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Determination of spore concentration in *Bacillus thuringiensis* through the analysis of dipicolinate by capillary zone electrophoresis

Jin He[a,b], Xiaofeng Luo[b], Shouwen Chen[b,c], Lili Cao[b], Ming Sun[a], Ziniu Yu[a,b,c,*]

[a]*Key Laboratory of Agricultural Microbiology, Ministry of Education, Wuhan, Hubei 430070, China*
[b]*College of Life Science and Technology, Huazhong Agricultural University, Wuhan, Hubei 430070, China*
[c]*National Engineering Research Center of Microbial Pesticides, Wuhan, Hubei 430070, China*

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Abstract

A new capillary zone electrophoresis (CZE) method for the analysis of dipicolinic acid, a specific component found in spores but not in vegetative cells, was used to determine spore concentration in *Bacillus thuringiensis* according to the relationship between the spore concentration and the content of dipicolinate. The quantitative relationship was established by using purified spores. Electrolyte conditions that affected the separation efficiency of dipicolinate and the reproducibility were investigated. With 10 m*M* phosphate, 10 m*M* ethylenediaminetetraacetic acid and 0.25 m*M* tetradecyltrimethylammonium bromide at pH 6.2 as the carrier electrolyte, dipicolinate can be determined within 8 min at an applied voltage of −25 kV (anode at detector) and a capillary temperature of 25 °C. The method has a high separation efficiency with which the number of theoretical plates is above 300 000 plates m^{-1}. The relative standard deviations for migration time and peak area are less than 0.5% and 2.0%, respectively. The detection limit for dipicolinate was 10 ng ml^{-1}, which corresponds to $7.2 \cdot 10^5$ spores ml^{-1}. The method was used to determine spores in fermentation broths, and the results obtained agreed well with the values obtained by plate counting.

Keywords: *Bacillus thuringiensis*; Dipicolinic acid

1. Introduction

Bacillus thuringiensis is a crystalliferous spore-forming bacterium, which is used as an insecticide on a large scale. The insecticidal activity is due to its ability to produce entomocidal parasporal crystals, also known as δ-endotoxins or Cry proteins. Its spores have synergism to the insecticidal activity [1,2], so the concentration of spores is an important quality index in *B. thuringiensis* preparation. Bacterial endospore concentrations are difficult to determine. The main method of plate counting is slow, tedious and inaccurate. The aim of this work was to develop a rapid method for the determination of spores from *B. thuringiensis* through analysis of a specific component of the bacterial spores.

Pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) is a unique constituent of all endospores from

*Corresponding author. Fax: +86-27-8739-3882.
E-mail address: yz41@public.wh.hb.cn (Z. Yu).

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Bacillus and *Clostridium* species, and represents a substantial amount of the dry weight in bacterial spores (5–14%) [3]. DPA is localized in the spore core, and chelated with divalent cations [4]. It has never been detected in vegetative cells. DPA is thus often used to detect the presence of bacterial spores.

Although there are many methods available for analyzing DPA in bacterial spores [5–27], none of these has yet gained acceptance as a standard method. A colorimetric method which involves the formation of a ferrous complex of DPA is convenient but lacks sensitivity [5]. Methods involving ultraviolet spectrophotometry of the calcium complex of DPA (Ca–DPA) [6], a difference spectrum of DPA and Ca–DPA [7] and a derivative spectroscopy of Ca–DPA [8] are more sensitive, but interference by other ultraviolet-absorbing materials is a major drawback. GC [9] and HPLC [10,11] are free from interferences and are more sensitive than the spectroscopic analyses; however they require intricate sample preparation. The complex of DPA with terbium is highly fluorescent which gives a lower detection limit [12–17] than the chromatographic procedures, but the fluorimetries are also laborious and suffer the interferences which cannot be controlled easily.

