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Short communication

Quantitative analysis of thuringiensin by micellar electrokinetic capillary chromatography

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Abstract

A method for quantitative analysis of thuringiensin is established using micellar electrokinetic capillary chromatography (MEKC). Tryptophan was used as an internal standard. The running buffer contained phosphate–borate (20 m*M*) and sodium dodecyl sulfate (100 m*M*) with 10% acetonitrile. Electrophoresis was conducted under a voltage of 15 kV for 20 min. The peak area ratio of thuringiensin to tryptophan was calculated from the resultant electropherogram. Results indicated a high correlation between the concentration of thuringiensin and the peak area ratio of thuringiensin to tryptophan. The general equation of linear regression line is very similar to the equation obtained from HPLC results established previously in our laboratory. However, MEKC is a faster, simpler and less expensive assay than the traditional HPLC method.

Keywords: Thuringiensin; Toxins; Tryptophan; Pesticides

1. Introduction

Thuringiensin is a β-exotoxin, derived from the bacterium *Bacillus thuringiensis*. This exotoxin is heat-stable and its chemical structure is similar to that of nucleotides (Fig. 1). It is especially effective as a fly control and often has been referred to as ''fly factor''. The mechanism of insecticidal action is to inhibit the production of DNA-dependent RNA polymerase by competition with ATP [1]. This toxic action generally applies to some orders of insects, such as Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Neuropera, Hemiptera and Acari in the families *Tetranychidae* and *Phytoseiidae* [2–5]. The toxicity of thuringiensin is much less than that of most chemical insecticides, therefore it shows great potential to become a very useful insecticide for a wide range of insects. Recent studies have shown that the production of thuringiensin could be improved by a net-draft-tube modified airlift reactor

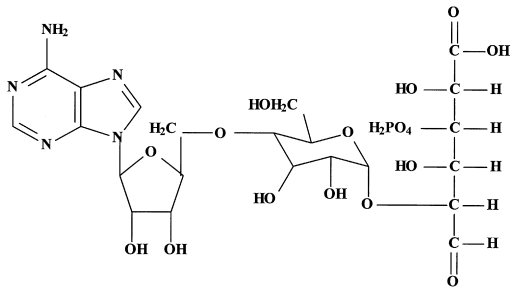

Fig. 1. Structure of thuringiensin (β-exotoxin) from *Bacillus thruingiensis*.

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[6]. The production of thuringiensin can be even further improved by a fed-batch culturing of *Bacillus thuringiensis* in the modified airlift reactor [7]. During the course of fermentation, penicillin-G may enhance the production [8]. These findings make thuringiensin more practical for mass production.

The insecticidal effect of thuringiensin can be assessed by bioassay. However, bioassay is an expensive and time-consuming method. In addition, many factors may affect the accuracy of the bioassay, such as the age of the test insects and the environmental conditions. Several investigators have reported that use of high-performance liquid chromatography (HPLC) may substitute the conventional bioassay [9–12]. Hsu et al. [13] demonstrated that results from bioassay and HPLC are strongly correlated. They monitored the concentration of thuringiensin during the course of fermentation by bioassay and HPLC in a side-by-side study.

Capillary electrophoresis (CE) is a new analytical technique, which provides a simple and rapid analysis with high efficient separation [14]. However, for some neutral hydrophobic molecules, this basic mode requires modification. Terabe et al. [15] demonstrated a method to separate neutral hydrophobic molecules by so-called micellar electrokinetic capillary chromatography (MEKC). The basic separation mechanism is based upon the interaction between analytes and micelles.

In this research, the MEKC mode of CE was used. Under optimized conditions a running buffer containing 0.1 *M* sodium dodecyl sulfate (SDS) was used for thuringiensin analysis. In this report, quantitative analysis of thuringiensin by HPLC and MEKC are compared. The HPLC procedure was described previously [16]. Adenosine monophosphate (AMP) was used as the internal standard for the quantitative HPLC method. In this report, tryptophan was used as the internal standard for MEKC. The results indicate that internal standard may provide a consistent and accurate method to measure the concentration of thuringiensin in the solution. The concentration of thuringiensin is correlated with the peak area ratio of thuringiensin to nucleotide peak area. From the general equation of linear regression, the concentration in broth or in semi-purified product can be calculated. Linear regression lines from HPLC and MEKC were calculated. MEKC provides a faster, simpler and less expensive assay than HPLC.

