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Determination of poly-β-hydroxybutyric acid in *Bacillus thuringiensis* by capillary zone electrophoresis with indirect ultraviolet absorbance detection

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Abstract

A new capillary electrophoresis method for determining poly-β-hydroxybutyric acid (PHB) in *Bacillus thuringiensis* was established. Poly-β-hydroxybutyric acid in samples was hydrolyzed by sulphuric acid and neutralized by $Ba(OH)_2$. The content of produced β-hydroxybutyrate was then determined by capillary zone electrophoresis (CZE) with indirect UV detection at 254 nm. With 5 m*M* *p*-hydroxybenzoate and 0.5 m*M* tetradecyltrimethylammonium bromide (TTAB) at pH 8.0 as carrier electrolyte, β-hydroxybutyrate can be determined within 6 min. Standard regression equation was made by β-hydroxybutyrate, and the linear range was 2–1000 μg/ml. The relative standard deviations (RSDs) for migration time and peak area are both less than 1.0%. The detection limit for β-hydroxybutyrate was 0.2 μg/ml, which is two to three orders of magnitude lower than that of the gas chromatography (GC) method. The capillary electrophoresis method was successfully applied to determine poly-β-hydroxybutyric acid in fermentation broth and single colony. The added standard recovery was 96%.
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1. Introduction

Poly-β-hydroxybutyric acid (PHB) is a common intracellular polymer involved in bacterial carbon and energy storage. It plays an important role in the course of metabolism [1]. PHB is similar to plastic in its physical characters. As one of the most interesting biodegradable materials, it has a promising application in medicine, material science and agriculture, etc. It has been found in activated sludge samples from conventional wastewater treatment plants [2]. Simple and reliable methods for the determination of bacterial PHB are thus needed.

Currently used methods for the determination of PHB are as follows: gravimetry [3], turbidimetry [4], spectrophotometry [5,6], infrared spectroscopy (IR)

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[7], and gas chromatography (GC) [2,8,9]. The GC method is widely used, which is based on the acid hydrolysis and transesterification of PHB with alcohol and sulphuric or hydrochloric acid. Nevertheless, the GC method requires many purification steps and is not suitable for analysis of a large number of samples [10].

Capillary zone electrophoresis (CZE) is used more and more as a standard analytical tool for many ionic compounds. The several advantages of ion analysis using CZE include good separation efficiency and resolution, fast analysis, reduced sample preparation and low runcost. Therefore, during the course of fermentative engineering research of *Bacillus thuringiensis*, we established a new method of CZE with indirect UV detection to determine the PHB content, according to the acid hydrolysis of PHB and determination the content of β-hydroxybutyrate.

2. Experimental

2.1. Reagents

Standard monomer (β-hydroxybutyric acid sodium salt) was obtained from BDH (Poole, UK), tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were purchased from Aldrich (Milwaukee, WI, USA), *p*-hydroxybenzoic acid and sodium hydroxide were analytical reagent grade chemicals.

2.2. Purification and identification of PHB

PHB was self-made by using the *Bacillus thuringiensis* gene engineering strain 833-2-1 (constructed by our laboratory [11]). The bacterial strain was cultivated on PM medium (g/l; tryptone 10, yeast extract 2, glucose 5, KH_2PO_4 1, $MgSO_4 \cdot 7H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 0.02, $ZnSO_4 \cdot 7H_2O$ 0.02, $MnSO_4$ 0.02, pH 7.2) for 16 h at 30 °C in shaken flasks (230 rpm). After centrifugation (6000 *g* at 5 °C for 5 min) with CR22G centrifuge (Hitachi, Tokyo, Japan), the wet precipitate obtained from 500 ml fermentation broth was washed three times with 200 ml of water, and one time with 100 ml of acetone. Afterwards, the wet precipitate was dried at 4 °C by Freeze Dry (Alpha I-5, Christ, Osterode, Germany). PHB was extracted and purified from the dry bacterial mass according to the methods described [4,12].

The granule obtained was white semitransparent plastic which is insoluble in water or ethyl ether but soluble in hot chloroform. The element analysis was performed on the element analyzer of model Vario ELIII (elementar, Hanau, Germany), the result was as the following: C, 55.93; H, 7.11; O, 37.04%, which was consistent with the calculated value of PHB: C, 55.80; H, 7.03; O, 37.13%. The IR spectra measured using a model 260-10 IR-Spectrophotometer (Hitachi, Tokyo, Japan) was identical with previous report [12]. The melting point of the granule was 172–174 °C, which indicated that the monomer is single β-hydroxybutyric acid. On the basis of the above results, the granule from strain 833-2-1 was identified as PHB. It was used as standard in the following experiments.