In order to decrease analysis time, there has been a recent move toward analyzing the bacteria directly without extracting the DPA. The methods of UV Raman spectroscopy [18] and Fourier-transform infrared spectroscopy [19–21] have been used to differentiate between spores and vegetative bacteria. Flow cytometry [22] and pyrolysis gas chromatography–ion mobility spectrometry [23] have also been explored for the rapid detection of spores. These methods are mostly only qualitative. Mass spectrometric analyses of DPA directly from spores offer quick and sensitive methods for the detection of spores [21,24–27]; however, the equipment is not commonly available.

Capillary zone electrophoresis (CZE) has become a popular analytical tool for many ionic compounds. Several advantages of ion analysis using CZE include high separation efficiency, good resolution, fast analysis, reduced sample preparation, and low operational costs. The CZE method described here requires minimal sample preparation, and is rapid, sensitive, and free from interference.

2. Experimental

2.1. Reagents

DPA, tetradecyltrimethylammonium bromide (TTAB) and cetyltrimethylammonium bromide (CTAB) were purchased from Aldrich (Milwaukee, WI, USA). Phosphoric acid, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, and other reagents were obtained from Shanghai Chemical Reagents (Shanghai, China). The chemicals used were of analytical reagent grade. Water was purified with a Barnstead EASYpure system (Sybron Barnstead, Boston, MA, USA).

2.2. Standard solution

A standard stock solution of dipicolinate (1000 μg ml^{-1}) was prepared by dissolving 101.2 mg of DPA in 100 ml water and stored at 4 °C. The stock solution was diluted to produce working standard solutions at different concentrations.

2.3. Preparation of spores and spore suspensions

Bacillus thuringiensis recombinant strain 833-2-1 (constructed in our laboratory) was grown under the conditions as described previously [28]. Cultures were monitored for spore release from vegetative cells by microscopy. Fermentation broths were harvested when the ratios of spores to vegetative cells reached >90% as determined by microscopic visual approximation. Spores were separated from cellular debris and parasporal crystals by differential centrifugation and tandem coupling of two aqueous two-phase systems [29,30]. The purified spores were lyophilized and stored at −20 °C.

Stock suspension of spores (stored at 4 °C) of 5000 μg ml^{-1} was prepared by weighing accurately 50 mg of the pure spores and suspending them in 10 ml of water. The stock suspension was diluted with water to obtain working spore suspensions ranging from 10 to 500 μg ml^{-1} (five points for each order of magnitude).

2.4. Plate counting of spores

Spore concentrations in various working spore suspensions were determined by serially diluting them in 0.1 *M* phosphate buffer (pH 6.9) plus 0.1% Tween 80 [2]. Tween 80 was added to minimize clumping of spores and consequently improve the accuracy of spore counts. Although aggregation and incomplete germination exist, 1 c.f.u. approximately equals to 1 spore.

After heating them up to 70 °C for 15 min to kill the vegetable cells, spores in fermentation broths were counted by the same method [2].

2.5. Sample preparation

Five aliquots of 1 ml of various working spore suspensions were transferred into respective eppendorf vials (1.5 ml). They were autoclaved at 121 °C for 15 min in a HVE-50 Autoclave (Hirayama, Tokyo, Japan). After cooling, they were centrifuged at 10 000 *g* for 10 min in a 5415D Centrifuge (Eppendorf AG, Hamburg, Germany), and the supernatants were analyzed by CZE.

A fermentation broth was diluted by a factor of 10. An aliquot of 1 ml of the diluted suspension was transferred into a 1.5 ml of eppendorf vial. It was centrifuged at 6000 *g* for 5 min. The supernatant was decanted and 1 ml of water was added. Each sample was repeated in five replicates. Afterward, they were autoclaved, centrifuged and analyzed by the method described above.

2.6. Capillary electrophoresis

Electrophoresis was performed with a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA), which was equipped with a P/ACE UV detector module or P/ACE diode array detector module, an autosampler and a temperature-controlled fluid-cooled capillary cartridge. A computer and MDQ software (Version 2.3) were used for instrument control and for data collection and processing.

