Analysis of Destruxins Produced from *Metarhizium anisopliae* by Capillary Electrophoresis

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Abstract

Destruxins are insecticidal metabolites of a fungus, *Metarhizium anisopliae*. These metabolites are usually secreted into the culture medium during growth. The structure of destruxins is classified as being a cyclic hexadepsipeptide. More than 35 different destruxins have been characterized with a wide range of insecticidal activities. In this report, the destruxins are extracted by acetonitrile and crystallization by lyophilization. The final crystal is subjected for capillary electrophoresis analysis. Because destruxins are relatively hydrophobic compounds, micellar electrokinetic capillary chromatography is used in this series of experiments. The borate-based running buffer is optimized according to (1) boric acid concentration, (2) sodium dodecyl sulfate (SDS) concentration, (3) acetonitrile concentration, and (4) the pH of the running buffer. Optimization is based on resolution and running speed. The results indicate that 20mM boric acid with 40mM SDS plus 10% acetonitrile with pH 9.24 is the best set of conditions for both resolution and running speed.

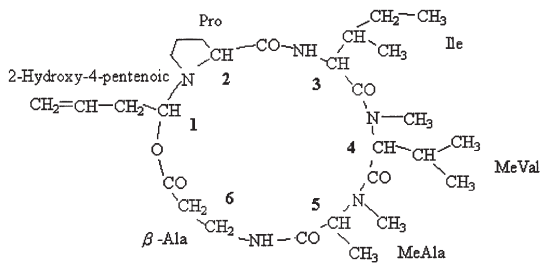
Introduction

The fungus *Metarhizium anisopliae* produces a family of cyclic peptide toxins termed destruxins, which exhibit various insecticidal activities to a wide range of insects. This entomopathogenic fungus is found in soils throughout the world. It was first recognized as a biocontrol agent in the 1880s. The cyclic peptide toxins are composed of five amino acids (proline, isoleucine, methyl-valine, methyl-alanine, and beta-alanine) and an α-hydroxy acid forming a cyclic hexadepsipeptide (Figure 1). To date, 35 destruxins have been found from different fungi (1–7). *M. anisopliae* is a major source for producing the destruxins (5). In addition to agricultural uses, some investigators have found that destruxins may induce erythropoietin production (8), antiviral effects (9–11), or antitumor effects (12). However, the preparative isolation, purification, and quantitative analysis of destruxins are required before practical usage. Hsiao and Ko (13) used high-performance liquid chromatography (HPLC) to analyze destruxins and cyclic peptide toxins from different strains of *M. anisopliae*. However, the results showed that resolution and sensitivity were not satisfactory. In addition, each run took 30 min to complete by HPLC (13). Capillary electrophoresis (CE) is a new technique that provides a simple and rapid analysis with high-resolution separation. This new analytical technique has been currently used widely in different areas. In our laboratory, this new technique has been applied for quantitative analysis of δ-endotoxin (14) and β-exotoxin (15) of *Bacillus thuringiensis*. In this report, four components of destruxin, DA (2-hydroxy-4-pentenoic acid), DB (2-hydroxy-4-methylpentanoic acid), DE (2-hydroxy-4,5-epoxy-pentanoic acid), and DMDB (desmethyl DB) from *M. anisopliae* fermentation broth, were analyzed by CE. The optimal condition for the analysis of destruxin is described in this report.

Experimental

Chemicals and reagents

Boric acid and sodium hydroxide were purchased from Katamaya (Osaka, Japan). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol were purchased from Mallinckrodt Baker (Paris, KY).



Destruxins	1 (hydroxy acid)	2(aa)	3(aa)	4(aa)	5(aa)	6(aa)
E	2-Hydroxy-4,5-epoxypentanoic	Pro	Ile	MeVal	MeAla	β-Ala
A	2-Hydroxy-4-pentenoic	Pro	Ile	MeVal	MeAla	β-Ala
DMDB	2-Hydroxy-4-methylpentanoic	Pro	Ile	Val	MeAla	β-Ala
B	2-Hydroxy-4-methylpnetanoic	Pro	Ile	MeVal	MeAla	β-Ala

Figure 1. Structure of destruxins from *Metarhizium anisopliae*.

