Quantitative Analysis of Thuringiensin by High-Performance Liquid Chromatography Using Adenosine Monophosphate as an Internal Standard

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Abstract

An analytical method for thuringiensin using adenosine monophosphate (AMP) as an internal standard is established. AMP, with high stability and availability, is an appropriate internal standard for thuringiensin quantitative analysis using high-performance liquid chromatography and ultraviolet absorbance detection at 260 nm. A good correlation between the concentration of thuringiensin and the peak-area ratios of thuringiensin to AMP is demonstrated. From this general equation of linear regression line, the concentration of thuringiensin can be assessed in fermentation broth or semi-purified product.

Introduction

Thuringiensin is a microbial pesticide derived from the bacterium *Bacillus thuringiensis*. This bacterial agent is a heat-stable β-exotoxin especially effective in the control of flies; therefore, thuringiensin is often referred to as "fly factor" (1). In general, thuringiensin is toxic to insects in the orders Coleoptera, Diptera, Hymenoptera, Isoptera, Lepidoptera, Orthoptera, Neuorptera, Hemiptera, and Acari in families *Tetranychidae* and *Phytoseiidae* (2–4). This bacterial exotoxin exhibits much less toxicity to humans than most other chemical insecticides. Recently, some investigators found that the production of thuringiensin can be enhanced by adding penicillin-G (5) or using a modified airlift reactor (6). Purification of thuringiensin can be achieved by micellar-enhanced ultrafiltration (7), and the insecticidal activity could be monitored using high-performance liquid chromatography (HPLC) (8). Because the quantity of purified thuringiensin in each fermented broth may vary from lot to lot, the quantitation of thuringiensin becomes an important factor in the prediction of the insecticidal activity and the final formulation of product.

This study was conducted using adenosine monophosphate (AMP) as an internal standard to assess the concentration of thuringiensin in each lot of broth. Thuringiensin and AMP have very similar chemical and physical characteristics, including structure, ultraviolet (UV) absorbance, and absorptive coefficient (9,10). The retention time of AMP is shorter than that of thuringiensin. This may be caused by either the partition constant or molecular size during reversed-phase HPLC. The concentration of AMP can be standardized by spectrophotometry. Using AMP as an internal standard, it is possible to determine the concentration and stability of each lot of thuringiensin.

Experimental

Chemicals and reagents

Analytical-grade potassium phosphate and phosphoric acid were purchased from Katamaya Chemical Company (Osaka, Japan). AMP, adenosine diphosphate (ADP), and adenosine triphosphate (ATP) were purchased from Sigma (St. Louis, MO). 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 of National Tsing Hua University. The solid particles of thuringiensin broth were removed by high-speed centrifugation (7500 × *g* for 30 min). The precipitate was then removed by first passing it through a 0.45-µm polycarbonate membrane (Amicon, Beverly, MA) and then passing it through a second 0.22-µm filter (Amicon). The thuringiensin was recovered using a micellar-enhanced ultrafiltration method (7). The purified and filtered thuringiensin was subjected to quantitative analysis. A portion of the purified thuringiensin was preserved by lyophilization as a standard specimen for future study.

Buffer solution and sample preparation

Phosphate buffer solution (50mM) was prepared by dissolving 13.6 g potassium dihydrogen phosphate into 2000 mL distilled and deionized water. The solution was sonicated using

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phosphoric acid to adjust the pH to 2.8. Two lots of standard specimens were dissolved in phosphate buffer and filtered with a 0.22-µm disc filter (Amicon). The clear solution was subjected to UV spectrophotometric and HPLC analysis.

Either AMP, ADP, or ATP (100 mg) was dissolved in 50 mL phosphate buffer as stock solution. After complete dissolution, the solutions were further diluted to 20-, 40-, and 80-fold with phosphate buffer. The diluted solutions were ready for UV spectrophotometric and HPLC analysis.

Procedure

For UV spectrophotometric analysis, all diluted nucleotides and thuringiensin were scanned for their UV light absorbance using a spectrophotometer set at a wavelength of 220–320 nm.