2. Experimental

2.1. Materials

Potassium phosphate, phosphoric acid, boric acid and sodium hydroxide were purchased from Katamaya (Osaka, Japan). SDS was purchased from Merck (Darmstadt, Germany). Acetonitrile was purchased from Mallinckrodt Baker (Paris, KY, USA). Thuringiensin reference standard was provided by Dr. H. De Barjac of the Institute Pasteur (Paris, France). Thuringiensin broth was obtained from Dr. W.T. Wu, National Tsing Hua University.

The broth was partially purified by high-speed centrifugation (7500 *g* for 30 min), and passed through a 0.45-μm membrane filter. For further purification, the thuringiensin was recovered by using a ''micellar-enhanced ultrafiltration'' method [12]. Both the semi-purified and purified thuringiensin were subjected to MEKC quantitative analysis. A portion of the purified thuringiensin was preserved by lyophilization as a standard specimen for future study. The amount of thuringiensin in the standard specimen (lot 2) is 67 mg per 1 g solid powder. It has been determined by comparison with the purified thuringiensin from the Institute Pasteur.

2.2. Apparatus

MEKC was performed on a P/ACE system Model 2100 (Beckman Instruments, Fullerton, CA, USA) controlled by System Gold software (Beckman). UV absorbance was monitored with a fixed-wavelength detector at 254 nm. Capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA). The dimensions of capillary were 47 cm×50 μm I.D. without coating. Samples were diluted with 20 m*M* phosphate and 20 m*M* borate buffer, pH 9.2 and injected by 10 s positive pressure. After injection, 15 kV potential was applied for electrophoresis.

2.3. Buffer solution and sample preparation

MEKC running buffer was prepared by adding

1.24 g boric acid and 7.16 g disodium phosphate into 850 ml of deionized distilled water. After complete dissolving 28.84 g of SDS was added, and the pH was then adjusted to 9.2 by 1 *M* sodium hydroxide. The final solution was then adjusted to a total volume of 900 ml with deionized distilled water. Acetonitrile, 100 ml, was added and mixed completely. The final buffer contained 20 m*M* boric acid, 20 m*M* disodium phosphate and 100 m*M* SDS, with 10% acetonitrile.

MEKC sample dilution buffer was prepared by adding 7.8 g potassium dihydrogenphosphate into 900 ml of deionized distilled water. After complete dissolving, the pH of the buffer solution was adjusted to 2.8 by phosphoric acid. Adjustment of the final volume to 1000 ml was then done by deionized distilled water.

The internal standard for MEKC was prepared by weighing 102.1 mg of tryptophan with 100 ml of sample dilution buffer. Which yielded a final concentration of 5 m*M*.

2.4. Procedure

Conventional HPLC was used as a reference method comparison with MEKC. In the MEKC experiment, 1 ml of tryptophan internal standard solution was mixed with an equal volume of thuringiensin solution containing varied amounts of thuringiensin (50.0, 25.0, 12.5 or 6.25 mg/ml). The mixtures were then subjected to a 10 s pressure injection. The electrophoretic field strength was 500 V/cm. The peak area ratios were integrated from the final electropherograms. To verify their reproducibility, each concentration was run four times. The resultant electropherograms were analyzed by SYSTEM GOLD software. The correlation between the ratio of peak area and the concentration was calculated by EXCEL software.

3. Results and discussion

MEKC with SDS was used for the analysis of normal and chemically modified nucleosides and nucleotides [17,18]. The running buffer was 20 m*M* sodium dihydrogenphosphate containing 100 m*M* SDS, pH 7 or 9.2, with or without acetonitrile. Sixteen nucleic acids and their analogues were separated in 25 min. In this report, the same running buffer with acetonitrile was chosen.

For quantification of thuringiensin, tryptophan was chosen as an internal standard. Tryptophan is an aromatic amino acid with strong UV absorption at 254 nm. The mobility of tryptophan is different from that of thuringiensin under the running conditions. A fixed volume of tryptophan (5 m*M*) was mixed with an equal volume of thuringiensin standard solutions (with varying amounts of thuringiensin) (Fig. 2). The thuringiensin peak and tryptophan peak appeared at 9–10 and 16 min, respectively, on the electropherogram. The peak area ratio between thuringiensin and tryptophan was calculated by SYSTEM GOLD. The correlation between the concentration of thuringiensin and peak area ratio was calculated by EXCEL. The results showed a good correlation coefficient ($r=0.9997$, $y=0.7854x-0.0119$) between two parameters. The standard deviations of each quadruplicate run are represented by a vertical bar at each point (Fig. 2).

