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Quantitative Analysis of Parasporal Crystal Protein from *Bacillus thuringiensis* by Capillary Electrophoresis

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ABSTRACT

The amount of parasporal crystal protein (δ-endotoxin) from the fermentation broth of the *Bacillus thuringiensis* is the best indication to assess the efficiency of the fermentation process or strain selection. Traditional methods for the assessment of insecticidal effect, such as bioassay or HPLC, were either time-consuming, inaccurate or inefficient. In this study, capillary electrophoresis (CE) was used for analyzing the amount of parasporal crystal protein after it was dissolved by adding a reducing agent, such as β-mercaptoethanol, to break the disulfide bonds. This soluble protein, δ-endotoxin, was then subjected to quantitative analysis by CE. The running buffer contained 300 mM boric acid, and pH was adjusted to 10.0 with 1 N NaOH. The dimensions of the capillary were 47 cm × 50 μm I.D. without coating. Lysozyme was used as internal standard for the quantitative assay of the δ-endotoxin. The migration time of the lysozyme peak was approximately 2 minutes earlier than that of the δ-endotoxin peak. The correlation between the concentration of δ-endotoxin and the ratio of the peak area of δ-endotoxin and the peak of lysozyme was calculated. The linear regression analysis showed that the correlation coefficient is equal to 0.9994, the slope is 0.4095 and the intercept is +0.0025. From this standard regression equation, the concentration of δ-endotoxin in fermentation broth or solution can be estimated easily by CE analysis.

Key words: *Bacillus thuringiensis*, parasporal crystal protein, capillary electrophoresis, quantitative analysis

INTRODUCTION

The Gram-positive soil bacterium *Bacillus thuringiensis* produces a parasporal crystal protein during sporulation. The sporulation of the bacteria is a process which produces large crystalline inclusion[1,2]. The crystal is toxic to insects when it is dissolved in the insect mid-gut and releases the δ-endotoxin protein. The δ-endotoxin will bind to phosphatidylcholine, sphingomyelin and phosphatidyl-ethanolainine, all of which are cell membrane components, thus leading to the disruption of membrane integrity and eventual cytolysis[3]. This bacterium comprises a number of different strains and subspecies, and may produce toxin that can kill specific insects. For example, toxin from *B. thuringlensis* subsp. *kurstaki* is toxic to lepidopteran larvae, such as moths, butterflies, and skipper larvae, cabbage worms, and spruce budworms. Toxin from *B. thuringiensis* subsp. *israelensis* kills diptera, such as mosquitoes, and blackflies. Toxin from *B. thuringiensis* subsp. *tenebrionis* (also known as *san diego*) is effective against coleoptera (beetles), such as potato beetles and boll weevils[4,5].

The δ-endotoxin produced by *B. thuringiensis* subsp. *kurstaki* is contained within a very large structure called the parasporal crystal, which is synthesized during bacterial sporulation[6]. The parasporal crystal comprises approximately 20 to 30% of dry weight of the sporulated culture and usually consists mainly of protein (95%) and a small amount of carbohydrate (5%)[7]. The crystal is an aggregate of protein that can generally be dissociated by mild alkali treatment into subunits[8,9]. The subunits can be further dissociated *in vitro* by treatment with β-mercaptoethanol, which reduces disufide linkages[10,11]. A protein is released when the parasporal is solubilized. The protoxin of the Cry I toxin group has a molecular mass of approximately 130 kilodaltons[12]. It has been well documented that the insecticidal potency is closely related to the amount of parasporal crystal or solubilized protoxin[7]. The traditional quantitative analysis method for insecticidal potency is spore counting[7]. However, recent investigations have found that the number of spores is sometimes not representative of the amount of parasporal crystal [8]. Also, some laboratories are using bioassay to assess insecticidal activity, but this method is time-consuming and lacks accuracy. Yamamoto *et al*. used HPLC to assess the concentration of protoxin and its digested peptides, and found that HPLC is very useful in characterizing these proteins[7]. However, there are some drawbacks in using HPLC for δ-endotoxin determination. For example, the HPLC column is expensive and the procedure is time-consuming as well. Capillary electrophoresis (CE) is a new analytical technique, which provides a simple and rapid analysis with high-resolution separation. In our laboratory, this new technique has been applied for quantitative analysis for δ-exotoxin (thuringiensin)[13]. The results indicated that the CE method is more accurate and rapid than that of HPLC. The present CE study for δ-endotoxin assay has demonstrated that it is a more convenient, rapid and efficient method than the conventional methods.