For HPLC analysis, a Hitachi HPLC workstation (Tokyo, Japan) consisting of a model 7400 UV detector (260 nm), an L-7100 pump HPLC workstation, and a reversed-phase C-18 µ-Bondapak column (30 cm × 3.9-mm i.d., 5-µm particle size) equipped with a 20-µL sample loop was used. A 20-mL aliquot of diluted AMP (25 µg/mL) was mixed with equal volumes of thuringiensin (50.0, 37.5, 25.0, or 12.5 mg/mL). Thus, the final concentration of each ingredient was only half of the original. The mixture was injected directly by a Rheodyne injector. Runs were performed at 25°C in 50mM KH_2PO_4 (pH 2.8) at a flow rate of 1.5 mL/min for the first 15 min, then shifted to 2 mL/min. The resultant chromatograms were analyzed by SISC software (Scientific Information Service Corporation, Taipei, Taiwan). The correlation between concentration and peak-area ratio was calculated by EXCEL software (Microsoft, Redmond, WA).

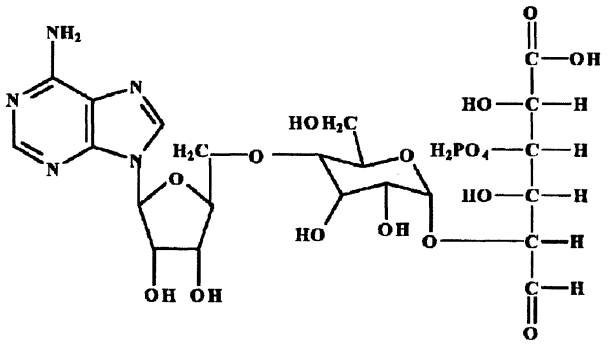

Figure 1. Structure of thuringiensin (β-exotoxin) from *Bacillus thuringiensis*.

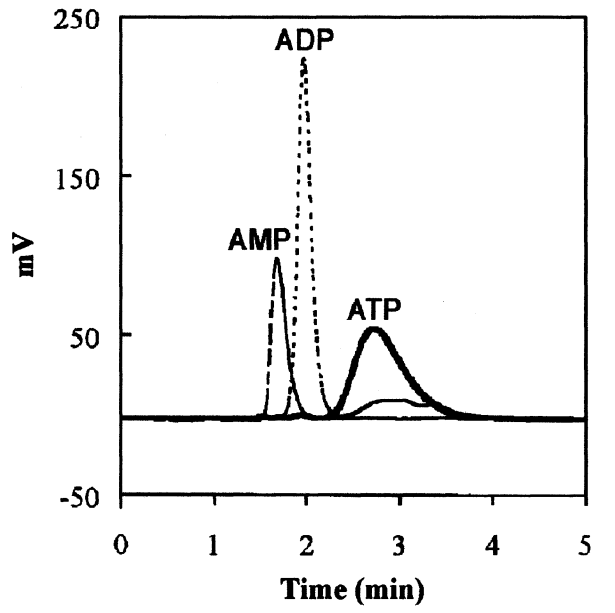

Figure 2. The HPLC chromatogram of AMP (50 µg/mL), ADP (100 µg/mL), and ATP (100 µg/mL). The samples were dissolved in phosphate buffer (50mM, pH 2.8) at a flow rate of 2 mL/min with detection at 260 nm. AMP showed a single and sharp peak with the shortest retention time.

