

# Development of an Enzyme-Linked Immunosorbent Assay for the $\beta$ -Exotoxin of *Bacillus thuringiensis*

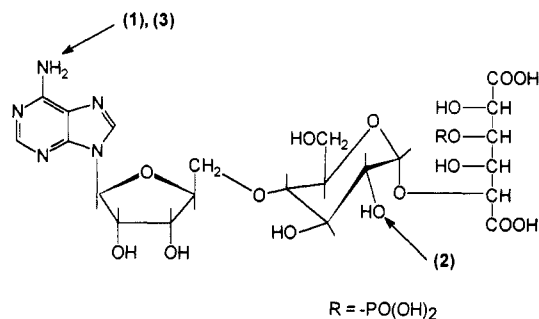
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A competitive enzyme-linked immunosorbent assay (ELISA) was developed to quantify the amount of  $\beta$ -exotoxin from *Bacillus thuringiensis* in solution and to evaluate the ability of the antibodies to distinguish among various natural and synthetic compounds related to the  $\beta$ -exotoxin. The  $\beta$ -exotoxin was coupled to several proteins via glutaraldehyde, diazotization, and periodate procedures. The antibodies used in the assay were obtained from antisera raised against  $\beta$ -exotoxin linked to keyhole limpet hemocyanin, and the ELISA coating antigens consisted of  $\beta$ -exotoxin-bovine serum albumin conjugates. Both homologous and heterologous ELISA systems were examined. The homologous systems were not useful because the free  $\beta$ -exotoxin did not inhibit antibody binding to the solid phase. The heterologous systems yielded the most sensitive assays, but antisera obtained from all of the immunogens were used successfully in developing ELISAs for  $\beta$ -exotoxin. With these sensitive ELISAs,  $\beta$ -exotoxin was detected in samples of commercial formulations at levels as low as 0.1 ng/mL. A good correlation was observed when culture media was fortified with  $\beta$ -exotoxin and analyzed in a blind fashion with both HPLC and ELISA. These data suggest that the ELISA could be a valuable tool for detecting and quantifying  $\beta$ -exotoxin.

## INTRODUCTION

$\beta$ -Exotoxin [O-5-L-deoxyadenosinyl-O- $\alpha$ -D-glucopyranosyl]-D-allaric acid 4-(dihydrogen phosphate) (Farkas et al., 1969; Figure 1) is a low molecular weight, heat-stable toxin with insecticidal activity, produced by some strains of *Bacillus thuringiensis* (BT) (Farkas et al., 1969). It is used commercially as an insect control agent in several countries and has been considered for registration as a microbially produced insecticide in the United States. It is also found as a contaminant in commercial formulations of BT. The toxicity of the  $\beta$ -exotoxin is due to the inhibition of RNA biosynthesis (Farkas et al., 1976). Due to the use of the  $\delta$ -endotoxin of BT and related proteins as selective insecticides, often in sensitive ecosystems, it is important to ensure that toxic levels of the  $\beta$ -exotoxin are not present in commercial materials. As we examine new conditions for production of BT using a variety of natural isolates and/or genetically engineered materials, it is important to have inexpensive, accurate, and precise analytical methods for the  $\beta$ -exotoxin both for quality control and for regulation. Current methods for the assay of heat-stable biological activity attributable to total  $\beta$ -exotoxin include a number of biological and biochemical tests based on its influence on insects and its inhibitory effect on DNA-dependent RNA polymerase from *Escherichia coli* and BT (Vanková, 1978). These assays are very reliable but are labor-intensive, time-consuming, and nonspecific. Recently, however, high-performance liquid chromatography (HPLC) was used as an alternative method for detection and quantification of the  $\beta$ -exotoxin (Oehler et al., 1982). Since then, there have been a number of modifications. Nevertheless, these methods lacked specificity for  $\beta$ -exotoxin in complex fermentation mixtures



**Figure 1.** Structure of the  $\beta$ -exotoxin from *B. thuringiensis*. Arrows show positions of conjugation by (1) diazotization, (2) periodate coupling, and (3) glutaraldehyde coupling.

(Johnson and Peterson, 1983), required long analysis times (Danilova and Kruglyak, 1983), or lacked the sensitivity necessary for environmental monitoring (Campbell et al., 1987; Levinson et al., 1990).

The demand for more cost-effective methods that are also efficient, rapid, and dependable may be partially filled by the successful use of enzyme-linked immunoassays (ELISA). ELISAs have been used to monitor the  $\delta$ -endotoxin. Early antibodies detected the protein but did not correlate completely to the bioactive components (Wie et al., 1982; Cheung et al., 1988). Monoclonal antibodies were developed against the specific toxin (Hofte et al., 1988) and against synthesized peptides of the conserved region to monitor the bioactive materials (Groat et al., 1990). The purpose of this study was to develop, optimize, and characterize a sensitive ELISA assay for the detection and quantification of the  $\beta$ -exotoxin.

