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A novel assay method for theanine synthetase activity by capillary electrophoresis

Ping Li[a], Xiao-Chun Wan[a,*], Zheng-Zhu Zhang[a], Jian Li[a], Zuo-Jun Shen[b]

[a] *Key Laboratory of Tea Biochemistry and Biotechnology, Ministry of Agriculture, Anhui Agricultural University, Hefei 230036, China*
[b] *Anhui Provincial Center for Clinical Laboratory, Anhui Provincial Hospital, Hefei 230001, China*

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Abstract

The determination of theanine has been performed by micellar electrokinetic capillary chromatography (MECC) using 2,4-dinitrofluorobenzene (DNFB) as a derivative reagent. To achieve the separation, a fused-silica capillary column was used with a borax buffer at 0.03 mol/L pH 9.8 (containing Brij35 and isopropanol) at 17 °C with detection wave length at 360 nm. The factors affecting the efficiency of the sample separation were examined simultaneously. A 40-min reaction at 35 °C between L-glutamate and ethylamine (with Tris–HCl buffer, pH 7.5) was investigated using the theanine synthetase from budding tea seeds. A novel method for the analysis of theanine synthetase activity based on MECC was established. The method shows mean recovery ranged from 87.1 to 105.3% and linearity ranged from 0.2 to 5.0 mmol/L.

Keywords: Micellar electrokinetic capillary chromatography; Theanine; Theanine synthetase; Activity assay

1. Introduction

In recent years, the studies have shown that theanine has an important effect in physiology, biochemistry, pharmacology and health care [1–3]. However, the theanine is a specific amino acid in tea plants and its availability is very limited. It only accounts for about 1–2% of the dry weight of fresh tea leaves. The biosynthesis of the theanine has become increasingly important. Theanine synthetase (EC6.3.1.6), which is also referred to as L-glutamic acid (Glu): ethylamine ($EtNH_2$) ligase is a special enzyme of tea plants. It plays an important role in the theanine biosynthesis. So, it is significant to come up with an accurate, rapid detecting method for the theanine synthetase activities, which would enable the further investigation of the enzyme.

The investigations on the theanine synthetase are scarcely reported. Sasaoka et al. first discovered that the theanine synthetase was the key enzyme during the theanine biosynthesis. They created an isotopic method to assay the activity of the theanine synthetase and investigated some properties of the enzyme. Subsequently, they studied the separation and purification of the theanine synthetase and their results were not very satisfactory [4–6]. Due to the complexities of the assaying methods of the enzyme and its biochemical properties, the further study of the enzyme was limited. The molecular biological characteristics of the enzyme have been largely unknown so far.

The high performance capillary electrophoresis (HPCE) is currently one of the most important means in analysis by separation. It has been widely used in many fields for its high efficiency, speed and resolution. It is also economic, environmental friendly and highly automated [7,8]. Not only may it be used to dissociate biological products such as protein, amino acid, organic acid and sugar [9–11], but it may also be used to analyze medicines such as the antibiotics and so forth [12,13]. In addition, it has been used to detect ion [14]. The use of this technique in assaying enzyme activity further demonstrated its merits in variety of applications

* Corresponding author. Tel.: +86 551 2823795x3401; fax: +86 551 5156265.
E-mail address: xcwan@ahau.edu.cn (X.-C. Wan).

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[15–17]. Horie et al. [18] has determined qualitatively the important components containing theanine in green tea infusions using capillary zone electrophoresis (CZE). Some major organic acids and amino acids in tea infusions were analyzed simultaneously by using CZE [19]. The separation of theanine by using micellar electrokinetic capillary chromatography (MECC) was also demonstrated [20]. Chen et al. used capillary electrophoresis to determine theanine in fresh tea leaves and oolong tea [21]. In this paper, we present a different way for determining theanine using MECC, and more importantly, a novel method for assaying theanine synthetase activity based on MECC.

