Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytica Chimica Acta 540 (2005) 293–297
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Enzyme-based microtiter plate assay for γ-aminobutyric acid: Application to the screening of γ-aminobutyric acid-producing lactic acid bacteria

Tadayuki Tsukatani [a,*], Tomoko Higuchi [a], Kiyoshi Matsumoto [b]

[a] *Biotechnology and Food Research Institute, Fukuoka Industrial Technology Center, Aikawa machi 1465-5, Kurume, Fukuoka 839-0861, Japan*
[b] *Division of Food Biotechnology, Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School Kyushu University, Fukuoka 812-8581, Japan*

Received 14 December 2004; received in revised form 17 March 2005; accepted 23 March 2005
Available online 19 April 2005

Abstract

An enzyme-based microtiter plate assay for γ-aminobutyric acid (GABA) was developed. GABA was quantified using γ-aminobutyrate glutamate aminotransferase and succinic semialdehyde dehydrogenase in the presence of $NADP^+$ and α-ketoglutarate. The NADPH produced by the series of enzymatic reactions was measured spectrophotometrically at 340 nm. A linear relationship between absorbance and the concentration of GABA was obtained in the ranges from 5.0×10^{-4} to 1.0×10^{-2} M. The relative standard deviation for 10 successive measurements was 0.9% at the 10 mM GABA level. This analytical method was applied to the screening of GABA-producing lactic acid bacteria in de Man–Rogosa–Sharpe (MRS) medium. The proposed method enables one to assay 96 samples for an hour without the pre-treatment of samples. The method is by far superior to the traditional HPLC method from the point of view of rapidity and simplicity.

Keywords: γ-Aminobutyric acid; Microtiter plate; Enzyme; GABase; Lactic acid bacteria

1. Introduction

γ-Aminobutyric acid (GABA) is well known as a neurotransmitter that regulates inhibitory neurotransmission in mammalian central nervous systems [1]. In addition, GABA has been proved to be effective for lowering the blood pressure of mammals [2]. Milk products fermented by lactic acid bacteria, which are enriched with GABA, decreased the blood pressure in spontaneously hypertensive rats [3], and in mildly and moderately hypertensive patients [4,5]. Therefore, the effects of GABA on human health are also of current interest in food production. Currently, the screening of GABA-producing lactic acid bacteria and the production of food enriched with GABA by the lactic acid bacteria are being done actively [6–8].

GABA can be quantified by high performance liquid chromatography (HPLC) with pre-/post-column derivatization [9,10]. However, it is difficult to assay a large number of samples for the screening of lactic acid bacteria by HPLC because it requires a specific pre-/post-column reaction and is time consuming. Therefore, high sampling frequency is required for screening of the bacteria. A microtiter plate assay using enzymatic determination is a suitable method to meet this demand.

GABase, which contains two main enzymes, γ-aminobutyrate glutamate aminotransferase (GABA-T) and succinic semialdehyde dehydrogenase (SSDH), is known to catalyze the following two reactions:

$$\text{GABA} + \alpha\text{-ketoglutarate} \xrightarrow{\text{GABA-T}} \text{succinic semialdehyde} + \text{glutamate} \quad (1)$$

$$\text{succinic semialdehyde} + NADP^+ + H_2O \xrightarrow{\text{SSDH}} \text{succinate} + NADPH + H^+ \quad (2)$$

* Corresponding author. Tel.: +81 942 30 6644; fax: +81 942 30 7244.
E-mail address: tukatani@fitc.pref.fukuoka.jp (T. Tsukatani).

0003-2670/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2005.03.056

GABA has been quantified by UV spectrophotometry using GABase based on the absorbance change caused by the conversion of $NADP^+$ to NADPH [11], but this method has not yet been applied to the screening of GABA-producing lactic acid bacteria. Therefore, we developed an enzyme-based microtiter plate assay for GABA and applied this method to the screening of GABA-producing lactic acid bacteria.