## MATERIALS AND METHODS

**Chemicals.** The proteins used in coupling, *p*-nitrophenyl phosphate substrate, adenine (6-aminopurine hydrochloride), AMP, ATP, NADP, DNA, guanine, D-glucose 6-phosphate, FAD, guanosine, Tween 20, alkaline phosphatase from bovine intestinal mucosa, and Freund's complete adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO). Diethanolamine was obtained from Aldrich Chemical Co. (Milwaukee, WI), and goat

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Table I. Summary of Titration Results Using Different Combinations of Antibodies with Different Coating Antigens<sup>a</sup>

coating antigen	antibody no./immunizing antigen <sup>b</sup>								
	939/D <sub>1</sub>	942/D <sub>1</sub>	948/G <sub>1</sub>	950/G <sub>1</sub>	952/P <sub>1</sub>	957/P <sub>1</sub>	959/P <sub>1</sub>	960/P <sub>1</sub>	961/P <sub>1</sub>
$\beta$ -exotoxin-D <sub>1</sub> -BSA	- <sup>c</sup>	-	-	-	+	-	-	-	++
$\beta$ -exotoxin-P <sub>1</sub> -BSA	-	-	+	++	++	++	+	-	+++
$\beta$ -exotoxin-G <sub>1</sub> -BSA	-	-	-	-	-	+	+	+	++
$\beta$ -exotoxin-G <sub>2</sub> -BSA	-	-	-	-	-	+	+	+	+

<sup>a</sup> Checkerboard titration assays were run with these coating antigen and antibody combinations as described under Materials and Methods.

<sup>b</sup> Rabbits 939 and 942 were immunized with  $\beta$ -exotoxin conjugated to keyhole limpet hemocyanin by the diazotization procedure (D<sub>1</sub>). Rabbits 948 and 950 were immunized with  $\beta$ -exotoxin conjugated to keyhole limpet hemocyanin by the glutaraldehyde method (G<sub>1</sub>). Rabbits 952, 957, and 959-961 were immunized with  $\beta$ -exotoxin conjugated to keyhole limpet hemocyanin by the periodate procedure (P<sub>1</sub>). <sup>c</sup> Titers were ranked as follows utilizing data from a coating antigen concentration of 20  $\mu$ g/mL and an antibody dilution of 1:500 of the final bleed: 0.0-0.3, low titration (-); 0.4-1.0, moderate titration (+); 1.1-1.6, high titration (++); 1.7-2.6, extremely high titration (+++).

anti-rabbit IgG alkaline phosphatase was obtained from Miles Laboratories (Elkhart, IN). The reference standard  $\beta$ -exotoxin (lot 12-313-BD, 80.7% purity) was a gift of the Chemical and Agricultural Products Division, Abbott Laboratories (N. Chicago, IL). The *E. coli* extract (lot B609) was purchased from Promega Co. (Madison, WI), and the *Bacillus subtilis* extract was a gift from Dr. Roy Doi (University of California, Davis). Dehydrated bacto brain-heart infusion, tryptone, bacto beef extract, bacto peptone, bacto yeast extract, dextrose, glucose, and bacto casamino acids were obtained from Difco Laboratories (Detroit, MI). Agar (ultrapure) was obtained from United States Biochemical Corp. (Cleveland, OH). BBL (trypticase soy broth) was obtained from BBL Microbiology Systems (Becton Dickinson and Co., Cockeysville, MD).

**Preparation of Hapten-Protein Conjugates.** Proteins used were bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). The  $\beta$ -exotoxin (hapten) was conjugated to both of these proteins by the diazotization (Nisonoff, 1967), periodate (Stollar, 1980) and glutaraldehyde (Voller et al., 1980) methods.

**Diazotization Method.** Briefly, 0.152 mmol of  $\beta$ -exotoxin was dissolved in 1 mL of 1 N HCl. An equimolar amount of NaNO<sub>2</sub> was added. This hapten solution was added dropwise to the protein solution (20 mg/mL in 0.05 M borate buffer, pH 9.6) on ice while stirring. The pH was maintained at 9-9.5 with 10% NaOH. After the protein hapten solution had stirred for 2 h, the conjugate was dialyzed exhaustively against phosphate-buffered saline (PBS). These conjugates were termed  $\beta$ -exotoxin-D<sub>1</sub>-protein.

**Periodate Method.** The  $\beta$ -exotoxin (0.152 mmol) was dissolved in 1.5 mL of NaIO<sub>4</sub> with stirring for 25 min at room temperature. This hapten solution was added to 5 mL of a 20 mg/mL solution of KLH or BSA in 0.1 M carbonate buffer, pH 9.6, and the pH kept at 9.5-9.7 with 10% NaOH. After the protein hapten mixture had stirred for 60 min and the pH had stabilized, 100 mg of NaBH<sub>4</sub> in 5 mL of water was added and then stirred for 2 h on ice. The reaction mixture was then dialyzed exhaustively against PBS to remove any material that did not react with the protein. These conjugates were termed  $\beta$ -exotoxin-P<sub>1</sub>-protein.

**Glutaraldehyde Method.**  $\beta$ -Exotoxin (0.152 mmol) was added to 5 mL of a 20 mg/mL solution of KLH or BSA in PBS. To this mixture was added 0.65 mL of 1% glutaraldehyde with stirring for 1 h and with the pH maintained at 9.5, followed by dialysis of the protein-hapten conjugate against PBS. This method was performed on two different days. These conjugates were termed  $\beta$ -exotoxin-G<sub>1</sub>-protein or  $\beta$ -exotoxin-G<sub>2</sub>-protein.