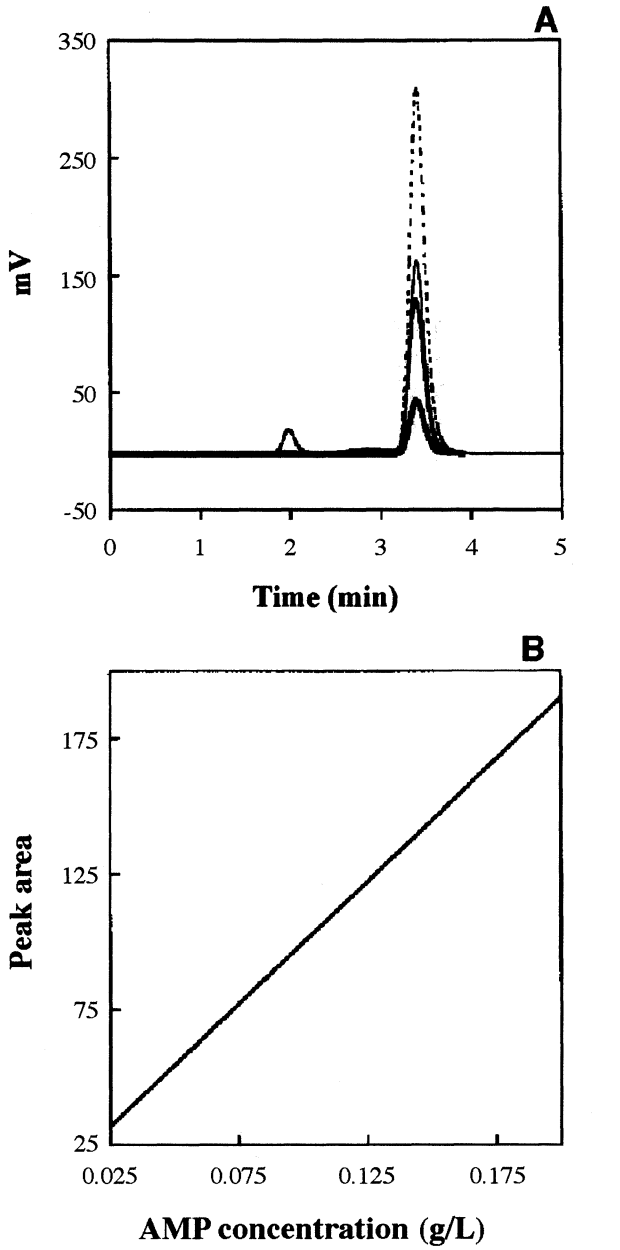

Figure 3. The HPLC chromatogram of 200, 100, 50, and 25 µg/mL AMP (A). The multiple regression analysis (B) showed that the concentration of AMP was well correlated with the peak area (r^2 = 0.9992; slope, 1.10; and intercept, 1.007).

Results and Discussion

Thuringiensin is a nucleotidic ATP analogue (11) with a molecular weight of 701 daltons. The chemical structural (Figure 1) and physical properties of thuringiensin are very similar to AMP. The absorption spectra of three nucleotides and two thuringiensin specimens were scanned using a UV spectrophotometer at wavelengths from 220 to 320 nm. Among the three nucleotides, AMP exhibited the highest molar absorption coefficient (ε_{260} = 16170), whereas ADP and ATP exhibited ε_{260} values of 13632 and 13895, respectively. Suortii et al. (12) used the adenosine nucleotide absorption coefficient as an index to estimate the purity of thuringiensin. The absorption coefficient described here is nearly the same number they used. The molar absorption coefficient of the second lot of standard specimen was approximately half of the absorption coefficient of this nucleotide. The first lot of standard specimen was even lower with approximately one-third of the absorption of nucleotide. These results indicated the standard specimens were still contaminated with some impure substance. Because the second lot possessed relatively more pure thuringiensin, it was used for the following experiments.

In HPLC analysis, the resultant chromatogram (Figure 2) showed that AMP not only possessed the shortest retention time (less than 4 min) but also a sharper peak than either ADP or ATP. To further evaluate the feasibility of AMP as an appropriate