2. Experimental

2.1. Reagents

GABase [a mixture of γ-aminobutyrate glutamate aminotransferase (GABA-T, EC 2.6.1.19) and succinic semialdehyde dehydrogenase (SSDH, EC 1.2.1.16), from *Pseudomonas fluorescens*] was bought from Sigma (St. Louis, MO, USA). $NADP^+$ was purchased from Oriental Yeast (Tokyo, Japan). GABA, dithiothreitol, α-ketoglutarate and tris(hydroxymethyl)aminomethane (Tris) were obtained from Wako Chemical Co. (Osaka, Japan). de Man–Rogosa–Sharpe (MRS) mediums were bought from Difco (Detroit, MI, USA) and Oxoid Limited (Basingstoke, Hampshire, UK). *Lactobacillus brevis* NBRC 3345, 12,005 and 12,520 were purchased from the National Institute of Technology and Evaluation (Chiba, Japan). Microtiter plates (96-well, UV plate) were obtained from Corning (Corning, NY, USA). All other chemicals were of analytical reagent grade and were used without further purification.

2.2. Microtiter plate assay for GABA

A Tris–HCl buffer (80 mM, pH 9.0) containing 750 mM sodium sulfate, 10 mM dithiothreitol, 1.4 mM $NADP^+$, 2.0 mM α-ketoglutarate and GABase (30–120 μg) as a final concentration in a total volume of 90 μl was added to each well (96-well microtiter plate). Then, the standard or sample solution (10 μl) was added to the well and incubated at 30 °C for 60 min. The formation of NADPH was measured as absorbance at 340 nm with a microplate reader (VersaMax, Molecular Devices Co., Sunnyvale, CA, USA). The blank value was measured in the absence of GABase. The concentration of GABA in samples was calculated from the calibration curve of the standard solutions.

2.3. Growth of lactic acid bacteria

For the screening of GABA-producing lactic acid bacteria, *L. brevis* NBRC 12,005 and other bacteria isolated in our laboratory were grown in MRS medium at 30 °C for 1 day. The medium was filtered with a membrane filter (0.45 μm) and then stored at −40 °C until use. To produce GABA at a high concentration, *L. brevis* NBRC 3345, 12,005 and 12,520 were cultivated in MRS medium containing 0–5% sodium glutamate at 30 °C for 3 days.

2.4. GABA determination with HPLC

GABA and other amino acids were measured with HPLC (PICO-TAG amino acid analysis system, Waters, Milford, MA, USA). Samples (20 μl) were dried, by adding 20 μl of methanol–water–triethylamine (2:2:1) to each tube. Then, 20 μl of derivatization reagent [methanol–water–triethylamine–phenyl isothiocyanate (7:1:1:1)] was added to each redried sample. After the derivatization for 20 min at 25 °C, the derivatization samples were dried, by adding 100 μl of 5% acetonitrile solution containing sodium acetate to each tube. The prepared samples (5 μl) were injected onto the HPLC.

3. Results and discussion

3.1. Optimization of GABA determination

It is usually necessary to measure a large number of samples all together to find lactic acid bacteria having high productivity of GABA. GABase, which is used in the proposed method, is relatively expensive. In order to maximize GABase activity and thus reduce consumption of the enzyme in the assay, the conditions of the enzymatic reaction were optimized.

The enzymatic reaction has been accomplished in the presence of sodium sulfate [11], but its effect on the reaction has not been studied in detail. Thus, it was necessary to determine the optimum concentration of sodium sulfate to promote the enzymatic reaction. We studied the effect of the concentration of sodium sulfate on the enzymatic reaction with 10 mM GABA as a sample solution in the presence of GABase in the range of 12–120 μg/well (Fig. 1). The enzymatic reaction was finished within 30 min regardless of the concentration of sodium sulfate when GABase was used at 120 μg/well (Fig. 1A). In the presence of GABase at 30 and 60 μg/well, the rate of the enzymatic reaction depends remarkably on the concentration of sodium sulfate, and the rate was promoted with increasing the concentration (Fig. 1B and C). However, the enzymatic reaction was not finished in 60 min in the presence of GABase at 12 μg/well (Fig. 1D). These results show that sodium sulfate works as an effective activator of the enzymatic reaction and that increasing the concentration of sodium sulfate enables one to reduce the amount of GABase used in the assay.

In addition, the effect of pH on the enzymatic reaction was studied using Tris–HCl buffer (pH 7.5–9.0) in order to determine the minimum amount of GABase required in the assay. As shown in Fig. 2, the enzymatic reaction was promoted by increasing the pH and could be finished in about 30 min at pH 9.0 in the presence of GABase at 30 μg/well and 750 mM sodium sulfate. We selected a Tris–HCl buffer (pH 9.0) because the enzyme activity and the NADPH formed are unstable at higher pH [12].