The coupling was assessed by UV absorbance spectrophotometry at 260 and 280 nm (for protein determination). The hapten density for periodate-BSA was larger than that for G<sub>1</sub>, G<sub>2</sub>, or D<sub>1</sub>-BSA. The molar ratio of hapten to BSA was 9.9:1, 0.66:1, and 0.54:1 for periodate, glutaraldehyde, and diazotized conjugates, respectively. The antibodies were obtained from antisera raised to a conjugate of  $\beta$ -exotoxin linked to KLH, while  $\beta$ -exotoxin was conjugated to BSA for use as a solid-phase antigen in an indirect competitive ELISA.

**Immunization of Rabbits.** Rabbits 938 and 942 were immunized intradermally with the diazotized conjugate, rabbits 948 and 950 with the glutaraldehyde conjugate ( $\beta$ -exotoxin-G<sub>1</sub>-KLH), and rabbits 952, 957, and 959-961 with the periodate conjugate (Table I). After initial subcutaneous immunization with 100  $\mu$ g per rabbit of hapten-protein conjugate in Freund's

complete adjuvant, boosts of 100  $\mu$ g of the same conjugate in Freund's incomplete adjuvant were administered three times, approximately every 4 weeks. Ten days after each boost, test bleeds were taken and examined for antibodies that recognized  $\beta$ -exotoxin coating antigens. Following the final boost, the animals were terminally bled. All reported ELISA studies were carried out using the antisera from this final bleed.

**Enzyme Immunoassay and Competitive Enzyme Immunoassay.** Following the principles of solid-phase immunoassays (Voller et al., 1976) ELISAs were conducted in a competitive format. Plates were coated by adding  $\beta$ -exotoxin-BSA conjugates in 0.5 M carbonate buffer, pH 9.6, at concentrations of 20, 10, 5, 2.5, and 1.25  $\mu$ g/mL to wells of a microtiter plate (100  $\mu$ L/well). Plates were sealed with adhesive plate sealers and incubated at 4 °C overnight. On day 2, the coated plates were washed five times with PBSTA (phosphate-buffered saline plus 0.05% Tween 20 plus 0.02% sodium azide) buffer to remove unbound coating antigen, and then serial dilutions of antiserum in PBSTA were added at 100  $\mu$ L/well, followed by incubation at room temperature for 2 h. The plates were again washed five times, and goat anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBSTA (1:2500) was added at 50  $\mu$ L/well and then incubated at room temperature for 2 h. After another five washes, phosphatase activity was measured by adding 100  $\mu$ L/well of *p*-nitrophenyl phosphate (1 mg/mL) in 10% diethanolamine buffer, pH 9.8, and incubated for 30 min; the color intensity was read at two wavelengths (405-650 nm) with a V<sub>max</sub> microplate reader. The absorbances at 650 nm were subtracted from the absorbances at 405 nm to minimize signal from distortions in the plastic of the 96-well microtiter plate.

For competitive inhibition ELISA, optimal concentrations of coating antigens and antisera were obtained from the results of checkerboard titrations as described above and used in the inhibition studies. The procedure for competitive ELISA was essentially the same as that for the indirect ELISA described above except that dilutions of  $\beta$ -exotoxin or sample containing exotoxin in distilled water were incubated overnight at room temperature with the anti- $\beta$ -exotoxin antiserum in PBSTA. The concentration of  $\beta$ -exotoxin that could inhibit the assay by 50% was designated the IC<sub>50</sub>. In determination of the antibody specificity, compounds structurally similar to  $\beta$ -exotoxin were analyzed. The cross-reactivity of these compounds is reported as

$$\left[ \frac{\text{(amount of } \beta\text{-exotoxin at the assay midpoint)}}{\text{(amount of structurally related compound at the assay midpoint)}} \right] \times 100 \quad (1)$$

Cross-reactivity tests were done on rabbit sera 948, 957, and 959-961. The periodate conjugate was used as the coating antigen for rabbit 948, the glutaraldehyde conjugate with antibodies 957, 959, and 960, and the diazotized conjugate with antibody 961.

**Instruments. Plate Reader.** Enzyme-linked immunosorbent assays were carried out in 96-well plates (Nunc, Roskilde, Denmark) and read with a V<sub>max</sub> microplate reader (Molecular Devices, Menlo Park, CA) at dual wavelength (405 minus 650 nm). The software package Softmax (Molecular Devices) was used for fitting the 12-point sigmoidal standard curve based on a four-parameter logistic method of Rodbard (1981).

**HPLC Conditions.** A Varian 9010 HPLC system with a 9050 UV-visible detector set at  $\lambda = 260$  nm and a C<sub>18</sub> (15 cm  $\times$  4.6 mm

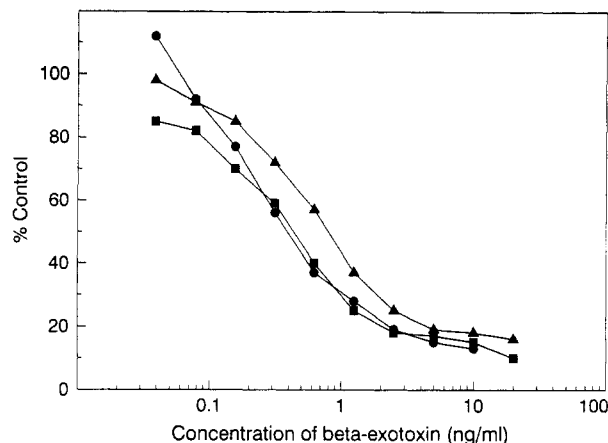
i.e., 5- $\mu\text{m}$  packing) column from Supelco, Inc. (Bellefonte, PA), was used to fraction samples according to the method of Campbell et al. (1987). The injection volume was 10  $\mu\text{L}$ , and fractions were collected every minute from 0 to 32 min. A mobile phase of 50 mM  $\text{KH}_2\text{PO}_4$ , pH 3.0, with a flow rate of 2 mL/min provided adequate conditions to separate the  $\beta$ -exotoxin components.

