo, Japan) with 3 L of the fermentation medium, and inoculated with 300 mL of the seed culture. The agitation speed was 100 rpm, temperature was maintained at 32°C, and pH was maintained stable at 5.0 with addition of 10 N H_2SO_4 [19]. At 12 and 24 h, 280 and 112 g of glutamate, respectively, were fed into the bioreactor and the fermentation time was 48 h.

Preparation of the resin

A 150-mL sample of 732# strong cation-exchange resin (sulfonate-polystyrene resin) in the sodium form (Shanghai Shanpu Chemical, Shanghai, China) was immersed in distilled water for 12 h, then the water was drained off; this process was repeated twice. The resin was packed into a glass column (25 × 4 cm), treated by passing 0.45 L of 1 N HCl solution at a flow rate of 0.9 L/h, followed by rinsing with 1.5 L of distilled water. Subsequently, the resin was successively treated with 3 × 0.45 L of 1 N NaOH and 1.5 L of distilled water; 0.45 L of 1 N HCI and 1.5 L of distilled water, as per the above HCl and water treatment. The resin was converted into the hydrogen form for the following use.

Purification of GABA from the fermented broth

The fermented broth was centrifuged at 8,000*g* for 10 min, and 200 mL of cell-free supernatant was withdrawn for the GABA separation. Active carbon (4 g) was added into the supernatant and stirred uniformly then maintained in 80°C for 20 min with a stir at 10 min. The solution was then filtered through filter paper, and concentrated to 60 mL by rotary evaporation under vacuum at 60°C. The condensed solution was desalinated by the addition of 140 mL anhydrous ethanol and set aside for 1 h at room temperature. The solution was filtered, ethanol was removed by rotary evaporation under vacuum at 60°C, then diluted to 400 mL with distilled water for IEC through the column packed with 732# cation resin. Each IEC test included three steps: loading, washing, and elution. The parameters for the IEC are shown in Table 1. The eluate was detected by the NTS method described in Sect. "Analytical methods" and the fraction containing GABA was collected. The GABA-containing eluate was condensed by rotary evaporation under vacuum at 60°C until the volume was almost unchanged; 200 mL anhydrous ethanol was then added into

Table 1 Operating conditions of the IEC

Operation step	Volume (L)	Flow rate (L/h)
Loading	0.4	0.6
Washing with distilled water	0.6	1.2
Washing with 0.1 M NH_3	0.3	1.2
Elution with 1.0 M NH_3		0.6

the condensed eluate and the mixture was kept at room temperature for 30 min, the precipitate was harvested, dried at 60°C, and weighed.

Analytical methods

GABA concentrations in the culture broths were determined by pre-staining paper chromatography [16]. Glucose concentration was determined by the 3,5-dinitrosalicylic acid method [21]. TLC was performed according to the method described by Qiu et al. [24]. Purity of the product was determined by the HPLC method described previously [16] using Agilent Technologies 1200 series (Agilent Technologies, America). The NTS method was developed for detecting GABA online as follows. A filter paper was cut into strips. One end of a strip was impregnated with acetone solution of 0.1% ninhydrin and the other end was a blank area for holding. One drop of sample was placed on the end with ninhydrin and dried; the resulting color indicated if there were amino acids in the sample, i.e., if Ruhemann's purple appeared.

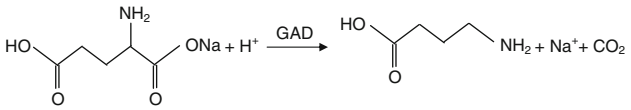
Results and discussion

Preparation of GABA-containing fermented broth

In our previous study, a fed-batch fermentation method was developed for the production of GABA by *L. brevis* NCL912. Though a high yield of GABA (reaching 1,006 ± 48 mM at 48 h) was obtained in the process, a large amount of glutamate (134 ± 24 mM) and glucose (15.28 ± 0.51 g/L) still remained in the medium [19]. It would waste material resources and not be beneficial to purify the GABA from that culture broth. Therefore, the amounts of glucose and glutamate in the medium needed to be reduced. In the current work, we used the same fermentation medium and process as previously [19], except that glucose in the medium and glutamate supplemented at 24 h were adjusted to 35 g/L and 112 g, respectively. The results showed that the modified fermentation process still has a powerful capacity for production of GABA (reaching 997 ± 51 mM), which was almost equal to that obtained before (1,006 ± 48 mM) [19], while glutamate was nearly completely converted to GABA, and no glucose could be detected at 48 h. The combination of *L. brevis* NCL912 and the current fed-batch method therefore has great potential for use in large-scale fermentations for the production of GABA.

Purification of GABA from the fermented broth

The fermented broth contained cells, proteins, pigments, and other impurities. In addition, a large amount of Na_2SO_4 accumulated in the broth as a result of the decarboxylation of glutamate. In LAB, glutamic acid decarboxylase (GAD) is responsible for the decarboxylation of glutamate to GABA [8, 13, 22, 30]:



The decarboxylation results in the stoichiometric release of the end product GABA and the consumption of a proton. The net effect of this reaction is to increase the alkalinity of the cytoplasm and environment [25, 28]. In the course of the fermentation, a large amount of H_2SO_4 was added into the broth to offset any pH rise from the decarboxylation. About 0.5 M Na_2SO_4 was produced according to the number of moles of produced GABA or converted glutamate [19]. The characteristics of the fermented broth allowed a series of separation steps like centrifugation, filtration, decoloration, desalination, IEC, and crystallization to be performed. A colorless transparent supernatant was obtained by centrifugation and decoloration. On the basis of our observation, the GABA could be dissolved in 70% ethanol solution (the solubility of GABA was more than 15 g/100 g of 70% ethanol solution) whereas Na_2SO_4 could not. This difference was therefore utilized to separate GABA from Na_2SO_4. The above decolorized solution was condensed to 60 mL. A large amount of white precipitate (Na_2SO_4) appeared after the addition of 140 mL anhydrous ethanol. Almost all the proteins in the broth were denatured by 70% ethanol and high temperature in the active carbon decoloration, and were simultaneously removed by the filtration. Ethanol was separated from the filtrate by rotary evaporation under vacuum at 60°C. The desalted solution was diluted to 400 mL and applied to IEC through the column packed with 732# strong cation-exchange resin. The interactions in IEC are due to the electrostatic force between ions in the solute and oppositely charged ligands on the matrix. The pH value of the fermented broth was 5.0, which was below the isoelectric point (7.19) of GABA, so GABA could bind with the negatively ligands. The nonspecifically bound impurities were washed out with distilled water and 0.1 M NH_3 solution. To desorb GABA from the resin, the forces between the charged species must be weakened. This is usually done either by increasing the ionic strength or by increasing the pH (which reduces the charge or changes the polarity of GABA). If GABA is eluted by the former approach, a new inorganic salt will be introduced into the GABA-containing solution, thereby deviating from the original intention to obtain a high purity of the GABA product. As a cheap and available reagent, ammonia can easily increase the pH and easily be removed from the solution, and so 1 M NH_3 was utilized to elute

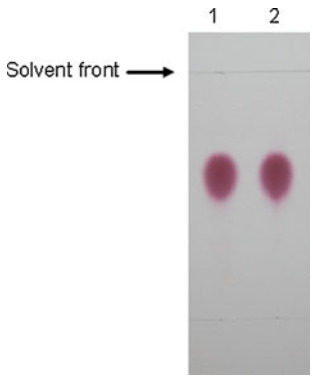


Fig. 1 TLC of the purified GABA product (*lane 1*) and GABA standard (*lane 2*). The concentration and sampling volume of the both were 10 g/L and 0.5 μL, respectively

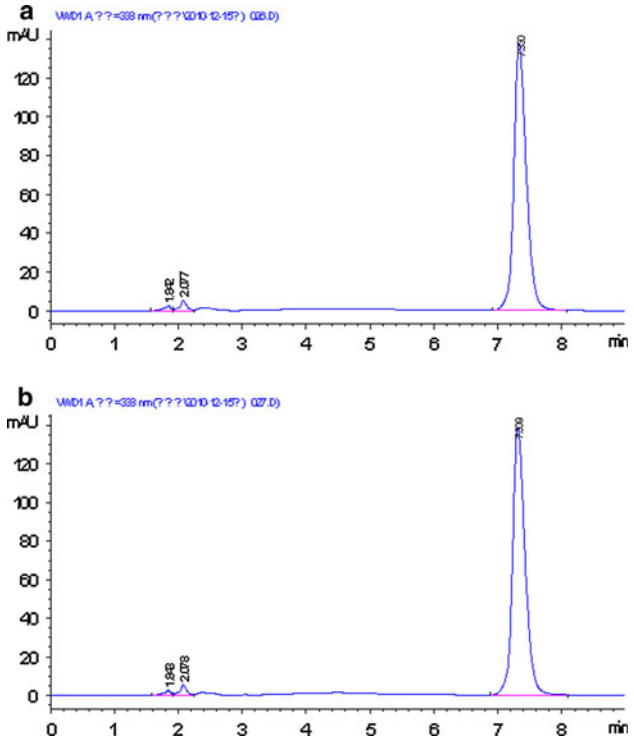


Fig. 2 HPLC chromatograms of the purified GABA product (**a**) and GABA standard (**b**). The concentration of the both was 0.4 g/L

GABA from the column. In this study, a quick NTS method was developed to monitor GABA during IEC in real time. Fractions containing GABA were collected and condensed until the volume was almost constant. A large amount of white precipitate (GABA) was produced after 200 mL ethanol was added into the concentrated solution; this was filtered and dried to give 10.76 ± 1.25 g of GABA as a white powder. The recovery rate of GABA was about 50% for the whole purification process. The purity of GABA product was analyzed using TLC (Fig. 1) and HPLC (Fig. 2) and reached 98.66 ± 2.36%. The purification of GABA from the fermented broth is summarized in Table 2. To our knowledge, this is the first report about the purification of GABA from fermentation broth.

Table 2 Purification of GABA from the fermented broth

Purification step	Recovery (%)	Purity (%)
Cell-free supernatant	100	53.89 ± 1.47
Decoloration	87.73 ± 1.08	49.52 ± 2.38
Desalination	79.38 ± 1.73	76.52 ± 1.94
IEC	99.39 ± 0.71	85.58 ± 1.28
Crystallization with ethanol	72.37 ± 7.03	98.66 ± 2.36

Conclusions

An efficient fed-batch fermentation process was successfully developed for the production of GABA by *L. brevis* NCL912. The fermented broth contained 997 ± 51 mM GABA and with little glutamate and glucose residuals. To separate high-purity GABA from the fermented broth, a process including centrifugation, filtration, decoloration, desalination, IEC, and crystallization was performed. Desalination using 70% ethanol was applied for the first time, and NTS was designed for real-time monitoring of GABA during IEC. The purity of the GABA product separated with this process was 98.66 ± 2.36%. The results proved that the combination of the above techniques was suitable for purifying GABA from the fermented broth of *L. brevis* NCL912.

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