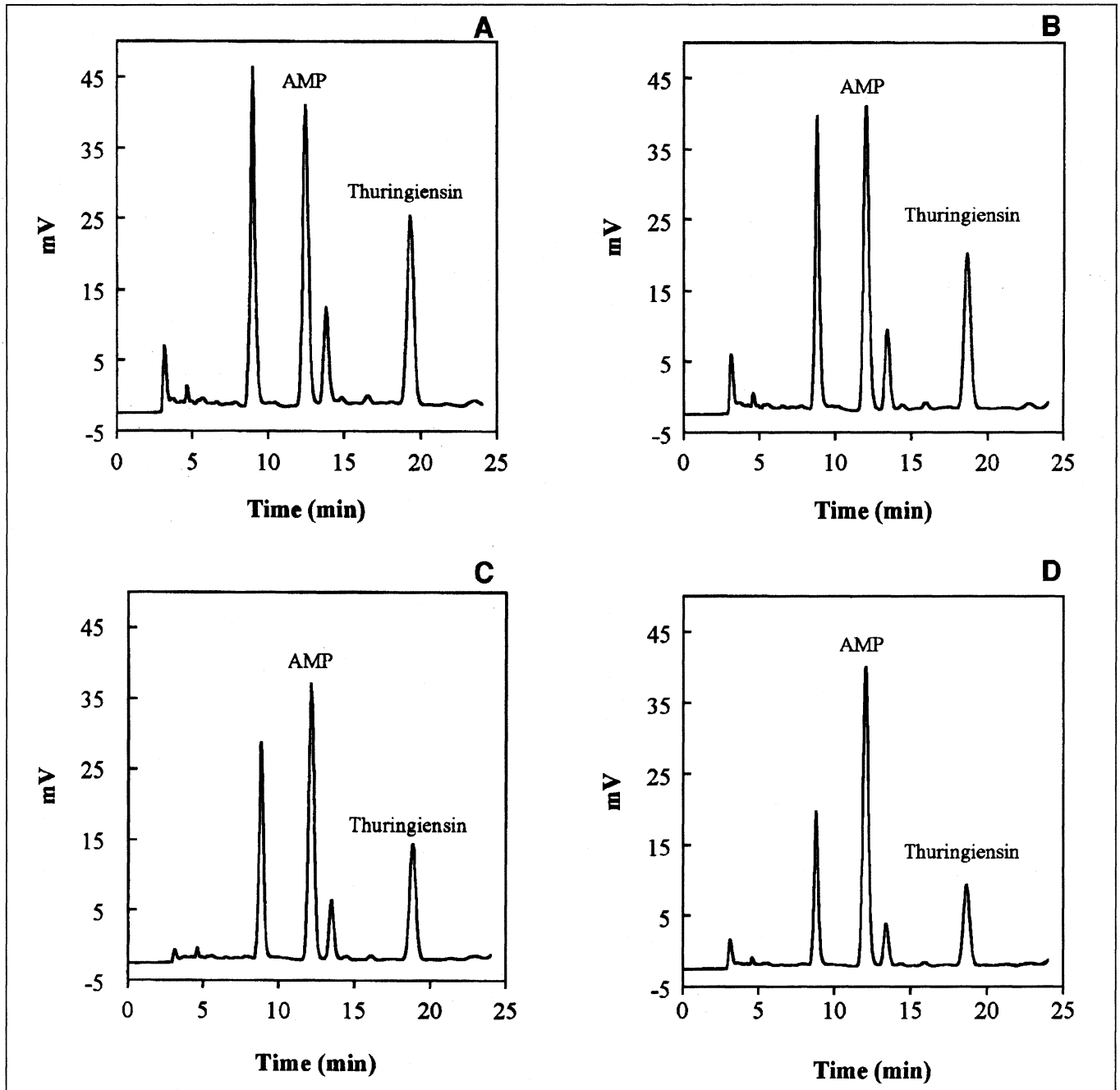

Figure 4. The HPLC chromatograms from four doses of standard thuringiensin specimens (A, 25 mg/mL; B, 18.75 mg/mL; C, 12.5 mg/mL; and D, 6.25 mg/mL) mixed with fixed amount of AMP (12.5 µg/mL).

internal standard for thuringiensin analysis, an experiment was performed by injecting 4 doses of AMP (200, 100, 50, and 25 µg/mL) and integrating the peak area. The results showed that the relation between peak area and concentration correlated quite well under regression analysis (correlation coefficient r^2 = 0.9992) (Figure 3).