## RESULTS AND DISCUSSION

Using a checkerboard titration, the nine antisera were each tested against four different coating antigens. As shown in Table I, in heterologous systems (those in which the immunizing antigen differs from the coating antigen by both type of protein and coupling method) rabbits immunized with conjugates from the periodate method ( $\text{P}_1$ ) bound well to the glutaraldehyde-conjugated coating antigens ( $\text{G}_1$  or  $\text{G}_2$ ). In contrast, the rabbits immunized with either diazotized conjugate ( $\text{D}_1$ ) or glutaraldehyde conjugate generally yielded antibodies that bound poorly to heterologous hapten-protein conjugates. Exceptions to this trend were rabbits 948 and 950 (anti- $\beta$ -exotoxin- $\text{G}_1$ -KLH), which appeared to have moderate and high titers, respectively, when  $\beta$ -exotoxin- $\text{P}_1$ -BSA was used as coating antigen. In homologous systems (in which the immunizing antigen and the coating antigen differ in the type of protein but not the coupling method) rabbits 952, 957, 959, and 961 (anti- $\beta$ -exotoxin- $\text{P}_1$ -KLH) had significant binding to the corresponding coating antigen. The titer is defined for this study as the absorbance obtained at an antiserum dilution of 1:500 and a coating antigen concentration of 20  $\mu\text{g}/\text{mL}$ . When absorbance yielded by this antibody-antigen concentration was less than 0.3 OD, it was designated low titer, greater than 1.0 OD was termed high titer, and all values in between were termed moderate.

Specificity differences due to different immunogens clearly play a role. When poor binding of an antiserum to the coating antigen resulted in an absorbance lower than 0.3 or the use of uneconomically high amounts of antibody (dilution > 1:500), the system was not screened further. Systems having the highest titers on the largest number of heterologous antigens were favored for further development. These combinations of antibody and ELISA antigen were advanced to the next level of screening as described below. The antibodies having high titers only in homologous systems were generally not useful in competitive inhibition assay. The best assays were based on positional heterologous systems. These data indicate that assays of still greater sensitivity and specificity could be generated in the future using paired immunizing and coating antigens with positional homology and a heterologous coupling chemistry and/or handle.

**Antibody Selection and Assay Optimization.** Recognition of free  $\beta$ -exotoxin was tested using the optimal concentrations for the  $\beta$ -exotoxin conjugates and antiserum determined from the checkerboard titration results described above. The ability of  $\beta$ -exotoxin to inhibit the binding of anti- $\beta$ -exotoxin antibody to antigen-coated plates is shown in Figure 2. The results are expressed as percent control of uninhibited antibody plotted against a variable concentration of analyte. This figure shows that differing sensitivities for the analyte may be obtained by simply changing the coating antigen. Other rabbits immunized with the same immunogen as the rabbit shown in Figure 2 produced antibodies of varying sensitivity for the  $\beta$ -exotoxin, even when tested on the same coating antigen. For example, the antisera against  $\beta$ -exotoxin- $\text{P}_1$ -KLH using  $\beta$ -exotoxin- $\text{G}_1$ -BSA as coating antigen gave the following  $\text{IC}_{50}$  values: 0.36, 1.72, 5.56, 38.8, and 52.8 ng/mL for antibodies 957, 959, 960, 952, and 961, respectively.

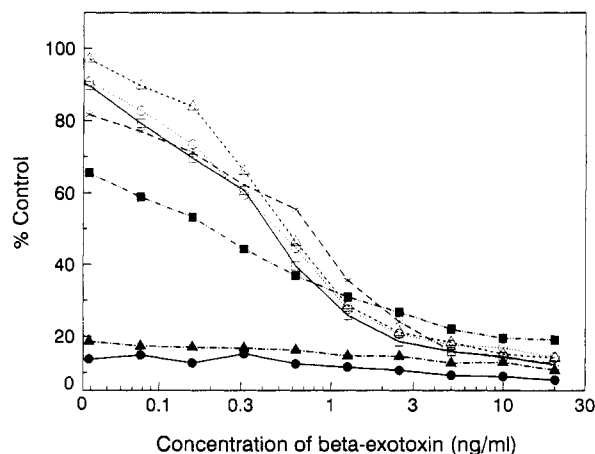


**Figure 2.** Standard curves of the  $\beta$ -exotoxin from *B. thuringiensis* in PBSTA using antibody 957 with  $\beta$ -exotoxin- $\text{G}_1$ -BSA ( $\blacksquare$ ),  $\beta$ -exotoxin- $\text{G}_2$ -BSA ( $\blacktriangle$ ), and  $\beta$ -exotoxin- $\text{D}_1$ -BSA ( $\bullet$ ) as the coating antigen.  $\text{IC}_{50}$  values for these curves were 0.4, 0.46, and 0.83 ng/mL, respectively.