The analysis was performed in an uncoated fused-silica capillary of effective length 50 cm×75 μm I.D. (Beckman Coulter, Fullerton, CA, USA). UV detection was employed at 200 nm (anode at detector). The samples were introduced into the capillary by pressure mode (1.0 p.s.i. for 10 s; 1 p.s.i.= 6894.75 Pa). Before each injection, a 3 min purge of capillary with carrier electrolyte was programmed. 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.

3. Results and discussion

3.1. Electrophoretic conditions

DPA can not be availably separated by CZE with a buffer such as phosphate, borate, or Tris along with an electroosmotic flow (EOF) modifier (TTAB or CTAB) at any pH value. However, it was found that DPA can be effectively separated by adding EDTA as a modifier to the carrier electrolyte composed of phosphate and TTAB only under a narrow pH range and special concentration ranges of phosphate, EDTA and TTAB. So, the carrier electrolyte primarily consisted of 50 m*M* phosphoric acid, 10 m*M* EDTA, and 0.5 m*M* TTAB. Moreover, sodium hydroxide was added to the carrier electrolyte to adjust the pH value. Reproducibility in migration time and peak area from run-to-run is very important in CZE analysis. Parameters that affected the separation efficiency and reproducibility were investigated respectively, including the pH of the carrier electrolyte, ionic strength of phosphate, concentrations of EDTA and TTAB, applied voltage and column temperature. With the diode array detector, detection sensitivity was found optimal at the absorbance maximum of 194 nm (Fig. 1a) [31], but fixed wavelength detection at 200 nm with the UV detector resulted in only a 12% reduction.

The pH value of a carrier electrolyte is an important parameter that can be manipulated to optimize the selectivity in CZE because it has a significant effect on the surface characteristics of the fused-silica capillary and also influences the electric charge of analyte. DPA is a weak acid with pK_1 2.16 and pK_2 6.92 [31–33]. Since the best separation of a

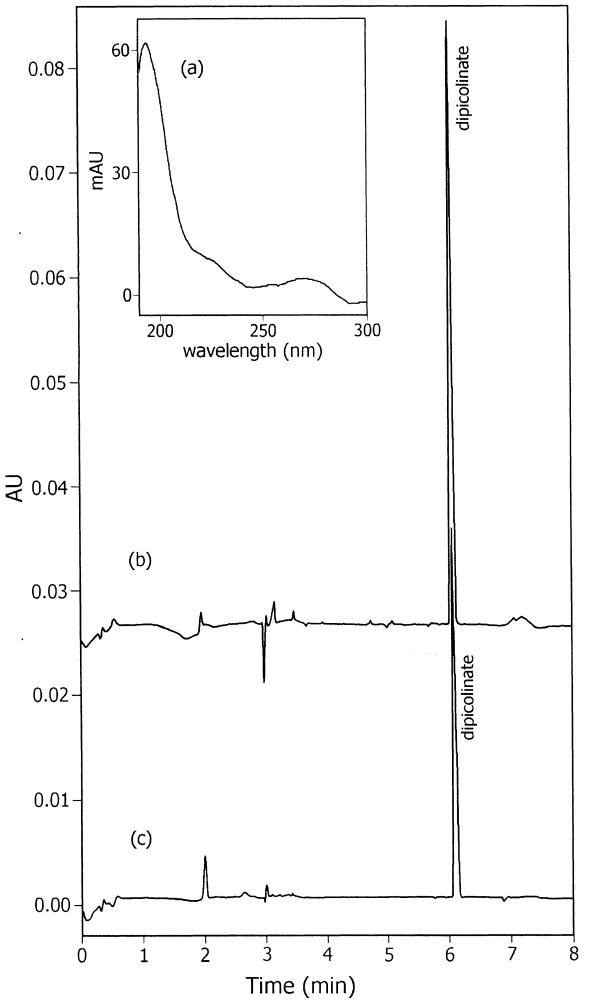

Fig. 1. Electropherograms of a diluted fermentation broth (b) and a working spore suspension (c). Experimental conditions: carrier electrolyte: 10 m*M* phosphate, 10 m*M* EDTA, 0.25 m*M* TTAB at pH 6.2; separation voltage: −25 kV; temperature: 25 °C; UV detection: 200 nm; pressure injection: 1.0 p.s.i. for 10 s. Sample preparation was described in the Experimental section. Dipicolinate in (b) and (c) were 13.70 μg ml^{-1} and 8.85 μg ml^{-1}, respectively. Insert (a) is the spectrum of dipicolinate in the carrier electrolyte, which is obtained by the diode array detector in the sample (b) assay.

