JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Separation and Purification of γ-Aminobutyric Acid from Fermentation Broth by Flocculation and Chromatographic Methodologies

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ABSTRACT: To date, the multifunctional γ-aminobutyric acid (GABA) is mainly produced by microbial fermentation in industry. The purpose of this study was to find an effective method for separation and purification of 31.2 g/L initial GABA from the fermentation broth of *Enterococcus raffinosus* TCCC11660. To remove the impurities from fermentation broth, flocculation pretreatment using chitosan and sodium alginate was first implemented to facilitate subsequent filtration. Ultrafiltration followed two discontinuous diafiltration steps to effectively remove proteins and macromolecular pigments, and the resulting permeate was further decolored by DA201-CII resin at a high decoloration ratio and GABA recovery. Subsequently, ion exchange chromatography (IEC) with Amberlite 200C resin and gradient elution were applied for GABA separation from glutamate and arginine. Finally, GABA crystals of 99.1% purity were prepared via warm ethanol precipitation twice. Overall, our results reveal that the successive process including flocculation, filtration, ultrafiltration, decoloration, IEC, and crystallization is promising for scale-up GABA extraction from fermentation broth.

KEYWORDS: *γ-aminobutyric acid, fermentation broth, flocculation, ultrafiltration, decoloration, ion exchange chromatography, crystallization*

INTRODUCTION

γ-Aminobutyric acid (GABA) (CAS no. 56-12-2), a non-proteinaceous amino acid widely distributed in various organisms, has been applied in many foods and pharmaceuticals as a bioactive compound. Due to its well-known physiological functions, such as inhibition of neurotransmission, tranquilization, and antihypotensive and diuretic effects,[1−6] to date, increasing attention has focused on the benefits of GABA for human health. In industry, GABA can be produced by chemical synthesis, enrichment from plant tissues, fermentation, and bioconversion.[7] Many kinds of GRAS (generally regarded as safe) microorganisms, such as some lactic acid bacteria, *Aspergillus* species, and yeasts, have been reported to produce GABA by catalyzing the glutamate decarboxylation reaction for acidic tolerance and ATP production.[8−10] Therefore, it is a promising project to use glutamate decarboxylase-expressing microbes as cell factories for GABA production because of the mild reaction conditions, safety for food and medicine, and environmental compatibility. However, few studies have emphasized the separation and purification of GABA from the fermentation broth because it is a very complicated heterogeneous system which contains microbial cells, metabolites, pigments, colloidal matrix, etc. Furthermore, these complex components bring many difficulties to the downstream extraction process for GABA and make the separation−purification process be the bottleneck for the industrial production of high quality GABA. At present, only Li et al.[11] have reported bench-scale preparation of GABA crystals from the fermentation broth of *Lactobacillus brevis* NCL912 by multiple-step routine unit operations.

In our previous work, the bacterium *Enterococcus raffinosus* TCCC11660 with high GABA-producing ability was screened from Chinese traditional pickled vegetables, and then a fed-batch fermentation process was developed for the efficient biosynthesis of GABA.[12] After fed-batch fermentation, some surplus glutamate substrate remained in the broth and other components also accumulated. To remove these impurities from the complex components, first, pretreatment by flocculation and solid−liquid separation were conducted. Then the supernatant was subjected to the following successive unit operations: ultrafiltration, decoloration with macroporous adsorption resin DA-201CII, ion exchange chromatography (IEC) with Amberlite 200C resin, and gradient elution. On the basis of its low operation cost and low energy consumption, ultrafiltration technology has been widely used in various bioseparation processes.[13−15] Macroporous adsorption resin, which possesses many advantages such as large adsorption ability and easy desorption, has been widely used in the separation of targeted components from other impurities.[16−18] In recent decades, the ion exchange and adsorption technology has been used for numerous applications in the purification of lactic acid, L-phenylalanine, etc.[19−21] In this study, we aimed to provide a feasible method for the effective separation of GABA from fermentation broth using coupled technologies.