Using these observations, a fixed amount of AMP (25 µg/mL) was mixed separately with four different concentrations of thuringiensin standard (50.0, 37.5, 25.0, and 12.5 mg/mL). The final concentrations of AMP and thuringiensin were only half of the concentration described previously. Each of the four mixtures with AMP (final concentration, 12.5 µg/mg) or AMP alone were subjected to HPLC analysis. In the initial experiment, results showed that there was a small peak with the same retention time as AMP which could interfere with AMP peak area calculation. To separate the small peak from the AMP peak, the flow rate was changed from 2 to 1.5 mL/min. After slowing the flow rate, the AMP peak was separated from the small peak. The resultant chromatograms (Figure 4) showed that the AMP peak appeared at 12 min and the thuringiensin peak appeared at 19 min (identified by using the purified standard from the Pasteur Institute). The peak areas were analyzed by SISC chromatogram analysis software. The peak area of AMP was not affected by the addition of thuringiensin. The peak area under the chromatogram of injected AMP was 531184, and the average AMP peak area of the chromatograms of four mixtures was 536847 + 12320 (mean ± standard error, relative standard deviation = 2.3%). Thus, the concentration of thuringiensin was well correlated with the peak-area ratio between the thuringiensin peak and the AMP peak. The correlation coefficient was 0.9952, the slope was 0.0281, and the intercept was 0.064 (Figure 5). The amount of thuringiensin was estimated by adding an equal volume of 25 µg/mL AMP and subjecting the mixture to HPLC analysis. Each experiment was repeated at least three times to verify its reproducibility. The peak-area ratio of thuringiensin to AMP was then calculated. The ratio minus 0.064 and divided by 0.0281 was equal to the estimated concentration of thuringiensin in the standard specimen (lot 2).

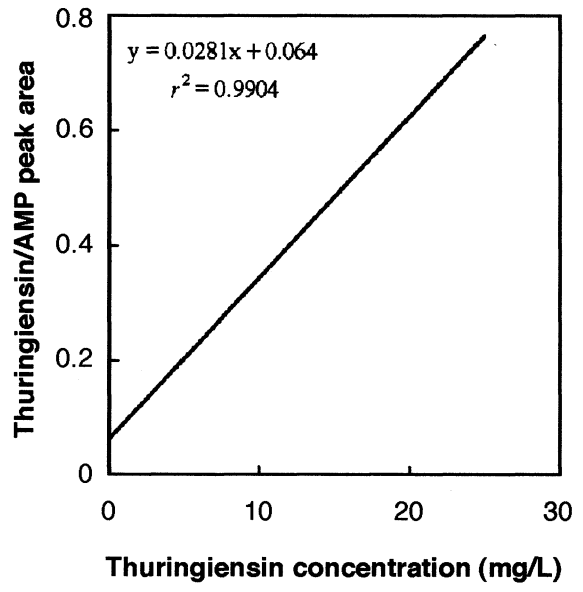

Figure 5. Correlation between the concentration of standard thuringiensin and the peak-area ratio of thuringiensin to AMP.

From this method and equation, the concentration of thuringiensin can be assessed from broth or semi-purified product. Because purified thuringiensin solution and lyophilized powder exhibit a short shelf life (confirmed by examining their physical appearance and checking their peak areas using chromatography), it may be advantageous to substitute AMP for purified thuringiensin as an internal standard during quantitative analysis. The AMP working solution should be diluted from the same lot of stock solution and its UV absorbance checked by spectrophotometry to make sure that the stock solution is still stable before mixing with the test specimen.

Conclusion

In summary, the use of AMP as an internal standard for thuringiensin analysis by HPLC may provide a consistent and accurate method to assess the concentration of thuringiensin in broth from bacterial fermentation. Because AMP is a stable and commercially available compound, it is much easier to use than purified thuringiensin, which may have a limited shelf life.

Acknowledgments

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