weak acid is achieved at a pH near to its pK_a [28,34], the pH values of the carrier electrolytes were at first tested with the range from 2.0 to 7.5. A better separation with the number of theoretical plates of 230 000 was obtained at pH 6.0.

Changing the pH values from 5.2 to 6.4 had a remarkable effect on separation efficiency. Although the separation efficiency was not the highest, the best reproducibility of both migration time (<0.5%) and peak area (<1.0%) and the best baseline were obtained at pH 6.2; so this was selected as the optimal pH.

With 10 m*M* EDTA, 0.5 m*M* TTAB, and phosphate at various concentrations as a carrier electrolyte (pH 6.2), the number of theoretical plates decreased from 232 000 to 203 000 plates m^{-1} when the concentrations of phosphate increased from 5.0 to 50.0 m*M*. The RSDs for migration times and peak areas were lower than 0.5 and 1.5%, respectively, in the concentration range. In general, a higher ionic strength had better buffering capacity, resulting in good precision. However, when the concentration was higher than 75 m*M*, the separation efficiency and reproducibility were greatly lowered by the Joule heating produced. While the concentration was at 10 m*M*, the baseline drift was almost negligible. So, the concentration of phosphate at 10 m*M* seemed to be an acceptable compromise.

Since the migration direction of anions is contrary to that of the EOF, a cationic surfactant TTAB was added to the carrier electrolyte in order to reverse the EOF allowing a short analysis time [33,35]. It was tested over the concentration range from 0.1 to 0.6 m*M*. The result showed that 0.25 m*M* TTAB as the EOF modifier was more effective. Nevertheless, efficient separation cannot be achieved if the EOF modifier was replaced by CTAB.

DPA was difficult to separate, as a broadening and tailing peak was observed in phosphate buffer with TTAB in our early experiment and also in another study [33], although the study was to develop an analysis method for Cr (III) and Cr (IV). The cause was probably related to the interaction between dipicolinate and the wall of the capillary. Furthermore, the determination of dipicolinate in real samples was complicated. Because it has the characteristic to bind divalent cations, multiple peaks and incomplete recoveries were thus yielded. However, a sharp and symmetrical peak can be obtained by adding EDTA as a modifier to the carrier electrolyte. There are two possible reasons: (1) avoid the interaction; (2) replace and release free dipicolinate from the chelates with cations, because the formation constants [32] of EDTA with cations are higher than

that of DPA with cations. For example, log K_1 of Ca–EDTA is 11.0, while log K_1 of Ca–DPA is 4.6. The effect of different concentrations of EDTA on separation efficiencies were studied in the concentration range from 5 to 15 m*M*. As a modifier, the higher concentration can get better separation efficiency, but it would simultaneously lower the sensitivity because of its UV absorbance. The result showed that the concentration of 10 m*M* was appropriate for sample analysis.

From the above results, the best separation (theoretical plates >300 000 plates m^{-1}) was obtained with an electrolyte containing 10 m*M* phosphate, 10 m*M* EDTA and 0.25 m*M* TTAB at pH 6.2.

In addition, the separation efficiency can be influenced by separation voltage and capillary temperature. The best separation was obtained with the voltage set at −25 kV and the capillary kept at 25 °C.

3.2. Validation of the CZE method

For the establishment of a standard curve, standard solutions of dipicolinate at various concentrations (0.05–50.00 μg ml^{-1}, five points for each order of magnitude) were analyzed under optimal conditions as mentioned above. The regression equation was: concentration (μg ml^{-1}) = $5.878 \cdot 10^{-5} \cdot$ area + 0.003719, r = 0.9996.