2.3. Sample preparation

A volume of 10.0 ml of fermentation broth was treated with ultrasonic at 100 W for 5 min (Sonifier 450, Danbury, CT, USA) and centrifuged (6000 *g*, 5 min). The precipitation was suspended in 10.0 ml of 10 *M* H_2SO_4 solution. Only 1.0 ml of the suspension was transferred into a ground-in test tube (10 ml). The test tube was then plugged with ground stopper tightly, and it was kept in an oven at 100 °C for 2 h. The mixture was shaken at the beginning and also during the incubator from time to time. After cooling to room temperature, it was transferred into a beaker, washed several times with 50 ml of water and transferred into the beaker, and then neutralized with $Ba(OH)_2 \cdot 10\ H_2O$ powder until pH 7.0–8.0 on a 90-2 Model stirrer of magnetic force (Shanghai Huxi Analysis Instrument Factor, Shanghai, China) with a 320-S pH meter (Mettler-Toledo, Shanghai, China). The mixture was made up to 100 ml, mixed up evenly, and filtrated with a quantitative filter paper (Hangzhou Xinhua Paper Industry, Hangzhou, China). After centrifugation (10 000 *g* for 10 min), a volume of 0.5 ml of centrifugate was analyzed automatically by the capillary electrophoresis system.

A single colony was suspended in a volume of 10.0 ml water and the bacterial suspension was then processed in the same way as fermentation broth

except that the precipitate of the bacterial cells was suspended in 1.0 ml of 10 *M* H_2SO_4 solution.

2.4. Apparatus

Electrophoresis was performed with P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA), which was equipped with a UV-detector, an autosampler and a temperature-controlled fluid cooled capillary cartridge. A computer and MDQ software (2.2 Version) were used for instrument control and for data collection and processing.

2.5. Analytical procedures

The analysis was performed in an uncoated fused-silica capillary of effective length 50 cm×75 μm I.D. (Beckman, 338454). Indirect UV detection was employed at 254 nm (anode at detector). The samples were introduced into the capillary by pressure mode (0.5 p.s.i. for 5 s). Before each injection, a 3-min purge of capillary with carrier electrolyte was programmed. The pH of the carrier electrolytes were adjusted to various values with sodium hydroxide. All carrier electrolyte solutions were filtered through a 0.22-μm syringe membrane filter (Shanghai Yadong Hitech, Shanghai, China) and all samples were centrifuged (10 000 *g* for 10 min) before they were introduced into the system.

All samples and carrier electrolytes were prepared using 18 MΩ/cm deionized water which prepared by a Barnstead EASYpure system (Sybron Barnstead, Boston, MA, USA).

3. Results and discussion

3.1. Electrophoretic conditions

The method of indirect UV detection was applied to detect β-hydroxybutyrate, which lacks UV absorption. The optimization of CZE with indirect UV detection consists of selection of a background electrolyte (BGE) with large molar absorptivity and effective mobility similar to that of the analyte anions [13–20]. Several indirect UV detection methods have been developed for the analysis of organic acids using various BGEs such as chromate [14–16,18,19], phthalate [15,16,18], pyromellitate [15,16,21], trimellitate [15], benzoate [14,15,18], *p*-hydroxybenzoate [15,19], *p*-anisate [14], mandelate [22], 2,6-naphthalenedicarboxylate [16,17,23,24], and pyridine-2,6-dicarboxylate [20]. Some of them with medium mobility were tested to be usable for the analysis of β-hydroxybutyrate. However, *p*-hydroxybenzoate is the most suitable for two reasons: (1) *p*-hydroxybenzoate has a high molar absorptivity (10 299 l mol^{-1} cm^{-1} at 254 nm) to allow sensitive indirect UV detection, since sensitivity is directly related to the molar absorptivity of the BGE [14,15]. (2) The separate efficiency and peak shape of an analyte can be affected by differences between its mobility and the mobility of the BGE [13]. Consequently, mis-matching the ionic mobilities of the BGE and sample ion can produce peak fronting or tailing [13,14,17,18,20]. The BGE anion (*p*-hydroxybenzoate) has mobility similar to that of analyte anion (β-hydroxybutyrate) as indicated by symmetrical peak shape shown in Fig. 1.