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MATERIALS AND METHODS

I. *Bacterial Strains and Media*

The rDNA *Bacillus thuringiensis* Yim 303 (obtained from Dr. K. F. Chak, National Yang-Ming University, Taipei) was used in this study. For the rDNA strain, *B. thuringiensis* subsp. *kurstaki Cry*B and HD73 were used as hosts for expression of the *cry* gene; *B. thuringiensis* s vector pSB909[14] was used to clone the *cotC* promoter-cryIAc and *crylC* genes fusion in *B. thuringiensis* host strain. HD-1 was purchased from the "Food Industry Research and Development Institute, (Shin-Chu, Taiwan). A3-4 was obtained from Dr. S. S. Kao, Taiwan Agricultural Chemicals and Toxic Substances Research Institute.

The culture media include the "Solid culture medium" which contains nutrient broth (0.8%), yeast extract (0.3%) and agar (5%) and the "liquid culture" which contains; glucose (3%), yeast extract (1.48), $(NH_4)_2S0_4$ (37%), K_2HP0_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.02%), $CaCl_2 \cdot 2H_2O$ (0.008%), $MnSO_2 \cdot 4H_20$ (0.005%). The formulation is modified from Acras' medium[15].

II. *Chemicals*

Boric acid (99.5%), sodium hydroxide (96%), di-basic sodium phosphate (85%) were purchased from Katayama Chemical Co. (Osaka, Japan). β-mercaptoethanol (>98%), Lysozyme (95%) were purchased from Sigma Chemical Company (St.Louise, MI, USA product number; L6876), and sodium bromide was purchased from Nihon Shiyaku Industries, Ltd. (Tokyo, Japan).

III. *Methods*

YIM 303, HD-1 and A3-4 clone vials from a liquid nitrogen tank were transferred to a solid culture medium by a platinum loop, and incubated at 30˚C for 72 hrs. The colonies were then transferred into a 500 mL flask with 200 mL liquid culture medium. The culture medium contained flask was shaken in an incubator at 200 rpm for 72 hrs.

This liquid culture medium was centrifuged 12,000 x g (4˚C for 20 min.). The precipitant was collected, washed with NaCl(1N),and centrifuged again. This step was repeated twice. Then the precipitant was washed with distilled water twice followed the same process which was described above (Figure 1). The washed precipitant was collected and mixed with a non-continuous sodium bromide gradient solution (30%, 35%, 40%) in a centrifuge tubing. This gradient centrifugation was conducted in an ultracentrifuge (Hitachi himac CP85 B), with rotor (P65A, P28S), under 4˚C and 121,000G for 2 hrs. After centrifugation, the precipitant in each layer of gradient was collected and examined by microscope with an oil lens. The collected parasporal crystal was pulled and then washed with 2D H_2O twice by the same method described above. The parasporal crystal was examined by using a phase contrast microscope (Olympus BX40, 400x amplification). The purified crystal was lyophilized and kept in a freezer (-20˚C) for future quantitative analysis. The purity and the concentration of protein content was verified by a "Bio-Rad Protein Assay" kit (Bio-Rad Laboratories, Hercules, CA, USA) after the lyophilized crystal was weighted and dissolved in an unit of solvent. This protein assay is based on the Bradford dye-binding procedure for measuring total protein concentration.

Fermentation broth
↓ Centrifuge 12,000 x g (4˚C for 20 min)
Crude parasporal crystal
↓ Wash with 1 N NaCl twice
↓ Wash with distilled water twice
Ist step purified parasporal crystal
↓ Sodium bromide gradient centrifugation
(121,000 x g, 4˚C for 2 h)
2nd step purified parasporat crystal
↓ Examined by microscope
↓ Wash with distilled water twice
Final purified parasporal crystal
↓ Dissolves ppt by β-mercaptoethanol for 12 h
Solublized crytal (concentration is determined by Bio-Rad protein assay)

Figure 1. The flow chart of d-endotoxin purification.

