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Rapid HPLC Assay for the β-Exotoxin of *Bacillus thuringiensis*

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A high-performance liquid chromatography (HPLC) assay has been developed for β-exotoxin in fermentation beers and formulation samples. The assay is rapid and specific, and results correlate well with the fly bioassay. Detector response is linear over a concentration range of 0.0–0.56 mg/mL β-exotoxin. The assay is capable of detecting both the phosphorylated and dephosphorylated forms of β-exotoxin.

A number of strains of *Bacillus thuringiensis* produce a low molecular weight, heat-stable insecticidal toxin known as β-exotoxin (Sebesta et al., 1981). β-Exotoxin (thuringiensen, $C_{22}H_{32}N_5O_{19}P_1 \cdot 3H_2O$) is a nucleotide composed of adenosine, glucose, and allaric acid (Figure 1). It is believed that the phosphate group on allaric acid is essential for activity and that the toxicity of β-exotoxin results from its inhibition of RNA biosynthesis by competing with ATP for binding sites (Farkas et al., 1976).

Fly bioassay (Ignoffo and Gard, 1970) up to now has been the most commonly used method of detecting the presence of β-exotoxin in fermentation beers. The bioassay monitors the ability of β-exotoxin to prevent pupae of housefly larvae from developing into normal complete adults. However, the fly bioassay suffers from the fact that it is quite variable and nonspecific, and it takes 9 days to complete a single assay.

In the last few years, two high-performance liquid chromatography (HPLC) assay methods for β-exotoxin have appeared in the literature (Oehler et al., 1982; Danilova and Kruglyak, 1983). Unfortunately the methods either lack specificity for β-exotoxin in complex fermentation mixtures (Johnson and Peterson, 1983) or require gradient elution techniques with long analysis times (Danilova and Kruglyak, 1983). Recently, we developed a rapid, specific, quantitiative HPLC assay for β-exotoxin. β-Exotoxin samples can be assayed in 20–30 min vs. an external β-exotoxin standard.

EXPERIMENTAL SECTION

Reagents and HPLC Standard. Analytical reagent-grade potassium dihydrogen phosphate (KH_2PO_4) and 85% phosphoric acid obtained from Mallinckrodt (Paris, KY) were used to prepare the mobile phase. Deionized water was obtained from a Milli Q purification system (Millipore Corp., Bedford, MA). Abbott Laboratories β-exotoxin reference standard (lot 635-19), having a purity of 80.9%, was used to assay β-exotoxin samples.

Liquid Chromatography. HPLC assays were performed isocratically on a Waters μ-Bondapak C_{18} column (30 cm × 3.9 mm i.d.) maintained at a constant temperature between 25 and 35 °C. A 50 mM KH_2PO_4 (pH 3.0) mobile phase and a flow rate of 2.0 mL/min were used. The mobile phase was filtered through a 0.4-μm polycarbonate membrane (Nuclepore Corp., Pleasanton, CA). Solid samples and standards were dissolved in deionized water. Solid samples that did not readily dissolve in deionized water were acidified to pH 3 with phosphoric acid. Liquid samples and dissolved solid samples were diluted to a concentration of approximately 0.1 mg/mL β-exotoxin and filtered through 0.45-μm disposable nylon 66 syringe filters (Alltech Associates Inc., Deerfield, IL). Samples were then injected into the HPLC. Instrumentation included an M6000A pump (Waters Associates, Milford, MA), a 501 autosampler (Beckman Instruments, Berkeley, CA) equipped with a 20-μL sample loop, a SF 770 variable-wavelength UV detector set at 260 nm (Kratos Analytical Instruments, Ramsey, NJ), and a Chromatopac C-R3A integrator (Shimadzu Corp., Kyoto, Japan).

Fly Factor Bioassay. A CSMA fly larva Medium No. 5060 (Ralston Purina Co., St. Louis, MO) was used for bioassay of β-exotoxin samples against 3-day-old larve of *Musca domestica*. Ten larvae in quadruplicate at each test level were transferred into a 4-dram shell vial containing 5 mL of diluted sample and 2 g of CSMA fly larvea media. Each vial was covered with a double thickness of paper toweling and secured with a rubber band. Vials were incubated at 27 °C for 9 days. The number of larve, pupae, normal adults, and deformed adults was noted. Deformed