**Table II.** Percent Cross-Reactivity of Various Structurally Related Compounds Relative to  $\beta$ -Exotoxin\*

inhibitor	$\text{IC}_{50}$ , mg/mL	% cross-reactivity
$\beta$ -exotoxin of <i>B. thuringiensis</i>	$4 \times 10^{-7}$	100
dephosphorylated $\beta$ -exotoxin		<0.4 <sup>b</sup>
adenosine 5-monophosphoric acid	1.84	<0.001
adenosine 5-diphosphate, Na-2H <sub>2</sub> O	0.067	0.004
adenosine 5-triphosphate, Na salt	0.111	0.006
nicotine adenine dinucleotide phosphate (NADP)	0.062	0.006

\* Cross-reactivity studies were conducted as described under Materials and Methods using rabbit 957 (anti- $\beta$ -exotoxin- $\text{P}_1$ -KLH) and  $\beta$ -exotoxin- $\text{G}_1$ -BSA (0.5  $\mu\text{g}/\text{mL}$ ). D-Mannitol, D-(+)-mannose,  $\alpha,\alpha$ -trehalose, D-glucose 6-phosphate, guanine, guanidine hydrochloride, adenine hydrochloride, guanosine 3,5-cyclic monophosphate sodium salt, adenosine 3,5-cyclic monophosphate sodium salt, flavin adenine dinucleotide disodium salt, and calf thymus DNA did not inhibit the assay at concentrations up to 5 mg/mL. <sup>b</sup> Cross-reactivity determined from HPLC; see discussion on validation of  $\beta$ -exotoxin assay.



**Figure 3.** Effect of different amounts of protein from lysed cells of *B. subtilis* on the standard curve of  $\beta$ -exotoxin using a combination of antibody 957 and  $\beta$ -exotoxin- $\text{G}_1$ -BSA as the coating antigen. Protein levels used were 0 ( $\square$ ), 10 ( $\Delta$ ), 100 ( $\circ$ ), 250 ( $*$ ), 500 ( $\blacksquare$ ), 1000 ( $\blacktriangle$ ), and 10 000 ( $\bullet$ ) ng/mL.

Previous studies have demonstrated the value of heterologous assays in improving ELISA sensitivity for environmental compounds (Wie and Hammock, 1984; Harrison et al., 1989). The most sensitive assays utilized antisera against the periodate antigen and either  $\beta$ -exotoxin- $\text{G}_1$ - or - $\text{G}_2$ -BSA or  $\beta$ -exotoxin- $\text{D}_1$ -BSA as the

Table III. Effect of Fermentation Media on BT  $\beta$ -Exotoxin Production

medium <sup>a</sup>	$\mu\text{g}$ of $\beta$ -exotoxin produced/mL of medium									
	6 h post-inoculation	12 h post-inoculation	18 h post-inoculation	24 h post-inoculation	30 h post-inoculation	36 h post-inoculation	42 h post-inoculation	48 h post-inoculation	60 post-inoculation	66 h post-inoculation
1	0.002 <sup>b</sup>	0.003	0.09	0.02	0.05	0.06	0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1
2	0.001	0.02	0.20 $\pm$ 0.02	0.4	0.6	3.9 $\pm$ 0.4	4.0 $\pm$ 0.5	9.0 $\pm$ 0.6	37.2 $\pm$ 2.6	86.0 $\pm$ 8.5
3	0.005	0.007	0.02 $\pm$ 0.08	0.06	0.1	0.2	0.5	0.4 $\pm$ 0.1	4.4 $\pm$ 0.4	71.0 $\pm$ 0.6
4	0.002	0.08 $\pm$ 0.02	0.20 $\pm$ 0.04	1.2 $\pm$ 0.1	3.8 $\pm$ 0.7	6.9 $\pm$ 3.5	16.5 $\pm$ 0.3	36.0 $\pm$ 2.0	72.0 $\pm$ 1.1	85.0 $\pm$ 13.4

<sup>a</sup> Media were inoculated with the same amount of BT. Media samples were taken at the indicated times. The cells were removed by centrifugation, and the assay was conducted on the supernatant. The data are the average and standard deviation of three ELISA assay replicates with each sample run in quadruplicate wells for each assay. Medium 1, Yostern-Rogoff; medium 2, trypticase soy broth; medium 3, brain-heart infusion; medium 4, casein. <sup>b</sup> Where standard deviations are not listed, the standard deviation was less than 5% of the mean.

coating antigen. We postulate that the periodate method produced a good yield of high molecular weight conjugates, which worked well in the ELISA (Nakane and Kawaoi, 1974). On the basis of this information and the high sensitivity, we chose antibody 957 (at a dilution of 1:500) with the coating antigen  $\beta$ -exotoxin-G<sub>1</sub>-BSA (at 0.5  $\mu\text{g}$ /well). For this antibody the  $\beta$ -exotoxin-G<sub>2</sub>-BSA also was an acceptable coating antigen. Although the only known difference in the G<sub>1</sub> and G<sub>2</sub> series of conjugate preparations is they were prepared at different times, one can see in Figure 2 that the antiserum can distinguish between these two preparations. For this reason, the two preparations were always used separately and identified by the G<sub>1</sub> and G<sub>2</sub> designations.