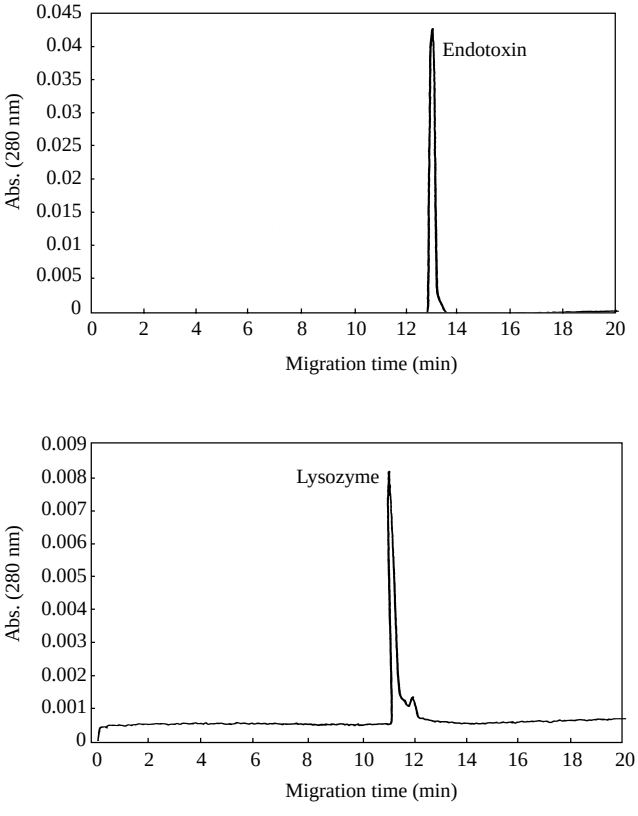

Figure 2. Electropherograms of the purified soluble δ-endotoxin (upper panel) and lysozyme (lower panel) from the CE analysis. Boric acid buffer (300 mM boric acid with l% mercaptoethanol, pH 10.0)was used as running buffer. The electrophoresis was conducted under 10 kV and injection time was 10 second positive pressure (0.5 psi).

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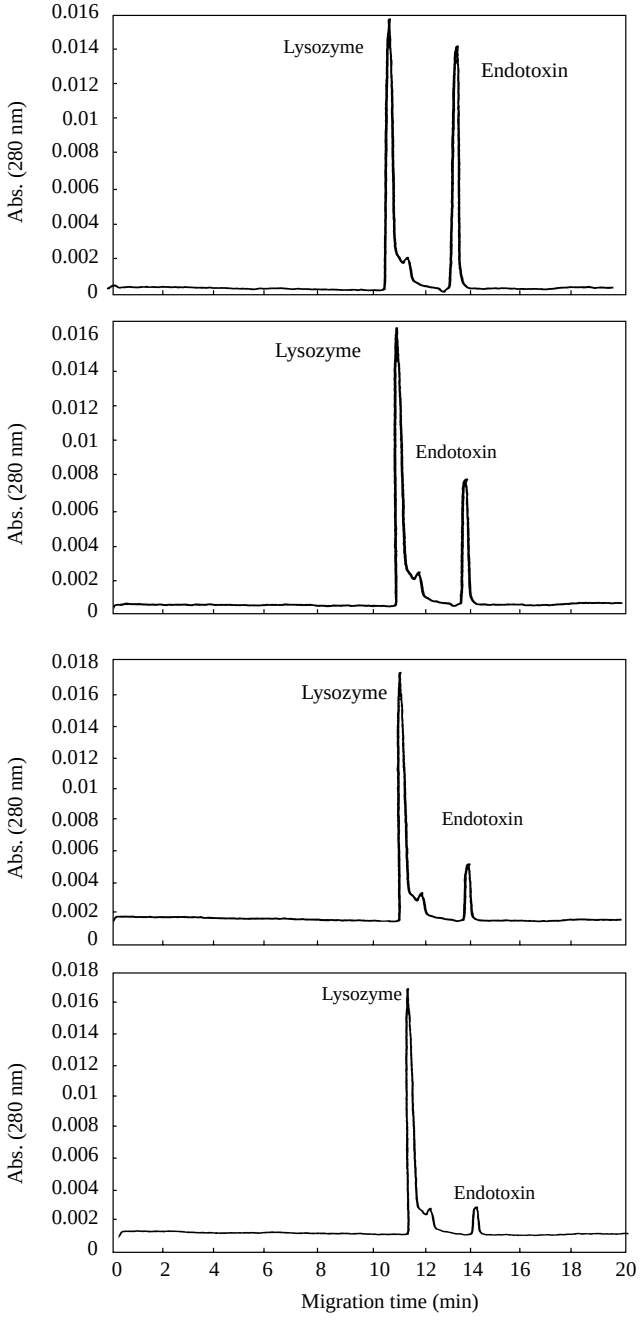

Figure 3. Lysozyme (5 mM) was mixed with differemt amount of δ-endotoxin. The concentrations range from 0.200 mg/mL, 0.100 mg/mL, 0.050 mg/mL to 0.025 mg/mL, and were subjected for CE analysis separately. The electropherograms are showed from high concentration (top) to low concentration (low).