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The broth was derived from *M. anisopliae* in a feed-batch cultivation in a modified airlift fermentation reactor. This process provided a high DB, DMDB, DA, and DE concentration. The broth was partially purified by high-speed centrifugation (8000 × *g* for 20 min, 4ºC). The supernatant was collected, and an equal volume of acetonitrile was added; this mixture was then mixed well with 5% NaCl and stirred for 20 min. After the salt dissolved completely, two layers were formed; the upper layer was the organic phase, and the lower layer was the aqueous phase. The organic phase was collected by a separating funnel and concentrated by lyophilization. The concentrated solution was crystallized by placing it in a 4ºC refrigerator overnight. The crystal was then subject for CE analysis.

Apparatus

CE analysis was performed on a P/ACE system Model 2200 (Beckman Instruments, Fullerton, CA) controlled by System Gold software (Beckman Instruments). UV absorbance was monitored with a fixed-wavelength detector at 214 nm. The capillary was purchased from Polymicro Technologies (Phoenix, AZ). The dimensions of the capillary were 47-cm × 50-µm i.d. without coating.

Running buffer

The running buffer used in this experiment was a boric acid-based running buffer, and SDS and acetonitrile were added to improve the solubility of destruxins. Boric acid ranged from 10 to 40mM, SDS ranged from 35 to 45mM, acetonitrile ranged from 5% to 15%, and the pH was adjusted from 8.7 to 9.7, depending upon the purpose of the experiment. The aim of this study was to optimize the running conditions for separating destruxins.

Procedure

Destruxins are heat stable and a highly hydrophobic class of compounds. The samples were dissolved with 100% acetonitrile and diluted to 10-fold concentration with double-distilled water and diluting the final 10% of acetonitrile. The samples were injected into P/ACE by 10-s positive pressure. The electrophoresis was conducted under 319 V/cm field strength, and the resultant electropherograms were analyzed by System Gold software.

Results and Discussion

Borate buffer-based running solution has been used for many analyses, including capillary zone electrophoresis (CZE) (16,17), as well as micellar electrokinetic capillary chromatography (MEKC) (18,19). Boric acid possesses the best buffering capacity in pH ranges between 9 and 10, and during this alkaline condi-