**Specificity of Antibody.** The specificity of the antibody was tested by using structurally related compounds as inhibitors. All of the chemicals were tested in the concentration range 9  $\mu\text{g}/\text{mL}$ –5 mg/mL. As summarized in Table II, all compounds tested showed no or minimal cross-reactivity. The percent cross-reactivity ranged from 0 to 0.006% with rabbit 957 (anti- $\beta$ -exotoxin-P<sub>1</sub>-KLH) and the  $\beta$ -exotoxin-G<sub>1</sub>-BSA coating antigen. This antiserum was at least 1000 times more specific for  $\beta$ -exotoxin than the strongest competitors, ADP and ATP. Several other antigen/antiserum combinations were tested for inhibition by ADP and ATP. IC<sub>50</sub> values ranged between 0.1 and 5 mg/mL. The curves obtained for ADP and ATP with each assay system were parallel to each other. However, these slopes were very different from the  $\beta$ -exotoxin. For example, the slopes of the four-parameter fit for ADP, ATP, and  $\beta$ -exotoxin with antibody 957 were 0.714, 0.693, and 1.74, respectively.

To determine the cross-reactivity of the antibody for other forms of the  $\beta$ -exotoxin, a dephosphorylated sample of  $\beta$ -exotoxin was prepared according to the method of Farkas et al. (1969). After 10 mg of the  $\beta$ -exotoxin standard with 1 mg of alkaline phosphatase from bovine intestinal mucosa was placed in 0.1 M diethanolamine buffer, pH 9.8, and incubated at room temperature, aliquots of the reaction mixture were removed over time (24, 48, and 73 h) and examined for immunoreactive response. The ELISA response dropped rapidly with time when the  $\beta$ -exotoxin was exposed to alkaline phosphatase. By 72 h the ELISA response was less than 0.2% of the original response. In buffer containing  $\beta$ -exotoxin, but no alkaline phosphatase, there was no change in immunoreactivity. (See also Figure 4 and discussion to follow).

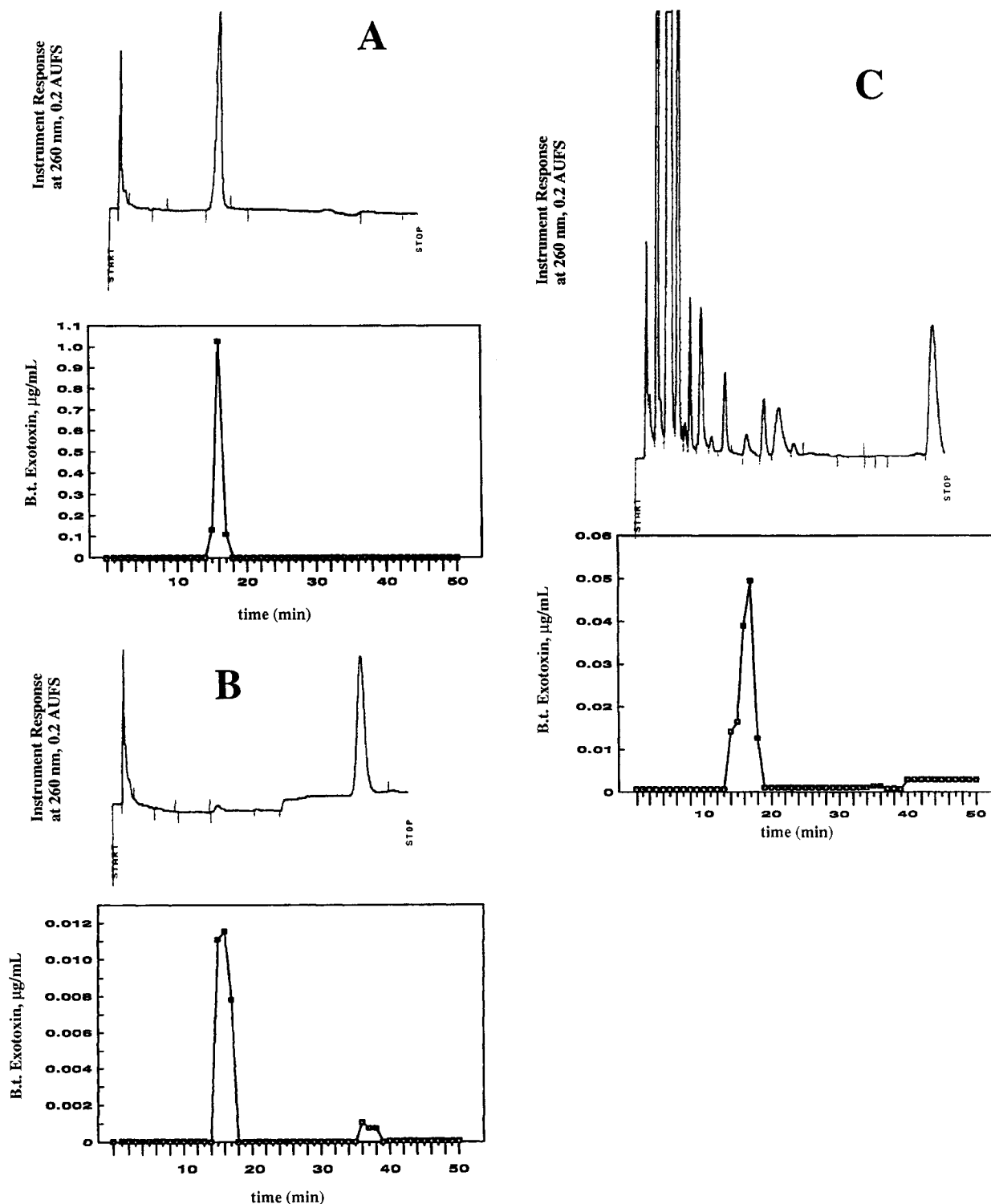
**Matrix Effects.** Ultimately this assay may be used to measure  $\beta$ -exotoxin in cultures of *Bacillus* or other bacterial species. Thus, one of the aims of this study was to determine the effect of the different concentrations of cell lysates of *B. subtilis* on the responses of the  $\beta$ -exotoxin ELISA. *B. thuringiensis* is often classified as a variant of *B. subtilis*. Figure 3 shows that the supernatant of lysed, washed log phase cells of *B. subtilis* had little effect on the determination of  $\beta$ -exotoxin when the protein