Received: November 8, 2012
Revised: January 24, 2013
Accepted: January 24, 2013
Published: January 24, 2013

MATERIALS AND METHODS

Bacterium, Media, and Cultivation. In this study, *Enterococcus raffinosus* TCCC11660 strain isolated from naturally pickled Chinese vegetables was used for GABA fermentation.[12] Briefly, the optimum composition of the seed medium was as follows: glucose 2%, peptone 1%, yeast extract 0.5%, sodium acetate 0.5%, K_2HPO_4 0.2%, $MnSO_4{\cdot}4H_2O$ 0.058%, $MgSO_4{\cdot}7H_2O$ 0.025%, ammonium citrate 0.2% (pH 7.0). The fermentation broth contained glucose 4%, yeast extract 2%, sodium acetate 0.4%, KH_2PO_4 0.2%, $MgSO_4{\cdot}7H_2O$ 0.02%, $Al_2(SO_4)_3$ 0.03%, monosodium glutamate (MSG) 5%, $(NH_4)_2SO_4$ 0.06%, Tween-80 2 mL/L (pH 6.5). Glucose, MSG, and the other components of the media were autoclaved separately at 115 °C for 20 min and mixed together prior to use. The fed-batch fermentation was carried out at an initial pH 6.5 and a controlled pH 4.5 with 3 M HCl from 14 to 72 h with a 3-L work volume in a 5-L fermentor (Biostat B, Sartorius AG, Goettingen, Germany) at 30 °C and 80 rpm. When the concentration of glutamate was less than 10 g/L, 250 g of MSG was fed into the fermentor until the GABA yield ceased. In this work, all the following unit operations for GABA purification were carried out at room temperature.

Pretreatment of the Adsorption Resins. To remove the residual porogenic agents and monomers during the resin synthesis process, macroporous adsorption resins including DA201-CII (Jiangsu Suqing Water Treatment Engineering Group Co., Ltd., Jiangyin, China), AB-8, ADS-17, D4006, H103, NKA-9, S-8, X-5, and XDA-1 (The Chemical Plant of Nankai University, Tianjing, China) were separately immersed in 1 M HCl and agitated constantly at 150 rpm for 6 h, followed by rinsing with deionized water to neutral pH, immersion in 1 M NaOH, agitatation at 150 rpm for 6 h, and rinsing with deionized water to neutral pH. Finally, the resin was immersed in 95% ethanol for 12 h and then washed with deionized water until ethanol was no longer detectable.[22] A 150-mL solid volume of cation exchange Amberlite 200C resin in the sodium form (Rohm and Haas Company, Philadelphia, PA) was immersed in anhydrous ethanol, agitated constantly for 12 h, and then rinsed with deionized water to desorb the impurities in the resin. This process was repeated twice, and then the resin was treated with 1 M HCl, followed by rinsing with deionized water. Subsequently, the resin was successively treated with 1 M NaOH and deionized water, 1 M HCl, and deionized water. Finally, the resin was converted into the hydrogen form.

Flocculation and Filtration of GABA from Fermentation Broth. To facilitate the solid−liquid separation, the fermentation broth was first pretreated by flocculation.[14,23] Considering that the fermentation broth is acidic and cationic, natural organic polymer flocculant chitosan with multiple applications in industry was selected for flocculation and sodium alginate as the coagulant aid.[24] The experiments for flocculation optimization were conducted in a constant temperature oscillator. A 100-mL bacterial suspension ($OD_{600\ nm}$ = 8.56) of 31.2 g/L initial GABA concentration was added to a 250-mL beaker. After addition of 50−250 mg/L chisotan, respectively, a rapid stirring (150 rpm) was implemented for 5 min to promote full contact between the flocculant and bacterium, and then slow stirring (25−30 rpm) for 15 min was maintained to allow floc growth. The following dose optimization of the coagulant aid, sodium alginate, was performed by the addition immediately after 150 mg/L chisotan to the bacterial suspension before the rapid agitation was started. Instead of flocculant and coagulant aid, the same volume of deionized water was added to the same batch bacterial suspension as control. After flocculation, the bacterial suspension was filtered by filter paper (Whatman No. 1) with precoated diatomite as a filter aid under normal pressure conditions. Based on the cake filtration model, filtration time and the volume of filtrate were recorded from time to time to calculate the filtration constant K from the following equation:

$$\frac{\tau}{q} = \frac{1}{K}q + \frac{2}{K}q_e \tag{1}$$

where τ is the filtration time (s), q is the volume of filtrate based on unit filter area (m^3/m^2), and K (m^2/s) and q_e (m^3/m^2) are the filtration constants, respectively.