2. Experimental

2.1. Reagents

2,4-Dinitrofluorobenzene (DNFB), Brij35, D-norleucine, L-glutamate (sodium salt), ethylamine (HCl salt), ATP (disodium salt), polyvinyl pyrrolidone (PVP) and bovine serum albumin were the products of the Sigma Chemical Company (USA). The theanine was purchased from Tokyo chemical company (Tokyo, Japan). Acetonitrile and isopropanol were chromatographic grade made in China. Other chemicals used were made in China and of analytical grade.

2.2. Instrument and analytical conditions

A Beckman (Fullerton, CA, USA) MDQ CE Instrument was used. UV absorption was detected indirectly at 360 nm and capillary temperature was maintained at 17 °C. Samples were injected into the capillary by pressure for 5 s. An open tubular fused-silica capillary was used throughout the experiments with an internal diameter of 50 μm and a length of 65 cm (60 cm from autosampler to detector). Electropherograms were obtained with 0.03 mol/L borax buffer containing 30% Brij35 and isopropanol (borax buffer:isopropanol:30%Brij35 = 825:150:25) prepared with de-ionized water (Millipore) and adjusted to the appropriate pH 9.8 with boric acid.

2.3. Sample treatment

2.3.1. Standard theanine sample

The standard theanine sample was dissolved by de-ionized water (Millipore), with the concentration of 0.5 mmol/L.

2.3.2. Tea seedlings

Tea seeds were sterilized by a 0.05% solution of benzalkonium chloride after removal of their shells, then soaked in running water overnight and germinated on sand in the dark at 20 °C for 2–3 weeks.

2.3.3. Preparation of acetone powder

One measure of the germinated tea seedlings were grounded in a pre-chilled mortar (−20 °C) with two measures of 0.1 mol/L potassium phosphate buffer (pH 7.5), one measure of PVP, and 20 measures of pre-chilled (−15 °C) acetone. After the removal of the acetone by centrifugation at 12,000 rpm for 20 min, the residue was washed two to three more times with acetone which amounts to twice its volume. The acetone was finally removed by vacuum filtration. The resulting acetone powder was stored at −20 °C for later use.

2.3.4. Extraction of enzyme solution (operation below 5 °C)

The dried acetone power was dissolved in 40 measures of 0.05 mol/L potassium phosphate buffer (pH 7.5) containing 0.01 mol/L $MgCl_2$ and 0.01 mol/L 2-mercaptoethanol. After filtration of the dispersion though gauze, the solution was centrifuged at 12,000 rpm for 20 min. The supernatant solution was collected and ammonium sulfate was added so it reached 35% saturation. This solution was allowed to stand for 15 min. The precipitate was then removed by centrifugation (12,000 rpm for 20 min). The supernatant solution was brought to 85% neutral saturation by adding additional ammonium sulfate. After 15 min, the precipitate was collected by centrifugation described above and was dissolved in 0.05 mol/L potassium phosphate buffer (pH 7.5) used above.

2.3.5. Determination of protein concentration

Protein concentration was determined according to the method of Coomassie using bovine serum albumin as the standard sample. The fractions eluted from the chromatographic systems were also monitored at 295 nm.

2.3.6. Derivative of samples

Different derivative systems were prepared in a tube. After shaking and stirring, the mixtures were incubated at 50 °C for 25 min.

2.4. CE analyses

2.4.1. Calibration curve for theanine detection

Dissolve the standard theanine sample in de-ionized water to create 8 different concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 3.0, 4.0 and 5.0 mmol/L). Analyze them by capillary electrophoresis after the derivatives are formed. The calibration curve for the theanine detection was then obtained.

2.4.2. Assaying enzyme activity

The theanine synthetase activity was measured in 1.0 ml of an assay solution containing 25 μmoles of L-glutamate, 10 μmoles of ethylamine, 10 μmoles of ATP, 25 μmoles of $MgCl_2$, 25 μmoles of 2-mercaptoethanol, 100 μmoles of Tris–HCl (pH 7.5), and 0.4 ml enzyme. The reaction was started with the addition of the enzyme and was carried out at 35 °C for 40 min. After the incubation, the reaction was

stopped by the addition of 3 ml of 99.5% ethyl alcohol. The reaction solution was centrifuged at 12,000 rpm for 20 min. The supernatant solution was treated with capillary electrophoresis after derivative reaction. One unit of enzyme activity (U) was defined as the amount of enzyme consumed to generate 1 μmol of product per minute, under the condition of a 40-min reaction at 35 °C.