IV. *Apparatus*

Capillary zone electrophoresis (CZE) was performed on a P/ACE System Model 2100 (Beckman Instruments, Fullerton, CA. USA). UV absorbance was monitored with a fixed-wavelength detector at 280 nm. Capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA). The dimensions of the capillary were 47 cm x 50 μm I.D. without coatings. The purified crystal was weighed and dissolved in a boric acid based electrolyte buffer solution (300 mM boric acid, 1% β-mercaptoethanol, pH adjusted to 10.0 with 1 N NaOH). The mixture was placed in a 30˚C water bath for 12 hrs in order to dissociate the disulfide bond. After the crystal was dissolved and degraded, an equal volume of lysozyme solution (10 mg/mL) was added as an internal standard. This mixture was the subject for CZE analysis. The mixture was injected to P/ACE by positive pressure (0.5 psi for 10 second). The electrophoresis was conducted under 10 kV for 20 min under 25˚C. The resultant electropherograms were analyzed by System Gold (San Ramon, CA,USA), Origin (Microcal Software Inc., MA, USA) and Microsoft Excel (Seattle,WA, USA).

RESULTS AND DISCUSSION

The parasporal crystal is composed of poorly soluble protein with a molecular weight of l30 kDa[12,16,17]. The poor solubility is likely due to disulfide bonds, which allows for crystallization in aqueous solution. The crystal can be dissolved by adding β-mercaptoethanol, which breaks the disulfide bonds. After dissociation, the soluble δ-endotoxin was subjected to CE for analysis. The running buffer contains 1% β-mercaptoethanol and is used to keep the δ-endotoxin out of recrystalization. High pH boric acid buffer has been used for protein analysis with excellent resolution and repeatable results[13]. Because it can provide the repulsion force between negative charged proteins and the fuse silica surface of capillary, and prevent the protein from adhering to the surface of the capillary. Figure 1 showed that the δ-endotoxin peak appeared on the final electropherogram. The protein was detected at 280 nm, which the absorbance is proportional to the number of aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. The migration time of δ-endotoxin was approximately 13.2 min. The peak area was proportional to the amount of δ-endotoxin. Lysozyme is an intracellular enzyme with a molecular weight of approximately 14,400 Dalton (Merck Index). Lysozyme pertains to high isoelectric point (pI = 10.5-11.0, in contrast to δ-endotoxin which pI = 4.4) which bearing less negative charges than that of δ-endotoxin in pH 10 buffer environment. In addition, the lower molecular weight of lysozyme should move faster than

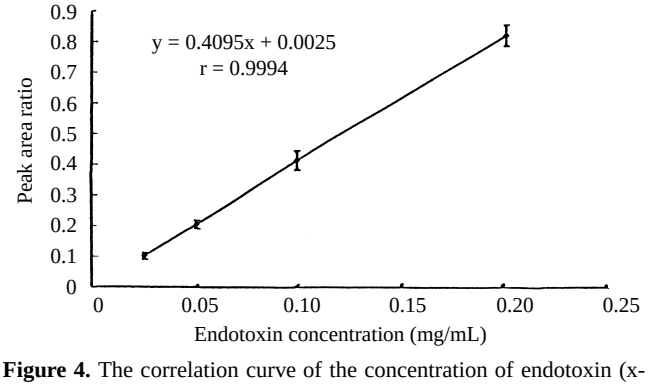

Figure 4. The correlation curve of the concentration of endotoxin (x-axis) and the peak ratio of endotoxin to lysozyme (y-axis). The correlation coefficient is 0.9994, with the slope of 0.4095, and the intercept + 0.0025. The endotoxin concentration in this analysis ranges from 0.025 mg/mL to 0.200 mg/mL.