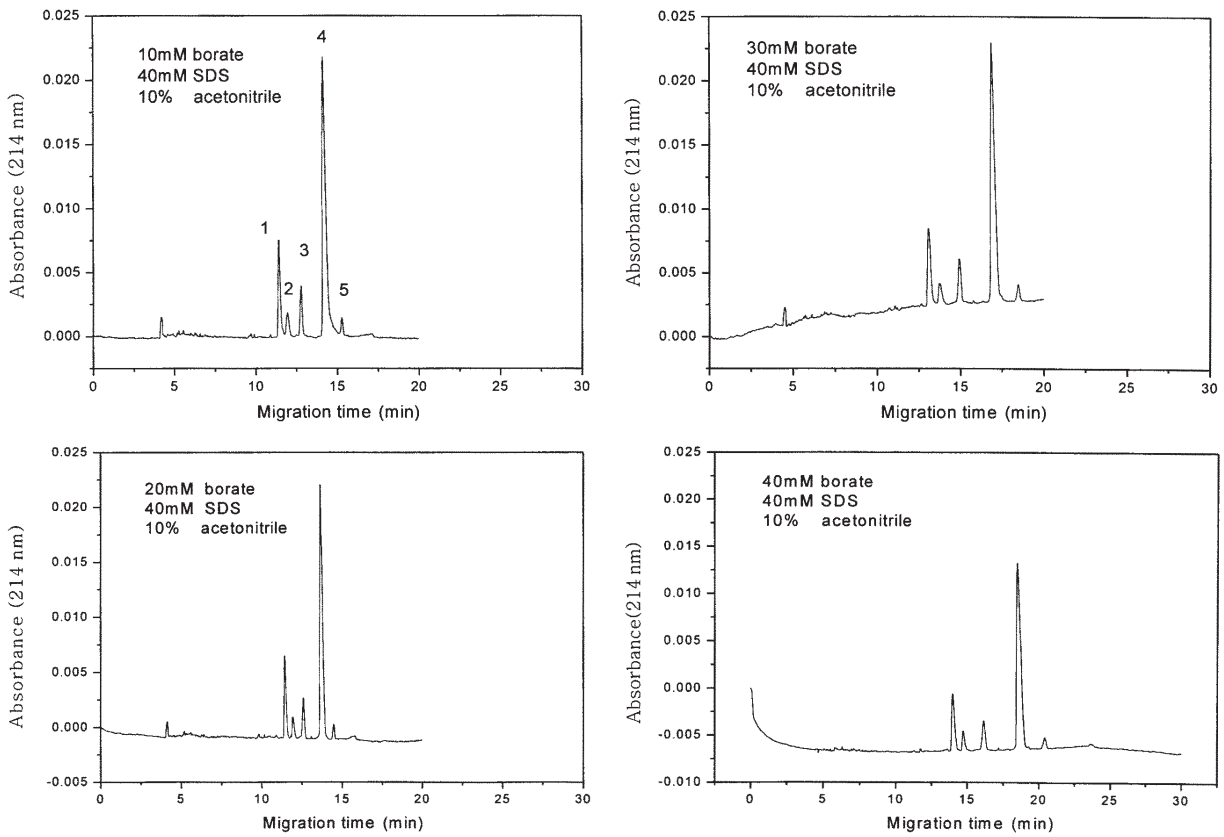

Figure 2. Effect of boric acid concentration on destruxins separation. Four concentrations of boric acid (10–40mM) were mixed with 40mM SDS and 10% acetonitrile as the running buffer (pH 9.24). Five peaks were identified: (1) DE, (2) DA, (3) DMDB, (4) DB, and (5) unknown (an unidentified peak may be expected as a new species of destruxin). A high resolution and short running time were shown on the electropherogram when 20mM boric acid was added in the running buffer (bottom left panel). The running conditions were set as 10 s pressure injection and 319V/cm electrophoresis on a 47-cm × 50-µm capillary.

tion, the interaction between analytes and capillary wall becomes minimal. The initial experiment was performed using various concentrations of borate buffer from 10 to 40mM and mixed with 40mM SDS and 10% acetonitrile. The resultant electropherograms showed that four distinct peaks (DE, DA, DMDB, and DB) were formed (Figure 2). When 20mM borate buffer was used as running buffer, it produced faster migration and a higher resolution between peak 1 (DE) and peak 2 (DA). In addition, its baseline was more stable than that of when 30 or 40mM boric acid was added. This phenomenon may be caused by the interaction between boric acid, SDS, and acetonitrile. It is possible that the higher ionic strength may generate more heat, causing the unstable baseline. However, the reason for this phenomenon has to be elucidated. Peak 5 is an unidentified peak; it possibly represents another species of destruxin or some other compound. In the separation of destruxins, CE has shown to be a faster and higher resolution method than HPLC (13). Figure 3 shows that in the relationship between borate concentration versus migration time, there was no difference between 10 and 20mM. However, the migration time was gradually increased beyond 20mM. In general, the ionic strength of the buffer has significant effects on solute mobility because the buffer contains a high concentration of anionic SDS, which may make the migration differentia become insignificant at a low ionic buffer below 20mM. Figure 4 shows the resolution (Rs) in each concentration of borate [Rs = $0.25(2)^{0.5}(\mu_{e1} + \mu_{e2})[V/(\mu_a + \mu_{eo}))^{0.5}$]. This result indicated that the effect of boric acid concentration on the mobility of destruxins on this running condition was very limited. However, the tailing peaks were found in the electropherogram when 10mM boric acid was added into the running buffer. This tailing may be simply be caused by the peak broadening lower ionic strength because no extra peak appeared when higher running buffers were used. When the buffer concentration reached 20mM, the tailing peak

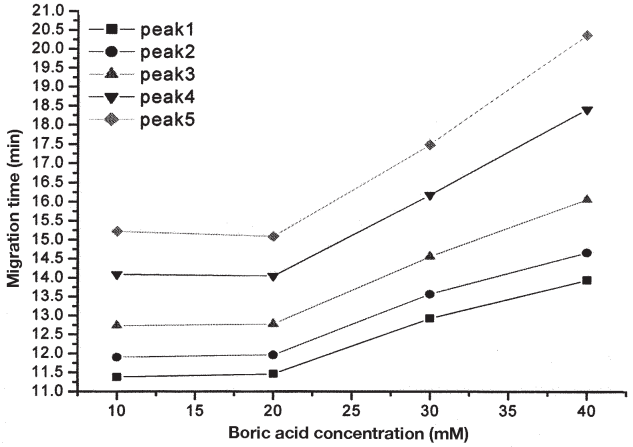

Figure 3. The boric acid concentration in running buffer versus migration time was plotted. Using 20mM boric acid showed improved resolution but without change in the migration time compared with 10mM. Peaks are identified in Figure 2.