A total 2-L volume of filtrate was obtained under optimized flocculation conditions for the ultrafiltration tests using the polyamide hollow fiber membrane filter (cutoff at 5000 Da; Shanghai Mosu Scientific Equipments Co., Ltd., Shanghai, China) to remove impurities. The applied ultrafiltration conditions were 0.1 MPa for the operating pressure and an initial pH 4.5 of the filtrate. After ultrafiltration and diafiltration, the permeate was treated with the selected macroporous adsorption resin DA201-CII. Then the above decolored solution was pumped into a glass column packed with 90.0 cm^3 of cation exchanger Amberlite 200C at 0.5−2.0 BV (bed volume)/h power flow rate. After rinsing with deionized water, gradient elution was implemented with 0.5 M ammonia and deionized water at the same flow rate.

For preparation of GABA crystals, in brief, the highly purified GABA eluent in corresponding fraction collection tubes was merged and concentrated using rotary evaporation. Afterward, GABA was crystallized from the concentrated solution by adding ethanol to final concentration of 75% at 60 °C and allowed to cool. By recrystallization with 90% warm ethanol, GABA crystals were collected by filtration and oven-dried at 80 °C for 3 h till constant weight.

Analytical Methods. The concentration of GABA was determined by HPLC (Agilent 1200 series, Agilent Technologies Co., Ltd., Palo Alto, CA). Samples were derivatized with 2,4-dinitrofluorobanzene (2,4-DNFB) for 50 min at 60 °C and then detected using a DAD UV detector at 360 nm at 30 °C (column temperature) with a reversed phase column (C-18, Agilent ZORBAX StableBond, 250 × 4.6 mm).[25] The qualitative analysis of related amino acids was performed using prestaining paper chromatography on Whatman No. 1 filer paper.[26] This paper chromatography process was developed overnight with a 2-μL loading volume for each sample and developing agent of *n*-butanol−acetic acid−water (6:1:3, v/v) containing 0.4% ninhydrin followed by directly drying for color generation in the oven at 50 °C. The molecular weight of crystals obtained was analyzed by HPLC-MS (Agilent 1200 series liquid chromatography interfaced with an Agilent 6100 mass detector). The electrospray source was operated at a spray voltage of 4.5 KV, a capillary voltage of 10 V, and a capillary temperature of 220 °C.

The concentrations of residual ethanol, glucose, and glutamate in all samples were assayed enzymatically after proper dilution by using a biosensor analyzer with immobilized enzymatic electrodes (SBA-40E; Biology Institute of Shandong Academy of Science, Jinan, China).

The flocculation efficiency of the supernatant was assessed as the percentage of relative reduction in turbidity measured spectrophotometrically at 600 nm by comparing with the original bacterial suspension. It is also called the flocculation ratio (FR):

$$\mathrm{FR}\ (\%) = \frac{OD_{cs} - OD_{su}}{OD_{cs}} \times 100\% \tag{2}$$

where OD_{cs} is the absorbance of original cell suspension at 600 nm, OD_{su} is the absorbance of supernatant after flocculation at 600 nm.

The decoloration efficiency of macroporous adsorption resin was measured by decoloration ratio (DR):

$$\mathrm{DR}\ (\%) = \frac{A_0 - A}{A_0} \times 100\% \tag{3}$$

where A_0 and A are the absorbance of fermentation broth at 390 nm before and after decoloration by resin, respectively.