Two examples for demonstrating this quantitative analysis of thuringiensin are described in the following. A new batch of fermentation broth was centrifuged and passed through a 0.45-μm pore size membrane filter to remove solid particles from the broth. An equal volume of standard tryptophan solution was added to the filtered solution. The mixture was then subjected to CE quantitative analysis. Fig. 3 (upper panel) shows the electropherogram of the mixture. In this electropherogram, the peak area ratio of thuringiensin to tryptophan was obtained by SYSTEM GOLD software (peak ratio=1.277). From this ratio the concentration of thuringiensin in the solution was calculated by the equation of linear regression. The concentration of thuringiensin in the broth was calculated as 1.641 mg/ml. Because this concentration is out of the linear range of the calibration curve (0.1–0.6 mg/ml), the sample was diluted with phosphate buffer and confirmed that the peak area ratios and concentration are well correlated by linear regression analysis. The limited linear range of calibration has been restricted by the concentration of thuringiensin in second standard specimen. The other example was the amount of thuringiensin estimated in a purified and lyophilized thuringiensin powder. Fifty mg of powder was

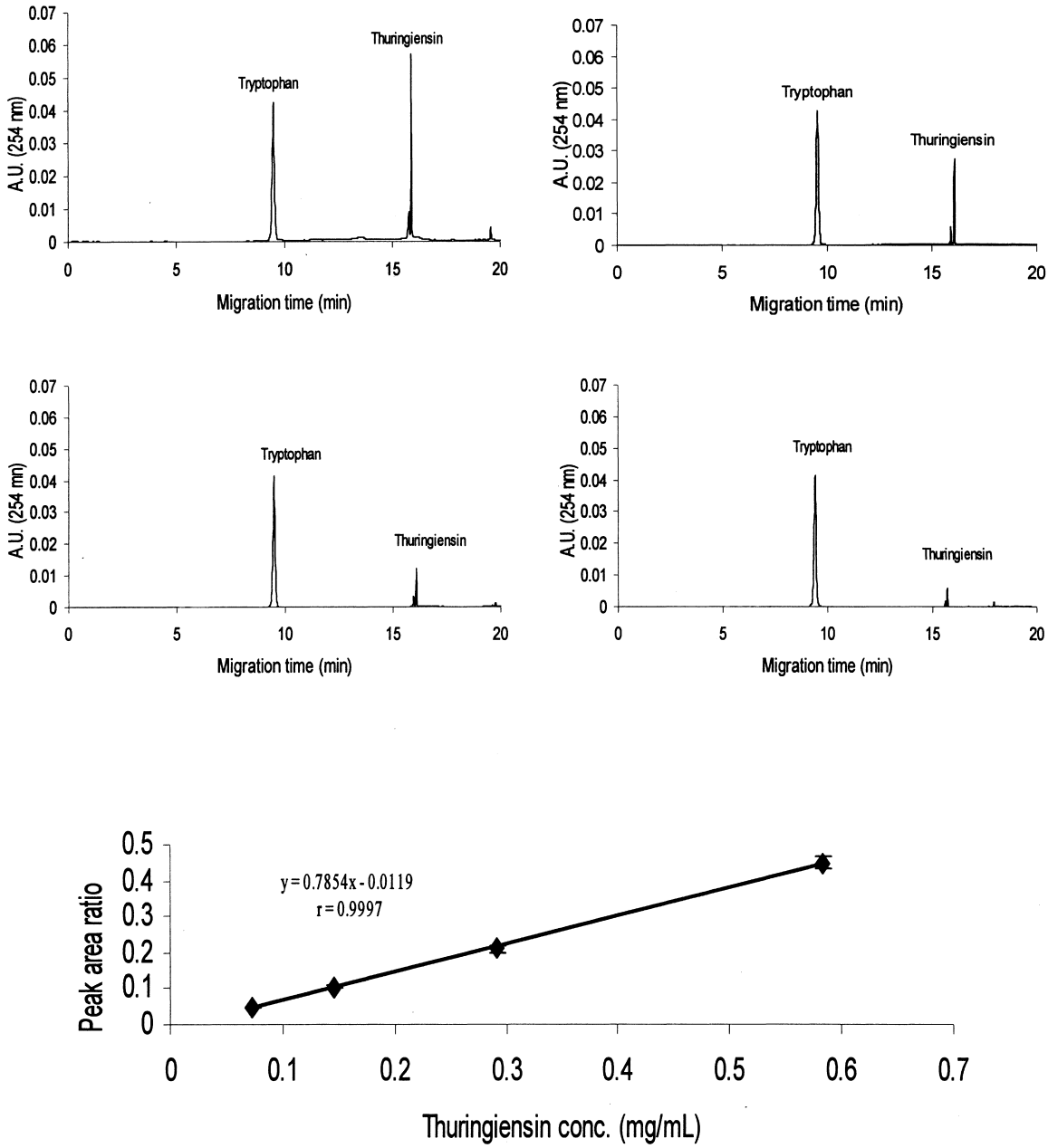

Fig. 2. Electropherograms (upper panel) from four concentrations of standard thuringiensin specimens (top left panel: 25 mg/ml, top right panel: 12.5 mg/ml, bottom left panel: 6.25 mg/ml and bottom right panel: 3.13 mg/ml) were mixed with a fixed amount of tryptophan (5 m*M*). The samples were introduced by 10 s pressure injection. The electrophoresis buffer is phosphate–borate buffer with 100 m*M* SDS and 10% acetonitrile. The peaks have been identified by spiking the purified tryptophan or thuringiensin (bottom panel). The relationship between the thuringiensin concentration and the peak area ratio of thuringiensin to tryptophan is represented on a linear regression curve. The vertical bars represent the standard deviation from each quadruplicate run.

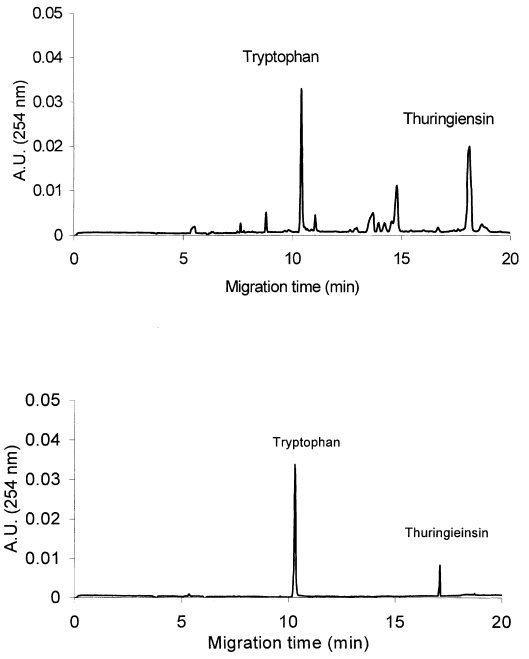

Fig. 3. Electropherograms of the fermentation broth (upper panel) and the purified thuringiensin (lower panel) were mixed with a fixed amount of tryptophan (5 m*M*). The same method was used as described in Fig. 2.