concentration of the lysate of *B. subtilis* was 10 and 100 ng/mL. A slight change in IC<sub>50</sub> was observed with the addition of 250 ng/mL of proteins from *B. subtilis* cell lysate. At 500 ng/mL of *B. subtilis* proteins, the interference was apparent. Complete inhibition was obtained when *B. subtilis* proteins were used at 1000 and 10 000 ng/mL.

It was not clear whether the interference was due to endogenous levels of  $\beta$ -exotoxin or to some other component of the bacterial extract. To test that hypothesis, we conducted the same experiment using the supernatant of an *E. coli* lysate. The standard curves remained unchanged until the protein concentration reached 1000 and 10 000 ng/mL, yet this shift in the standard curve was considerably less than seen with *B. subtilis*. Protein concentrations of the lysates were used to indicate the relative amounts of cell lysate added.

Another way to determine the nature of the matrix effect is to compare the parallelism of a dilution curve of the sample to the standard curve. If the curves are parallel, it is assumed the interference is specific (Perlstein, 1987). Thus, the data for the zero concentration of proteins used above were replotted and the slopes compared to the slope of the standard curve determined by the four-parameter fit. The *B* (or relative slope) values from the four-parameter fit for cell lysate of *B. subtilis* and *E. coli* were 4.26 and 7.02, respectively. These slopes appeared to be different from each other and from the slope of a standard curve (slope = 1.49), suggesting the interference found was of a much more complex nature, and the reader is cautioned to examine each matrix for interferences prior to quantitative analysis. The aim of this study was not to characterize the nature of the interference, and these studies are not intended to imply that the protein is the sole interfering material. This interference, however, may be reduced by diluting the sample prior to analysis.

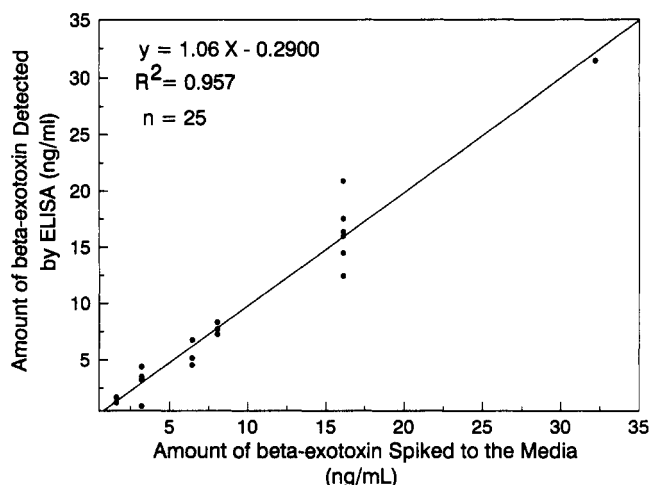
**Effect of Fermentation Media on BT  $\beta$ -Exotoxin Production.** In this set of experiments, the optimized ELISA was used to determine the  $\beta$ -exotoxin levels when BT was grown in different fermentation media. Two flasks for each of the four media (Table III) were inoculated with BT var. kurstaki (BTK) and incubated at 200 rpm in a shaking water bath at 34 °C. Aliquots from all flasks were removed at time intervals indicated in Table III and frozen until analysis. ELISA results demonstrate that BTK produced large amounts of  $\beta$ -exotoxin when grown in BBL trypticase soy broth and casein media, but, when grown in Yostern-Rogoff broth, significantly smaller amounts of  $\beta$ -exotoxin were produced. After 66 h,  $\beta$ -exotoxin produced in trypticase soy broth was almost equal to the amount produced in casein medium, but it was 366 times more than the toxin produced in Yostern-Rogoff broth and 12 times more than that of brain-heart infusion. The variation of  $\beta$ -exotoxin production could be due to the difference in media components. For instance, Yostern-Rogoff broth has higher carbohydrate