The content of protein impurity was measured with conventional BCA kits. The deproteinization efficiency of the ultrafiltration process was quantified by the following equation:[27]

$$\text{deproteinization efficiency}\ (\%) = \frac{C_0 - C}{C_0} \times 100\% \tag{4}$$

where C_0 and C are the protein concentrations in solution before and after ultrafiltration, respectively.

dx.doi.org/10.1021/jf304749v | J. Agric. Food Chem. 2013, 61, 1914−1919

RESULTS AND DISCUSSION

Flocculation and Filtration. In the current study, the dose effects of flocculant and coagulant aid on flocculation under different conditions were compared. Optimum flocculant dose was obtained by addition at a range of 50−250 mg/L chitosan to the fermentation broth. With the increase of flocculant dose, a significant improvement in flocculation ratio was achieved. As shown in Figure 1, when the chitosan concentration was up to

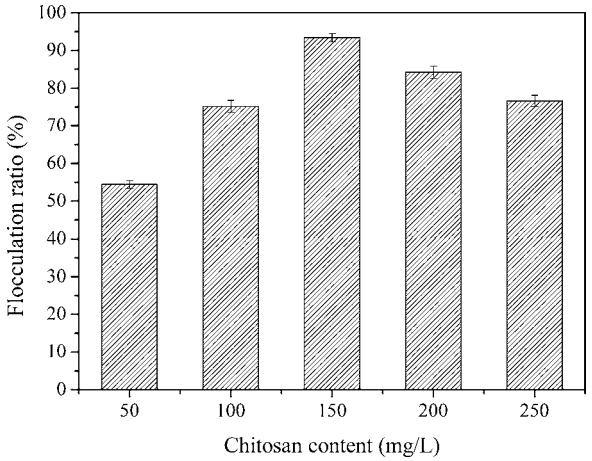

Figure 1. The effect of chitosan content on flocculation ratio. All the flocculation experiments were repeated three times, and the data are presented as the mean value with standard deviation.

150 mg/L, the flocculation effect was the best. However, more addition of flocculant led to the decline of the flocculation ratio. Figure 2 exhibits an optimum concentration ratio of flocculant

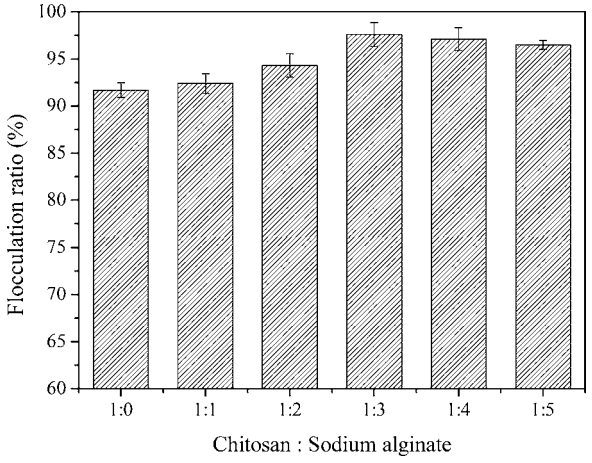

Figure 2. The effect of the ratio of chitosan to sodium alginate on flocculation ratio.

to coagulant aid of 1:3. Altering the pH value of the fermentation broth prior to flocculant addition had an obvious effect on flocculation at the selected flocculant doses (150 mg/L chitosan and 450 mg/L sodium alginate), and the best flocculation occurred at pH 4.5−5.5 (Figure 3).

The purpose of flocculation is to facilitate solid−liquid separation; thus, the influence of flocculation on filtration was investigated, and the relevant filtration parameters are shown in Table 1. The filtration time of flocculation scheme C was far less than that of the control experiment. Although the addition

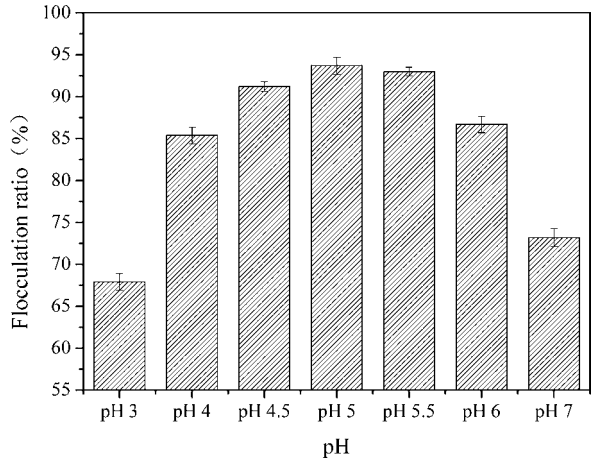

Figure 3. The effect of pH on flocculation ratio. Chitosan content, 150 mg/L; ratio of chitosan to sodium alginate = 1:3.