2.4.3. Evaluation of derivation recovery

Dissolve a known theanine sample in pure water to create solutions of concentrations of 0.62, 1.24 and 2.48 mmol/L. Then add standard theanine sample with the concentration of 0.70, 1.40 and 2.80 mmol/L, respectively. Stir evenly and allow derivative reaction to complete. Analyze the samples using instruments. The same procedure was repeated with the other two known theanine samples and then recovery was calculated.

3. Results and discussion

3.1. The electropherogram of samples

When theanine was mixed with other sixteen amino acids and D-norlucineis (IS) with derivative reaction, they could be isolated perfectly one by one through micellar electrokinetic capillary chromatography (Fig. 1). Peak 16 (IS) and Peak 19 (theanine) appeared at about 12.2 and 14.2 min marks, respectively. Then a suitable amount of enzyme solution was added to the enzyme reaction system. A peak appeared at about 14.2-min mark (Fig. 2), which presents the derivation of theanine. It proved that the theanine synthetase acted as a catalyst and also demonstrated the feasibility of this method for assaying the enzyme activity.

3.2. Stability of theanine derivation

The theanine derivation was evaluated under different time and conditions. The different temperatures of storing derivatives were 4 and 25 °C. In both cases, the results showed the theanine derivation maintained its stability in 5 days and

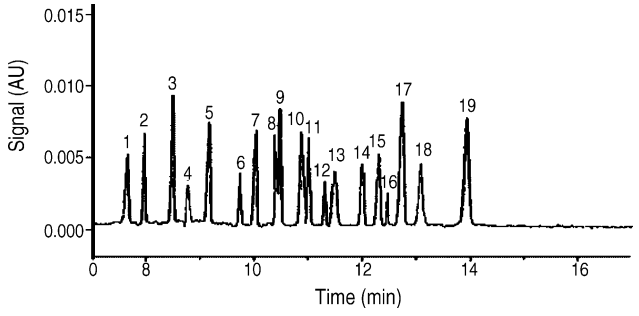

Fig. 1. Electropherogram of 17 amino acids and IS. Numbers (1–19) in this figure delegate amino acids and IS as follows: (1) Arg, (2) Tyr, (3) Lys, (4) unknown, (5) Trp, (6) His, (7) Phe, (8) Ile, (9) Leu, (10) Met, (11) Val, (12) Cys, (13) Ser, (14) Thr, (15) Pro, (16) IS, (17) Ala, (18) Gly, and (19) The.

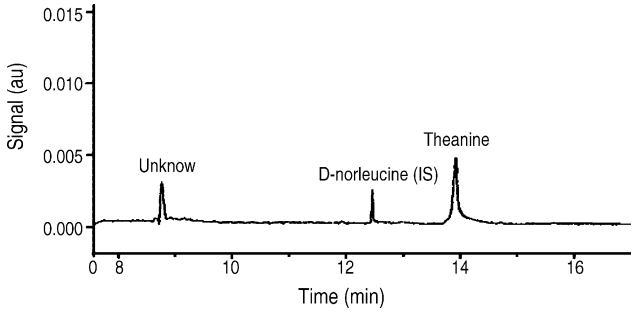

Fig. 2. Electropherogram of derivatized theanine sample.

the RSD (relative standard deviation) was 2.40 and 2.37%, respectively.

3.3. Calibration curve of the standard theanine

First, the standard theanine solution of different levels of concentration was separated by capillary electrophoresis. Then the measured values were processed at a CE workstation. The calibration curve of the standard theanine was obtained with the theanine level on the vertical axis Y and the peak area on the horizontal axis X. Linear regression was then performed to obtain the following equation: $Y = -1.26e-011x^2 + 2.25e-005x$ with $r = 0.995$, indicating a good linear relationship from 0.2 to 5.0 mmol/L. The minimal detection limit (S/N = 2) was 0.05 mmol/L.