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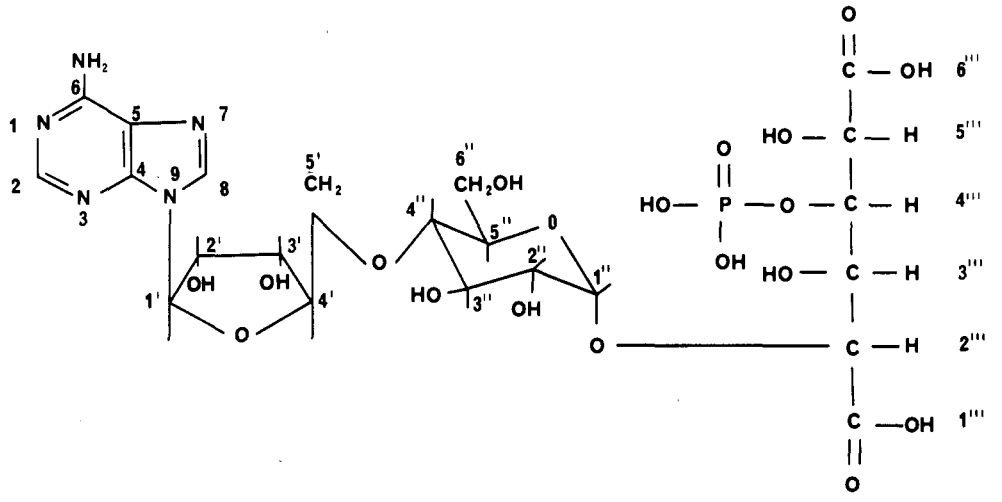

Figure 1. β-Exotoxin.

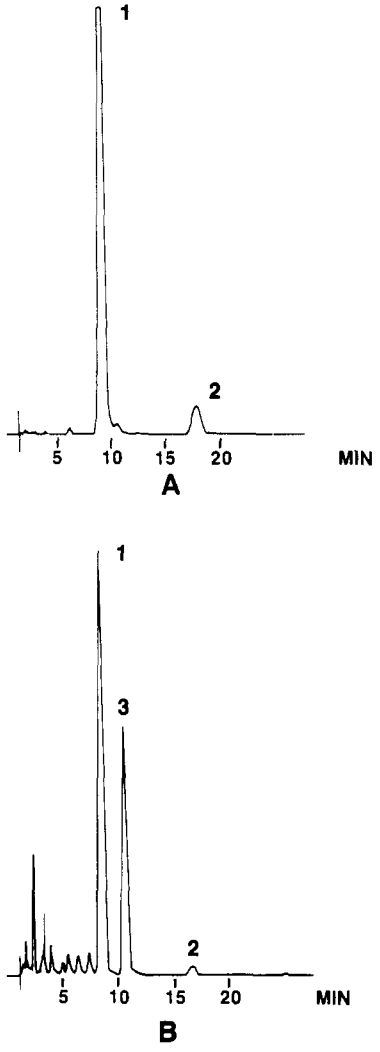

Figure 2. HPLC chromatograms of β-exotoxin standard (A) and a fermentation beer (B). Peaks: (1) β-exotoxin; (2) dephosphorylated β-exotoxin; (3) major adjuvant peak.

flies were calculated as nonemerged. LC_{50} values were calculated by Probit mortality analysis.

Comparison of the Fly Factor Assay with the HPLC Assay. The fly factor and HPLC assays were compared by assaying six autoclaved samples. Samples assayed represented both β-exotoxin calcium salts and fermentation beers. Calcium salts were dissolved in deionized water by adjusting to pH 3 with phosphoric acid. Samples were then diluted accordingly with deionized water and assayed.

RESULTS AND DISCUSSION

Typical HPLC chromatograms of Abbott β-exotoxin standard and a fermentation beer are shown in Figure 2. A peak having a retention time approximately twice that of β-exotoxin was observed in both the standard and beer chromatograms. This peak was later identified as dephosphorylated β-exotoxin by reacting the β-exotoxin with alkaline phosphatase in a manner similar to Sebesta et al. (1969) except using bovine alkaline phosphatase in diethanolamine buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). The resulting reaction mixture was then analyzed by HPLC (Figure 3).

Further confirmation of the identity of the peak was obtained by heating an aqueous solution of the β-exotoxin at pH 3 to cleave the phosphate ester. Results showed a decrease in the β-exotoxin peak and a concomitant increase in the dephosphorylated peak.

The HPLC assay was found to have excellent β-exotoxin specificity and has been used to examine a number of *B. thuringiensis* strains. HPLC results were found to correlate well with the fly bioassay for a variety of autoclaved β-exotoxin samples as shown in Figure 4. The assay is very specific and is capable of separating β-exotoxin from adenosine and other nucleotides such as adenosine 5′-triphosphate and adenosine 5′-monophosphate.

Detector response was linear over a 0.0–0.56 mg/mL concentration range from both peak height and peak area quantitation. Correlation coefficients were 1.0000, and response vs. concentration curves passed through the origin.

The precision of the assay was determined by assaying eight replicate sample preparations. The relative standard deviation of the assay was ±0.4% for both peak height and peak area quantitation.

β-Exotoxin recovery was examined by spiking in standard at levels ranging from 0.0 to 10% of the nominal concentration into a formulation sample. Recoveries were 97.0–100.4% from peak height and peak area quantitation.

The ruggedness of the assay was studied by varying the mobile phase and column temperature. A ±10% variation in the KH_2PO_4 concentration was found to have no effect on peak retention time, height, or area. A variation of ±1 pH unit in the mobile phase was found to have no effect on the assay from peak area or peak height quantitation.