Table 1. The Filtration Constant of Various Flocculation Schemes

	filtration time (s)		
volume of the permeate (mL)	scheme A[a]	scheme B[b]	scheme C[c]
10	120	105	67
20	521	294	169
30	1084	643	295
40	1779	1028	511
50	2825	1364	786
K (m^2/s)			
scheme A	scheme B	scheme C	
2.304×10^{-8}	5.556×10^{-8}	1.106×10^{-7}	

[a]Control experiment using deionized water instead of flocculant and coagulant aid. [b]Flocculation with 150 mg/L chitosan. [c]Flocculation with 150 mg/L chitosan and 450 mg/L sodium alginate.

of coagulant aid improved the flocculation ratio by 6.2%, filtration experiments found that the use of sodium alginate as coagulant aid promotes the filtration speed significantly (Figure 2). This may be due to the fact that the addition of coagulant aid leads to bigger floc. The filtration constant K is deduced by plotting of τ/q versus q and eq 1 (Figure 4). In addition, after the flocculation with chitosan and sodium alginate under optimized conditions, the filtration constant K was found to be 1 order of magnitude higher than that of the control. This result indicates that flocculation greatly improves the filtration speed and filtration quality with industrial potential.

Ultrafiltration Step. The results listed in Table 2 demonstrate that ultrafiltration is efficient for the purification of GABA. Discontinuous diafiltration (DD) twice followed by a single ultrafiltration was carried out to increase the recovery efficiency of GABA. Adding a 4-fold volume deionized water to a 0.4-L ultrafiltration concentrate resulted in a significant increase of GABA recovery, but the purity of GABA showed a slight decrease after the diafiltration step (Table 2). This result is due to the fact that small molecular weight impurities, which remained in the concentrate and on the ultrafiltration membrane, penetrated through the membrane into the permeate of the second DD. In addition, the protein content in permeate was reduced by 68.4%. On the basis of our observation, some macromolecular pigments were intercepted by the ultrafiltration membrane because the color of permeate

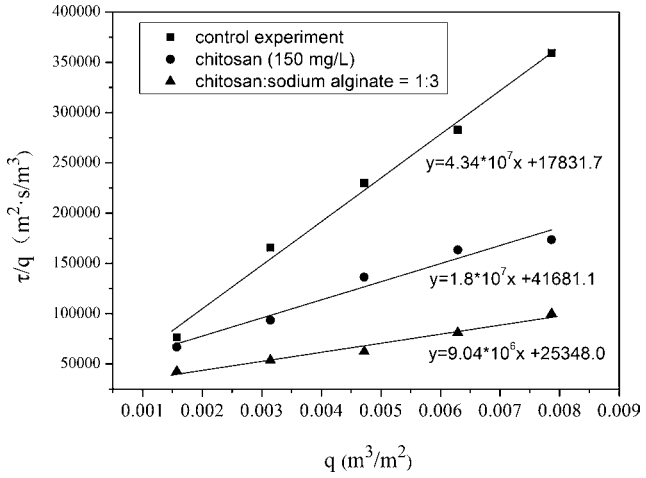

Figure 4. The effect of different flocculation methods on filtration constant *K*. Symbols: ■, control experiment; ●, chitosan (150 mg/L); ▲, chitosan:sodium alginate = 1:3.

Table 2. The Parameters of Ultrafiltration and Discontinuous Diafiltration

samples	volume (L)	C_{GABA}[a] (g/L)	recovery ratio (%)	total solid content (%)	purity of GABA[d] (%)
fermentation broth	2	30.4	–	5.86	51.87
permeate of the UF[b]	1.6	31.2	82.1	4.35	71.72
permeate of the first DD[c]	3.2	17.4	91.6	2.54	68.50
permeate of the second DD	4.8	12.3	97.1	1.83	67.21

[a]GABA concentration in solution. [b]UF: ultrafiltration. [c]DD: discontinuous diafiltration. [d]Purity of GABA (%) = C_{GABA}/total solid content × 100%.

became more transparent. The removal of these impurities had a significant benefit not only on GABA purity but also on the subsequent adsorption capacity of the resin.