δ-endotoxin during electrophoresis and had shorter migration time. It migrated approximately 2 minutes earlier than δ-endotoxin (Figure 3). From this experimental results, lysozyme has no interaction with δ-endotoxin and faster mobility. Based on these two advantageous factors it was used as an internal standard for the quantitative analysis of δ-endotoxin. In order to generate a correlation standard curve, the fixed amount of lysozyme (5 mM) was mixed with four serially diluted δ-endotoxin samples. (from 0.200 mg/mL, 0.100 mg/mL, 0.050 mg/mL and 0.025 mg/mL, the concentrations were determined by using "Bio-Rad protein assay" kit) (Figure 3). The small bump on the peak of lysozyme may have been due to either impurity or some degraded product of lysozyme. In this experiment, this small bump was included in the peak area of lysozyme. Nevertheless, it does not affect the results of the analysis significantly by calculating the peak area from the electropherograms. The correlation between δ-endotoxin and lysozyme was analyzed by linear regression using concentrations of δ-endotoxin versus the peak area of δ-endotoxin to lysozyme (Figure 4). Each concentration was run four times repeatedly and the standard deviation was expressed as the vertical bar at each point. The results indicated that the correlation coefficient equal to 0.9994 and the slope is 0.4095, with intercept at 0.0025 of y axis (y = 0.4095x + 0.0025). The amount of δ-endotoxin in solution or fermentation broth could then be assayed by the linear regression equation. In other words, from the resultant electropherogram, the peak area ratio of δ-endotoxin to lysozyme could be calculated by "System Gold" software. The concentration of the δ-endotoxin could be calculated from this ratio by the equation. Figure 5 is an example for this application. The endotoxin was produced from 3 different strains of *thuringiensis*, the wild types of HD-1 and A3-4 . The DNA recombinant strain YIM 303. The amount of δ-endotoxin in the solubilized solution which from the same volume of the fermentation broth can be predicted by adding a fixed amount of lysozyme and analyzing by CE. Resultant electropherogram shows those three strains of bacteria under the same fermentation condition and the yield of δ-endotoxin

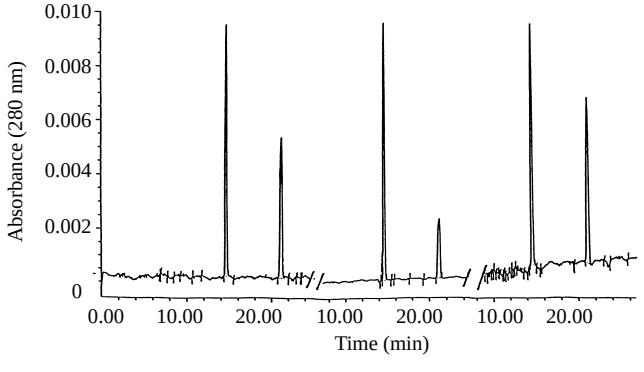

Figure 5. The combination of three electropherograins from three strains of *Bacillus thuringiensis*; HD-1, A3-4 and YIM 303 (from left to right). The high peak is lysozyme, and the short peak after the lysozyme peak is the δ-endotoxin. From the peak area ratio of endotoxin and lysozyme, the concentration of δ-endotoxin can be calculated as 1.665 mg/mL (HD-1), 0.8727 mg/mL (A3-4) and 1.919 mg/mL (YIM303) respectively.

estimated by this method. The amount of δ-endotoxin in HD-1, A3-4 and YIM 303 broth were assessed approximately as 1.665 mg/mL, 0.8727 mg/mL and 1.919 mg/mL respectively.

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利用毛細管電泳作蘇力菌素伴胞結晶蛋白之定量分析

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摘　要

蘇力菌素產生的伴胞結晶蛋白（δ-endotoxin）為目前最具潛力的生物農藥之一。在菌種的選擇及發酵方法的研發過程中，測試發酵液中伴胞結晶蛋白之量為一決定性的重要依據。傳統的方法是以生物檢測法或HPLC為主，這些方法一般較為耗時且準確度不高。本研究利用毛細管電泳分析經還原劑溶解後的伴胞結晶蛋白作定量之分析，以達到快而準確的分析。本實驗以較高濃度的硼酸（300 mM Boric acid）用1N的NaOH將pH調至10.0，用Lysozyme作為內在標準品，在注入已知量的結晶蛋白溶解液及定量Lysozyme之混合液至毛細管電泳儀，經過10 kV，二十分鐘之電泳後，從電泳圖譜上結晶蛋白溶解液及Lysozyme形成的波峰面積之比率與結晶蛋白之濃度作線性迴歸相關性分析。其結果顯示相關係數為0.9994，斜率為0.4095，交叉點為+0.0025，利用此一公式，可用定量之Lysozyme加入所要測試的結晶蛋白溶解液中作快速、經確之定量分析。本實驗用三種品系的蘇力菌（HD-1，A3-4，及YIM303）分析其發酵液中結晶蛋白之含量作為例證。

關鍵詞：蘇力菌，伴胞結晶蛋白，毛細管電泳，定量分析