The detection limit of dipicolinate was determined by injecting a low concentration of dipicolinate solution to produce a signal-to-noise ratio (S/N) of 3.

The recovery of dipicolinate from fermentation broth was determined by the addition of a known concentration standard solution. The analytical procedure was as described in the sample assays except that 1 ml of water was replaced by 1 ml of 2.0 μg ml^{-1} dipicolinate standard solution. The average percent recovery was 102.9% (n = 5, RSD = 2.65%), which indicates that the CZE method had a good accuracy.

3.3. Determination of spores

Dipicolinate can be extracted from spores by physical, germination, and chemical methods [17]. Among them, heating at 100 °C in a buffer [10] and autoclaving at 121 °C [5,6,11] were often used. The incomplete extraction of dipicolinate and the excessive extraction of other UV absorbing compounds from samples was achieved by heating at 100 °C in a phosphate buffer [10]. Nevertheless, few other UV absorbing anions were observed except for dipicolinate by extraction with autoclaving. The relative contents of dipicolinate extracted at 121 °C for 10, 15, 20, 25, and 30 min are 98.7%, 100.0%, 98.6%, 99.0%, and 99.5%, respectively. The trial showed that 15 min was adequate for complete extraction.

DPA is an invariable constituent in the spores of a bacterial species. In order to determine bacterial spores by CZE, the relationship between spore concentration and dipicolinate content must be established. In general, the quantitative relationship can be found directly by using a fermentation broth. However, the personal error of plate counting was important with a fermentation broth. In order to lower the error, the pure spores were used. The linearity relation between spore concentration (y, c.f.u./ml) and dipicolinate content (c.f.u. ml^{-1}) in the serial working spore suspensions was displayed as the following:

$$y = 7.175 \cdot 10^{7} \cdot x - 3.368 \cdot 10^{4},\ r = 0.9954$$

In brief, 1 μg ml^{-1} dipicolinate corresponds to $7.2 \cdot 10^{7}$ spores ml^{-1}.

The dipicolinate content was found to be 8.6% of spore dry mass by using a working spore suspension of 102.7 mg ml^{-1} (Fig. 1c).

A volume of 10 ml of fermentation broth was diluted to 100 ml. The diluted suspension was then determined and the dipicolinate content was 13.70±0.23 μg ml^{-1} (Fig. 1b). The spore concentration of the fermentation broth should be $9.9 \cdot 10^{9}$ according to the regression equation. The spore concentration, which was determined simultaneously by plate counting, was $(9.2 \pm 2.3) \cdot 10^{9}$. These results agreed with each other. However, the precision of plate counting was very low (RSD = 25.3%, n = 10). So, the CZE method was more accurate than the plate counting.

The CZE method has a high separation efficiency. However, the small dimension of the separation system and the small injection volume restrict its sensitivity. The detection limit of 0.01 μg ml^{-1} dipicolinate corresponds to $7.2 \cdot 10^{5}$ spores ml^{-1} determined by plate counting. Previous to this work,

the lowest detection limits reported for bacterial spores by a DPA assay were $7 \cdot 10^5$ spores ml^{-1} (*B. cereus*) [9], $4.4 \cdot 10^5$ spores ml^{-1} (*B. subtilis*) [13], $1.2 \cdot 10^5$ spores ml^{-1} (*B. globigii*) [14], and 10^4 spores ml^{-1} (*B. subtilis*) [15]. Certainly, the detection limit can be further decreased by simply increasing the injection quantity (volume or pressure) or applying on-line sample pre-concentration methods, such as field amplification injection [36] and isotachophoresis [37]. However, the sensitivity was high enough for the determination of spores in the fermentation broths.

4. Conclusions

A CZE method for the determination of spores in *Bacillus thuringiensis* was established. It is rapid, sensitive and accurate. It is expected that the method can be used to determine spore concentrations in other *Bacillus* species. DPA analysis is of importance in studies of sporulation, dormancy, resistance and germination. Therefore it can be also applied in biochemical areas.

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