dissolved in 1 ml of 0.3 *M* phosphate buffer (pH 7.0). After complete dissolving, the solution was passed through a disc membrane filter. The filtered solution was then mixed with an equal amount of tryptophan solution. The amount of sample in the mixture was diluted to half, that is, 25 mg/ml. The mixture was subjected to CE quantitative analysis. The peak area ratio of thuringiensin to tryptophan was equal to 0.1352. From this ratio (Fig. 3, bottom panel), the concentration of thuringiensin in this solution was calculated by the equation of linear regression and found to be equal to 0.1873 mg/ml. This indicates that every 25 mg of sample only contains 0.1873 mg of thuringiensin. The purity of the lyophilized powder is equal to 0.1873 mg/25 mg or 7.49 mg/g.

Comparing this method with the conventional HPLC method which was conducted in our laboratory [16], the two methods show similar results. However, MEKC proves a faster, simpler and less expensive method than HPLC. In addition, MEKC uses a much smaller sample volume HPLC. Linear regression analysis from the results of the two methods showed different slopes and intercepts ($y=0.0281x+0.064$ in HPLC vs. $y=0.7854x-0.0119$ in MEKC). This discrepancy is due to different internal standards and second standard specimens were used in two methods. Overall, the regression analysis showed that the results from the MEKC method are better than the HPLC method in terms of correlation coefficient and intercept.

4. Conclusion

The use of tryptophan as the internal standard for MEKC to quantify the amount of thuringiensin has been established. Tryptophan is a common chemical with relatively high purity and stability. It is an aromatic amino acid with relative strong UV absorption at 254 nm. Because the internal standard was mixed with thuringiensin sample in fixed amounts, the peak area ratio is always constant regardless of the variation of the amount injected. In addition, the purified thuringiensin is very difficult to prepare, and the stability of purified thuringiensin makes it even more difficult to use it in assessment for fermented broth or semi-purified product. In this study, we found that tryptophan is suitable as an internal standard for MEKC. The amount of tryptophan in fermented broth or purified solution was negligible. Tryptophan was moving at different speeds from thuringiensin in MEKC. This equation of linear regression is very similar to our previous report using HPLC with AMP as an internal standard. From these equations, the amount of thuringiensin in the sample can be estimated from MEKC by calculation of peak area ratio. MEKC provides a faster, simpler and less expensive method than HPLC.

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