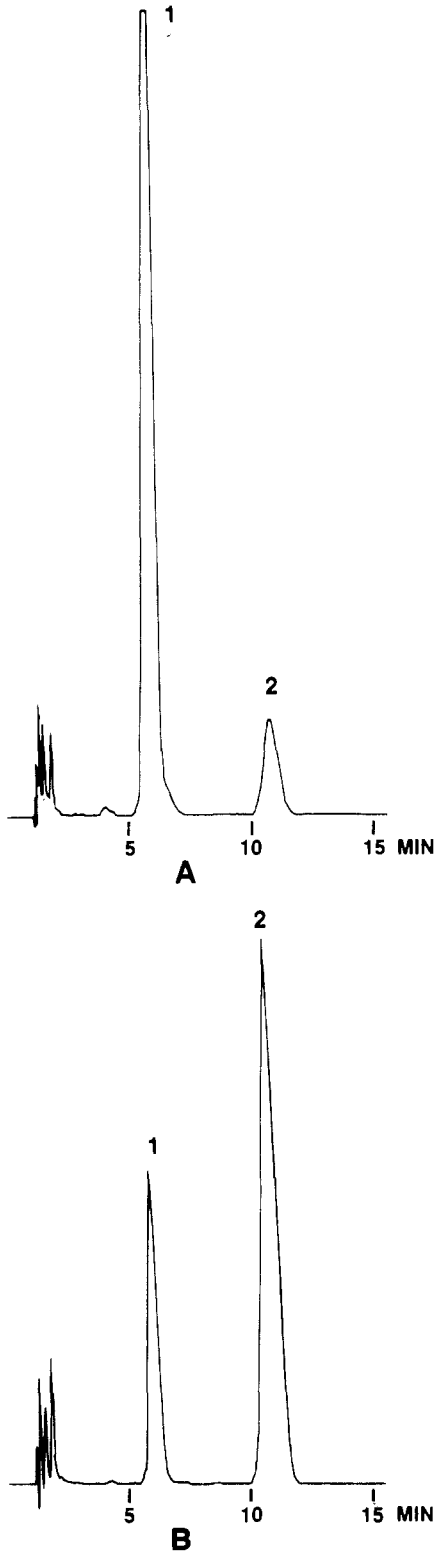

Figure 3. HPLC chromatogram of β-exotoxin standard degraded by alkaline phosphatase: (A) 0; (B) 21 h. Peaks: (1) β-exotoxin; (2) dephosphorylated β-exotoxin.

However, the β-exotoxin peak retention time was found to change from 5.9 min at pH 2 to 8.8 min at pH 4. This indicates that the ionic form of β-exotoxin is very sensitive to pH changes, thus allowing mobile-phase pH variations to be used to confirm the presence of β-exotoxin in complex mixtures.

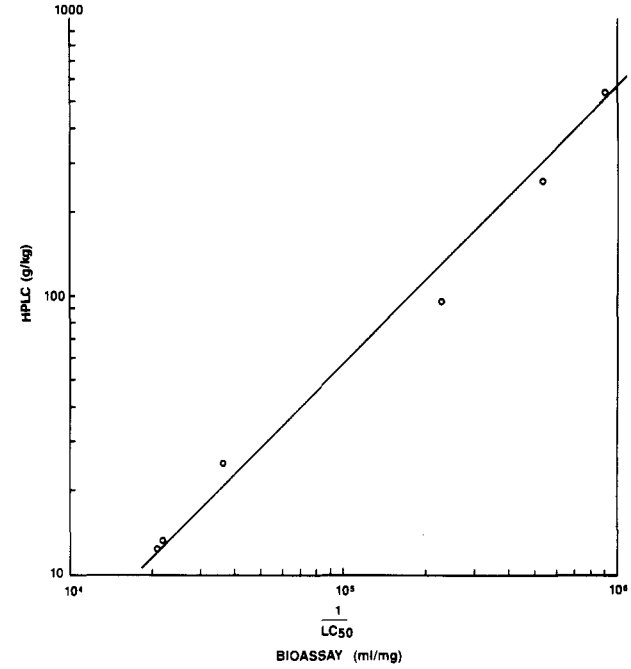

Figure 4. Correlation of fly bioassay and HPLC assay. Correlation coefficient 0.995.

Column temperature was found to have no effect on the β-exotoxin assay as long as the temperature was held at a constant temperature between 25 and 35 °C. The β-exotoxin peak and the major adjuvant peak (Figure 2B) were found to coelute at 15 °C, thus causing a positive bias in the assay.

The β-exotoxin HPLC assay is superior to the fly bioassay in accuracy, specificity, precision, and speed. The assay is very sensitive and is capable of detecting both the phosphorylated and dephosphorylated forms of β-exotoxin in complex beer formulation mixtures.

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Registry No. β-Exotoxin, 23526-02-5; dephospho-β-exotoxin, 23526-03-6.

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