The separation efficiency was influenced by the concentration of BGE within a range from 2.0 to 10.0 m*M* only to a minor degree. The BGE with high concentrations should be used for analytes with high contents in samples. However, it would simultaneously decrease the sensitivity, slow the migration velocity, and increase the Joule heating. It showed that 5.0 m*M* *p*-hydroxybenzoate was suitable for the separation of β-hydroxybutyrate whose concentrations were less than 1000 μg/ml.

Since the migration direction of anions is contrary to that of the electroosmotic flow (EOF), a cationic surfactant such as TTAB or CTAB was added to the BGE in order to reverse the EOF allowing a short analysis time [16,17,20,24–26]. They were investigated over the concentration range from 0.25 to 1.0 m*M*. The result showed that 0.5 m*M* TTAB as the EOF modifier was more effective than CTAB.

The pH value of a carrier electrolyte is an important parameter that can be manipulated to optimize selectivity in CZE because it has significant effects on the surface characteristics of the fused-silica capillary and also influences the electric charge of both analyte and electrolyte ions. The migration behavior of a weak acid in CZE can be described by the following relationship:

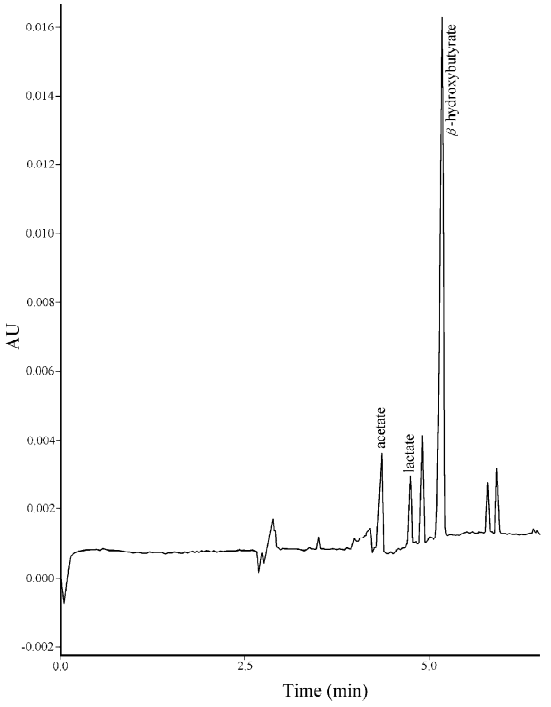

Fig. 1. Electropherogram of a fermentation broth at 16 h. Experimental conditions: carrier electrolyte: 5 m*M* *p*-hydroxybenzate with 0.5 m*M* TTAB at pH 8.0; separation voltage: −15 kV; column temperature: 30 °C; indirect UV detection at 254 nm; pressure injection: 0.5 p.s.i. for 5 s. Hydrolysis procedure and conditions were as described under Experimental section.

$$\mu = \mu_A^-(K_a/[H^+])/[1 + (K_a/[H^+])]$$

where μ is mobility at a given pH, μ_A^- is the mobility of the anionic form of the acid. β-Hydroxybutyric acid is a weak acid with pK_a value 4.41 [22,27]. Since the best separation of weak acids is achieved at pH values near to their pK_a values [22,28,29], the pH values of the carrier electrolyte (including BGE and EOF modifier) were tested within the range from 3.5 to 6.0 at first. The high separation efficiencies (exceeding 200 000 theoretical plates/m) were obtained. Unfortunately, β-hydroxybutyrate could not be completely separated with acetate (behind the large peak of β-hydroxybutyrate) at pH 4.5–5.0 and another impurity (not identified, before the large peak of β-hydroxybutyrate) at pH 5.5 in a real sample. Moreover, at a lower pH value, the migration time of β-hydroxybutyrate was long, and the ionic strength of the carrier electrolyte had a tendency to lower because *p*-hydroxybenzoate (pK_a = 4.57 [25,27]) was ionized partly.

Migration time reproducibility is dependent upon reproducible mobility which in turn is pH dependent. Therefore, the carrier electrolyte should have a good buffering capacity at the operating pH. Repetitive analyses (five consecutive injections) of a standard solution of β-hydroxybutyrate were performed at the concentration of 20.0 μg/ml. The results showed that the relative standard deviations (RSDs) for migration times and peak areas from run-to-run were high within the pH interval 3.5–6.0.

Changing the pH from 6.0 to 9.0 had no marked effect on migration times and the number of theoretical plates, for EOF changes in a narrow range and the anionic (deprotonated) form is dominant and therefore mobility is pH independent (approaches μ_A^-) at high pH ($>$pK_a + 1) [29]. The RSDs for migration times and peak areas were both less than 1.0% within the pH range from 8.0 to 9.0. So, a pH of 8.0 was selected as the optimal pH.