Decoloration Step. To guarantee the color gradation and GABA purity, the permeate of ultrafiltration still needs further decoloration to remove the small molecular pigments. In this study, resins DA201-CII and S-8 exhibited better adsorption ability for pigments at 87.5% and 77.2%, respectively; the other resins were not suitable because of their lower decoloration ratios. On the basis of the low GABA loss ratio, easy desorption of pigments, and good mechanical strength, DA201-CII resin was selected as the decoloration medium for the permeate. Static adsorption experiments disclosed that the decoloration ratio increased with the decrease of pH value of the permeate, but the effect of temperature on decoloration was not significant. Considering that a lot of ions need to be added into the permeate to adjust the pH value, the decoloration step was implemented at an approximate initial pH of 4.5 for the permeate, to preclude adverse effects on the subsequent ion exchange chromatography. The best conditions for decoloration were determined as a 3 BV/h flow rate for a 5-BV loading volume, and the decoloration ratio reached 90.6% with 92.5% GABA recovery.

IEC and GABA Crystallization. The decolored solution was applied to IEC through the column packed with cation exchange resin Amberlite 200C. Besides GABA, the decolored solution also contained another two kinds of amino acids, a small amount of surplus substrate glutamic acid (pK_{a1} = 2.19, pK_{a2} = 4.25, pI = 3.22) and a metabolite arginine (pK_{a2} = 9.04, pK_{a3} = 12.48, pI = 10.76). As amphoteric electrolyte, an amino acid acts as both base and acid at different pH values.[28] The pH value is one of the most important factors to be controlled in the IEC process. Our pre-experiments proved that a lower pH value of the solution led to the adsorption competition of H^+ against $GABA^+$, which resulted in the decrease of adsorption capacity for GABA by Amberlite 200C resin.

The pH value of decolored solution was approximately 4.5, between the isoelectric points of glutamic acid and GABA (pK_{a1} = 4.02, pK_{a2} = 10.35, pI = 7.19). At this pH point, glutamic acid is mainly negatively charged, which can hardly be adsorbed by the resin; meanwhile, GABA can be adsorbed due to its positive charge. The adsorption capacity of amino acids by ion exchange resin is also significantly influenced by the salt composition in the decolored solution. To avoid the introduction of salt ions, we chose not to adjust the pH of the decolored solution for dynamic ion exchange adsorption by Amberlite 200C resin. When the adsorption process achieves the breakthrough point (C/C_o = 0.05), which is defined as when the outflow concentration of GABA from the column (C) is 5% of the entrance concentration (C_o), continuing to load the decolored solution into the resin column will cause the loss of target product. Thus, the adsorption operation needs to be stopped when the adsorption reaches the breakthrough point and changes into the processes of impurities cleaning, adsorbate elution, and adsorbent regeneration.

In this paper, the dynamic ion exchange adsorption experiments demonstrated that a high flow rate is detrimental to GABA adsorption. $GABA^+$ ions can be absorbed on the functional sulfonic acid exchange groups of Amberlite 200C resin after film diffusion and intraparticle diffusion. By plotting the breakthrough curves for dynamic adsorption by Amberlite 200C at various flow rates, we found that it is easy to break through at the high flow rate due to the fact that the resin could not play its full role (Figure 5). There is not sufficient time for GABA adsorption if the flow rate is too high, but a low flow rate reduces the production efficiency. Accordingly, the following conditions were set up to obtain a good adsorption result at 1 BV/h flow rate for a 2-BV loading volume. After dynamic

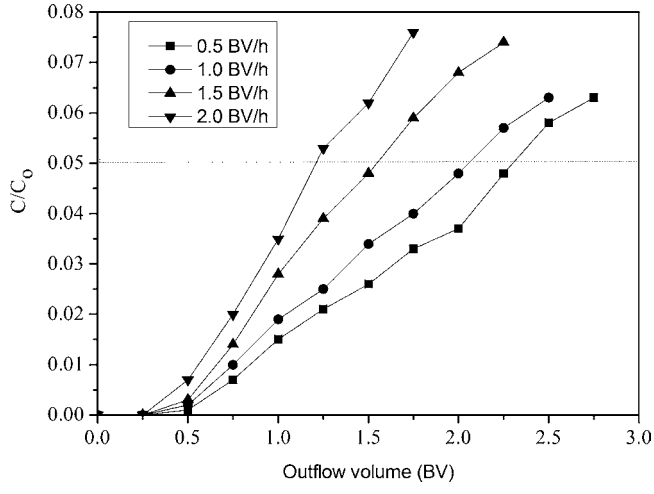

Figure 5. The breakthrough curves for GABA ion exchange by Amberlite 200C resin. Symbols (BV/h): ■, 0.5; ●, 1.0; ▲, 1.5; ▼, 2.0.