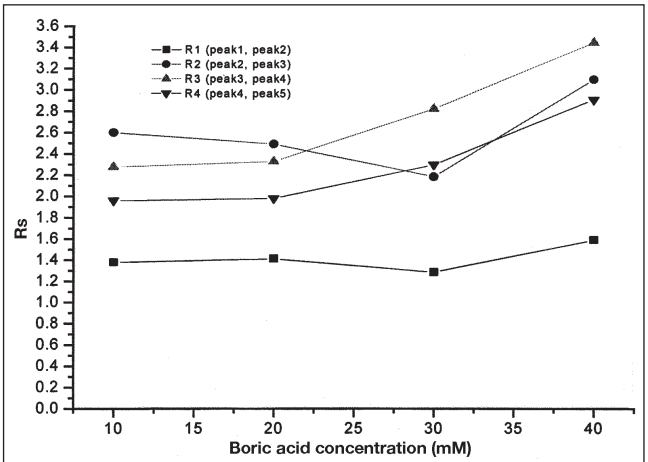

Figure 4. The plot of the boric acid concentration in running buffer versus peak resolution (Rs = 0.25(2)0.5(μe1 + μe2)[V/(μa + μeo)]0.5). Peaks identified in Figure 2.

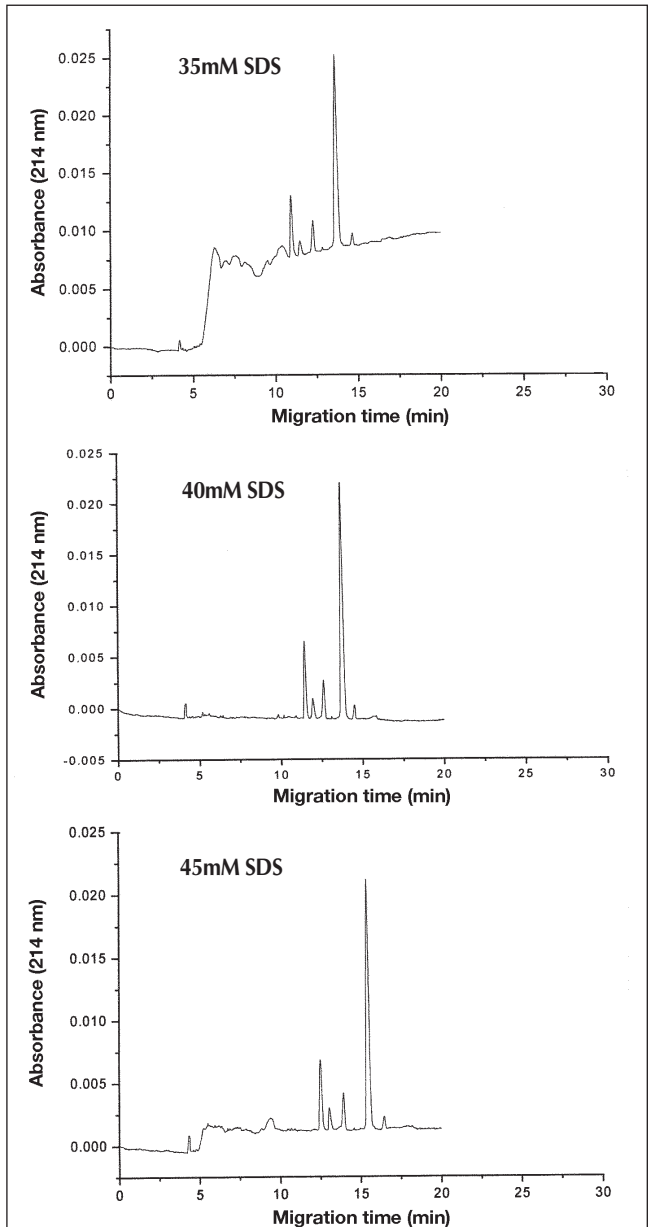

Figure 5. Effect of SDS concentration on destruxins separation. Three concentrations of SDS (35, 40, and 45mM) were added into the running buffer separately. The resultant electropherogams showed that a more stable baseline occurred when 40mM of SDS was added (middle panel). The running conditions are the same as in Figure 1.

disappeared. The baseline separation could be obtained among the five peaks. Further increasing the boric acid concentration to 30 or 40mM only prolonged the running time and did not significantly improve the resolution. The ionic strength of the buffer is inversely proportional to the coefficient of electroosmotic flow (EOF). Slower EOF may allow more space for analytes, but it also takes longer to complete a run. For compromising the running time without losing the resolution, borate buffer with a moderate concentration was chosen because it may create faster EOF than some other buffer, such as phosphate buffer. (20). In the Rs analysis, a biphasic curve was found on R2 trace at a boric acid concentration of 30mM. It is hard to explain the phenomenon; we speculate that it may relate to the different species of destruxin having their own solubility in this running buffer and the complicated interactions that may happen between boric acid, sodium, SDS, and acetonitrile. The real reasons needs to be elucidated in further studies.