3.4. Recovery of the derivation

In order to more accurately determine the enzyme, the recovery rates of derivation were assayed. As indicated in Table 1, the recovery rate of theanine ranged from 87.1 to 105.3%.

3.5. Results of theanine synthetase activity

Theanine synthetase activity was measured by the capillary electrophoresis and the results were shown in Table 2. Samples 1–4 came from the same source and hence contained the same enzyme solution and other reagents. However, the

Table 1
Recovery of theanine in samples

Samples (mmol/L)	Theanine standard (mmol/L)	Forecast value (mmol/L)	Actual value (mmol/L)	Recovery (%)
0.62	0.70	0.66	0.62	93.9
0.70	0.70	0.70	0.61	87.1
0.81	0.70	0.76	0.70	92.1
1.24	1.40	1.32	1.33	100.8
1.40	1.40	1.40	1.36	97.1
1.62	1.40	1.51	1.53	101.3
2.48	2.80	2.64	2.75	104.2
2.80	2.80	2.80	2.49	88.9
3.24	2.80	3.02	3.18	105.3

Table 2
The results of theanine synthetase activity

Samples	CE assay (U)
1	52.3
2	53.7
3	54.1
4	53.5
Average	53.4

four samples were put through the derivation process separately under the same experimental conditions. The resulting four derivatives were analyzed by MECC. The analysis results suggested that the enzyme activities in all four samples were consistent, which demonstrated that the method was applicable to assay theanine synthetase activity.

Many factors of the enzyme reaction system could affect the results of the dissociation of theanine using the capillary electrophoresis method. We were able to successfully eliminate all the negative factors one by one through experiments. This is due to the right selection of the derivative reagent. It also has to do with how the enzyme reaction ended. Specifically, when 99.5% ethyl alcohol was put into the reacting solution, the theanine was extracted while the remaining small amount of other residuals was precipitated.

Most of disturbance factors (for example, small molecular amino acids) in the theanine synthetase solution were removed by the prior dialysis treatment. The enzyme solution was then precipitated by 99.5% ethyl alcohol again. These procedures eliminated all factors that negatively impacted capillary electrophoretic dissociation in the reaction system. Therefore, the dissociation of the derivative was very effective.

In contrast to the methods used in Horie et al. [18–20] and Chen et al. [21], our MECC based approach has a surfactant Brij35 added to the buffer based on CZE. Then, a ball structure called micelle was formed. Adding the organic decoration agent isoproanol into the buffer may help reduce the interaction of between the dissolving matter and the micelle. It may also maintain the interaction among the surface-active agent molecules of the micelle structure and the molecules of the circulating water. Hence, it would accelerate the hromatogrhy electrokinetics, reduce electroosmosis flow (EOF), and increase the resolution.

In our experiment, we found that the effect of Brij-35 was better than sodium dodecyl sulfate (SDS) used in the previous works [18–21] in the selection buffer system of CE. The reason is that anionic surfactant SDS may increase the electroosmosis flow (EOF). The EOF is usually stronger than the electrophoretic migration of the micelle under neutral or alkaline conditions, and, therefore, the anionic micelle also travels toward the negative electrode at a retarded velocity [22].

In this paper, we presented a novel method for assaying theanine synthetase activity based on MECC. Our experiment demonstrated the simplicity and effectiveness of the method. Measuring enzyme activities using the classical isotopic method is both complicated and time consuming, whereas the prominent merits of the dissociation technology has been its simplicity and speed. It has been reported that the samples could be continuous sampling analysis [23,24]. This may further simplify the procedure of the capillary electrophoretic technology. There is no doubt that the capillary electrophoretic method could become a core technology for the dissociation studies and certainly be an efficient means to assay the enzyme activities.

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