Separation voltage has a great influence on migration time and separation efficiency. An increase in separation voltage resulted in reduction in migration time, as illustrated in Table 1. According to the CE theory, an increased field strength leads to improved separation efficiency [28,30]. However, this could not be confirmed by the results obtained. Low

Table 1
Effect of separation voltage on migration time and separation efficiency

	Separation voltage (kV)								
	−5.0	−7.5	−10.0	−12.5	−15.0	−17.5	−20.0	−22.5	−25.0
Migration time (min)	14.8	10.0	7.5	6.0	5.2	4.3	3.8	3.2	3.0
Number of theoretical plates ($\times 10^3$/m)	181	217	230	207	203	185	173	158	134

Experimental conditions: sample is β-hydroxybutyrate (20.0 μg/ml). Other experimental conditions as in Fig. 1.

voltage (−7.5 and −10.0 kV) increased operating time but the separation efficiency was better than that of high voltage (⩾−17.5 kV) while the latter may be used for fast analysis. In this experiment, the separation voltage of −15 kV was applied. It is a compromise between analysis time and separation efficiency.

In addition, the separation efficiency can also be influenced by column temperature of capillary, current and the baseline of an electropherogram were both the most stable when the column temperature of the capillary was controlled at 30 °C.

Fig. 1 represents the separation of a real sample of fermentation broth at 16 h on PM medium under optimal separation condition.

3.2. Linearity and detection limit

For the establishment of linearity, standard solutions of β-hydroxybutyrate in various concentrations (0.1–2000.0 μg/ml, five points for each order of magnitude) were analyzed automatically under optimal conditions as mentioned above (Section 3.1). The good linear range was 2–1000 μg/ml. The regression equation was:

$$\text{Concentration}\ (\mu g/ml) = 8.646 \times 10^{-4} \times \text{Area} + 0.2490, \quad r = 0.9999$$

With the regression equation, the detection limit was estimated to be 0.2 μg of β-hydroxybutyrate per milliliter, based on a signal-to-noise ratio (S/N) of three. The detection limit is 2–3 orders of magnitude lower than that of the GC method [2,8]. In other words, the sensitivity of the method is far higher than that of the GC method. Therefore, PHB can be determined in a single colony (see below).

3.3. Detection and computing the PHB content

Hydrochloric acid or sulfuric acid solution is often used in acid hydrolysis. If PHB in samples was hydrolyzed with HCl, Cl^- is not easy to be eliminated from the hydrolysate, so the prior large peak of Cl^- seriously interferes with the detection of β-hydroxybutyrate. However, hydrolysis with H_2SO_4 and then neutralizing with $Ba(OH)_2$ can avoid the interference. The hydrolyzed conditions were with 10 M H_2SO_4 at 100 °C for 2 h. They were obtained by using standard PHB and testified to be the optimal conditions for sample preparation.

The PHB contents in fermentation broth of *Bacillus thuringiensis* 833-2-1 were determined and then calculated using a standard regression equation: $\text{Concentration}\ (\mu g/ml) = 8.571 \times 10^{-4} \times \text{Area} + 0.5274$ ($r = 0.9995$). The standard regression equation was obtained by determining β-hydroxybutyrate solutions (10.0–100.0 μg/ml) concurrently with sample assays. The hydrolytic coefficient of 0.83 must be multiplied when calculate the contents of PHB in samples. The highest content was approximate 2 mg/ml at 16 h after inoculation, and the weight of PHB was about 30% of the dried weight of the cells.

The PHB contents in single colonies on PM solid medium (PM medium plus 2% agar) could be assayed, they fluctuated from 0 to 50 μg per colony in different growth period.

3.4. Recovery

A 100-μl volume of PHB solution (10.00 mg/ml, dissolved in chloroform) was transferred precisely into a ground-in test tube. When chloroform was volatilized, one share of 10 ml of fermentation broth was treated with ultrasonic and transferred partly (10%) into the test tube and then hydrolyzed and neutralized in the same way as sample assays. The mean recovery was found with five replicates to be 95.9% ($n = 5$, RSD = 2.4%).

4. Conclusions

The method of capillary electrophoresis for determining PHB in *Bacillus thuringiensis* was established. It has a high sensitivity, a wide linear range and an excellent fidelity. This method can detect the PHB contents from fermentation broth and single colony in *Bacillus thuringiensis* directly without drying sample, so the CE method has great advantage over the GC method. It can also determine PHB from other samples. Therefore, it can be applied in bacterial metabolism and biomaterial research.

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