**Figure 4.** Correlation between HPLC response and immunoreactivity of eluted fractions. (A) Chromatogram of 1.3  $\mu\text{g}$  of the  $\beta$ -exotoxin standard (top); immunoreactivity corresponding to HPLC fractions (2 mL) (bottom). (B) Chromatogram of 1.3  $\mu\text{g}$  of the  $\beta$ -exotoxin standard following treatment with alkaline phosphatase at room temperature for 24 h (top); immunoreactivity of corresponding fractions (2 mL) (bottom). The immunoreactive peak at 16 min corresponds to the  $\beta$ -exotoxin and that at 37 min to the dephosphorylated product. (C) Chromatogram of a casein media sample taken 66 h after inoculation with *B. thuringiensis* var. *kurstaki* (top); immunoreactivity of corresponding fractions (2 mL) (bottom).

content (10 g of glucose/L), which may have prolonged the vegetative exponential phase of BT, delaying sporulation and lysis when compared to trypticase soy broth, Difco brain-heart infusion, and casein medium. This hypothesis is supported by the experiment using casein medium, which has no glucose. BT produced a large amount of  $\beta$ -exotoxin through the period of 12–60 h compared to trypticase soy broth and brain-heart infusion. The other reasons for variation of  $\beta$ -exotoxin production

could be the cumulative effect of quality and quantity of protein and carbohydrate and their ratio in the respective media. Difco brain-heart infusion contains 200 and 250 g/L of calf brain and beef heart infusion. This high content of proteins may contain one or more amino acids which inhibit the production of  $\beta$ -exotoxin. The purpose of this study was to demonstrate the utility of the ELISA in monitoring  $\beta$ -exotoxin levels under different growth conditions. The ELISA, possibly if used in conjunction



**Figure 5.** Correlation between the amount of  $\beta$ -exotoxin-fortified media and the amount detected by ELISA.

with an immunoassay for the  $\delta$ -endotoxin (Cheung et al., 1988), could be used to screen culture conditions very quickly either to optimize or to minimize the production of the  $\beta$ -exotoxin.

**Validation of the  $\beta$ -Exotoxin Immunoassay.** To verify that the immunoreactivity detected was in fact  $\beta$ -exotoxin, several samples were fractionated by HPLC and then the fractions analyzed by the ELISA. Figure 4 shows typical HPLC chromatograms of (A) the  $\beta$ -exotoxin standard, (B) a  $\beta$ -exotoxin standard that was enzymatically dephosphorylated, and (C) a casein medium sample taken at 66 h of incubation with *B. subtilis*. ELISA analysis of fractions from the HPLC further supported the characterization of the immunoreactive material being  $\beta$ -exotoxin. Of the immunoreactive material applied to the HPLC column, 96, 104, and 115% was recovered by ELISA in the fractions corresponding to the  $\beta$ -exotoxin peak, respectively. In Figure 4B, 1.3  $\mu$ g of  $\beta$ -exotoxin was incubated with alkaline phosphatase for 24 h. The reaction mixture was then applied to the HPLC column. The peak corresponding to the dephosphorylated product contained 0.23% of the total immunoreactivity placed on the column. The  $\beta$ -exotoxin peak (by UV) accounted for approximately 0.013  $\mu$ g. Assuming that the  $\beta$ -exotoxin lost was converted to the dephosphorylated form, the peak corresponding to the dephosphorylated form (retention time of 37 min) should contain about 1.287  $\mu$ g of material. Immunoassay could only detect 0.003  $\mu$ g, implying the cross-reactivity for the dephosphorylated form to be about 0.2%.

To further characterize this assay in directly detecting  $\beta$ -exotoxin in culture media, a series of 25 media samples were fortified from 0 to 32.4 ng/mL and analyzed in a blind fashion. As seen in Figure 5, the correlation between the amount of  $\beta$ -exotoxin spiked to the media and that detected by the ELISA is excellent. In a similar study, 10 samples were obtained from Sandoz Agro Inc., drawn from their noncommercial strain collection, and preselected by Sandoz for the presence or absence of  $\beta$ -exotoxin based on HPLC analysis. The ELISA results indicated that samples 1–6 contained less than 2 ng/mL and that samples 7, 8, 9, and 10 contained  $0.45 \pm 0.01$ ,  $1.56 \pm 0.37$ ,  $3.64 \pm 0.26$ , and  $2.74 \pm 0.85$  mg, respectively, of  $\beta$ -exotoxin/mL of fermentation media. HPLC results subsequently received from Sandoz indicated samples 1–7 contained less than 20  $\mu$ g/mL and samples 8, 9, and 10 had 1.4, 2.3, and 1.5 mg/mL, respectively. Thus, under the conditions described, levels of  $\beta$ -exotoxin detectable by HPLC were similar to that measured by the ELISA. The level of

$\beta$ -exotoxin in sample 7 was below the detection limit by HPLC, whereas the ELISA clearly detected the compound.

## CONCLUSIONS

An indirect competitive ELISA for BT  $\beta$ -exotoxin was developed which showed low cross-reactivity to the dephosphorylated product. The assay has a lower limit of detectability of 0.1 ng/mL in buffer and is significantly more sensitive than high-performance liquid chromatographic methods (Oehler et al., 1982; Danilova and Kruglyak, 1983; Johnson and Peterson, 1983). The ELISA assay can be used to qualify and quantitate the *B. thuringiensis*  $\beta$ -exotoxin in fermentation broths and thus may be a useful tool for quality control.

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