SDS is the most commonly used surfactant in MEKC. In this experiment, SDS not only increased the solubility of destruxins, but it also may aid in separation by different partitioning with each species. Terabe et al. (21) plotted the reciprocal of the electrokinetic velocities versus SDS concentration up to 300mM. Fujiwara et al. (22) used the various concentrations of SDS (25–100mM). They found that the migration time is proportional to the concentration of SDS. The increased migration time may be because of the viscosity of the aqueous SDS solution instead of affecting zeta potential of the glass–solution interface. Both experiments agreed that the migration time generally increases with increased micelle content. These results are in concordance with the previous reports. In our experimental conditions, in the concentration range of SDS from 35 to 45mM, the results showed that 40mM might yield a stable baseline and shorter migration time. Figure 5 shows that the concentration might affect the baseline levels instead of resolution. The optimal concentration of SDS was 40mM, which resulted in the most stable and quiet baseline. This may be related to the aggregation of SDS in different concentrations under these running conditions. Aggregation may cause light absorption in the UV spectra.

Acetonitrile is an organic solvent that may slowly change EOF. This is because it has a weaker interaction with the capillary wall when compared with some other organic solvents. Addition of organic solvents to the electrophoretic buffer permits the analysis of some analytes that are not normally aqueous soluble by improving their solubility in the buffer. In this report, the concentration of acetonitrile from 5% to 15% was added into the running buffer; the results showed that a higher concentration of acetonitrile (above 12.5%) may reduce the peak number. The reason for this merging may reflect that the high concentration of acetonitrile may cause demixing of the microemulsion. These results agreed with the previous report by Altria et al. (23). For compromising the EOF and peak resolution, 10% of acetonitrile in borate-based buffer was used for the rest of experiments.

In addition to acetonitrile, the same concentration of some other organic solvents such as methanol, ethanol, and isopropanol were tested for their migration time and resolution. The results generally agree with the expectation that the EOF is dependent upon the chain length of the alcohol. Van Orman et al. (24) added ethanol or isopropanol in MES (2-[*N*-morpholino] ethane-sulfonic acid)-based buffer and found the EOF to be without significant difference. It seems implied that in addition to the chain length, the position of the alcohol group may also affect the EOF. From these experiment results, it is indicated that acetonitrile is better than some other organic modifiers such as methanol, ethanol, and isopropanol in terms of running time and peak resolution.

The other factor for influencing the EOF is buffer pH. Some observations demonstrated that an increase in pH of organic solvent increased EOF (26). This phenomenon is expected because the silanol groups dissociate more at a higher pH, causing the zeta potential to increase. Because the pH of boric acid is 9.24, some lower (8.70, 9.00, 9.14) and higher pHs (9.44, 9.54) were tested for improvement of resolution and running time. The results showed that raising the pH may shorten the running time; however when pH was over 9.24, it affected the EOF very insignificantly. The final optimized running conditions (20mM borate, SDS, and 10% acetonitrile) were performed in quadruplicate. The statistical analysis for reproducibility and migration time for these optimized running conditions and the results showed that the relative standard deviations are less than 0.5% and 5.6%, respectively.

Conclusion

The optimal running conditions for analysis of destruxins were established from these serious experiments. The boric acid-based running buffer is capable of separating the various destruxins. The optimal concentration of boric acid is in the range 20–40mM. The most optimal concentration for SDS is 40mM. At this concentration, the baseline is the most stable. Acetonitrile is the organic solvent with the least interaction with the capillary wall and the least delay of the migration time. In this study, we found that 10% acetonitrile may help increase the solubility and resolution, but it does not affect the migration time much. Finally, the optimal pH of the buffer is located at 9.24; in these alkaline buffer conditions, there is less interaction between destruxins and the capillary wall because of the repulsion of negative charges.

Acknowledgments

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