dx.doi.org/10.1021/jf304749v | J. Agric. Food Chem. 2013, 61, 1914−1919

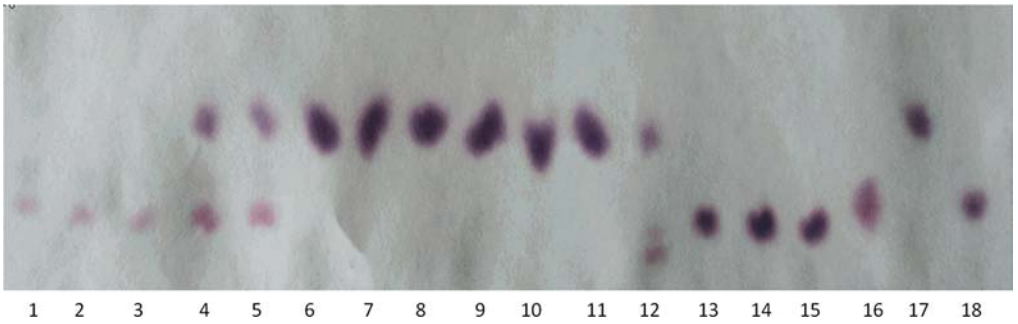

Figure 6. Paper chromatography for GABA gradient elution. Lanes 1−15: diluted samples of collection tubes nos. 1−15; lane 16: glutamic acid standard (2.0 μg/μL); lane 17: GABA standard (5.0 μg/μL); lane 18: arginine standard (1.5 μg/μL).

adsorption, a 2-BV of deionized water was pumped into the column for removal of impurities, especially for glutamic acid, followed by gradient elution. Meanwhile, the outflow composition was collected by a fraction collector. At pH 4.5, the order of binding force between amino acids and the strong acidic functional group in the cation exchange resin is the following: basic amino acids > neutral amino acids > acidic amino acids; the elution order is correspondingly reversed for these amino acids. Therefore, GABA eluted before arginine. Furthermore, there was only one spot which was confirmed as GABA in lanes 6−11 from the paper chromatography (Figure 6), which indicates that the target product is effectively separated from the other two amino acids.

Finally, the GABA crystals were produced from the merged fraction collection tubes nos. 6−11 via precipitation twice in warm ethanol. The obtained crystals were confirmed as GABA by HPLC-MS analysis, which gave the same molecular weight of 268.1 after being derivatized with 2,4-DNFB for both the obtained crystals and standard GABA. Furthermore, the GABA crystals were obtained in 99.1% purity as shown by HPLC analysis. This result also implies that a higher purity of GABA is available via the multiple circle of the crystallization process.

In this work, the preparation of GABA crystals mainly possesses the following merits: (1) the flocculation pretreatment efficiently promotes solid−liquid separation and also reduces the industrial equipment requirement and energy consumption; (2) by regeneration, macroporous resin DA-201CII can be repeatedly used for dynamic decoloration of the permeate, which is much closer to the real industrial process; (3) the strongly acidic ion exchanger Amberlite 200C expresses a high GABA adsorption ratio for potential industrial application. Therefore, the established successive processes will have a guiding significance to the separation and purification of GABA from fermentation broth for potential industrial-scale application under the careful precautions based on UHSA or other equivalent European requirements.

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Funding

This work was financially supported by National 973 Program of China (2013CB734004 and 2011CB707401), National 863 Program of China (2012AA021302), and the Key Project for Innovative Research of Tianjin Binhai New Area of China (2011-BK120014).

Notes

The authors declare no competing financial interest.

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