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Evidence for Two Immune Inhibitors from *Bacillus thuringiensis* Interfering with the Humoral Defense System of Saturniid Pupae

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Mutants of *Bacillus thuringiensis* lacking either β-exotoxin or γ-endotoxin were compared for their virulence using pupae of a giant silk moth. Known doses of viable log-phase bacteria were injected, and the response was followed as the number of viable bacteria in the hemolymph. The results obtained imply that, in the system used, neither the β-exotoxin nor the γ-endotoxin and the sporeforming ability are of importance for virulence. Results with sterile culture filtrate from *B. thuringiensis* have given evidence for the production of two inhibitors, A and B, which interfere with the humoral defense system in pupae of *Hyalophora cecropia*. Inhibitor A, which blocked the lysis of *Escherichia coli*, was precipitated by trichloroacetic acid and sensitive to heating. Inhibitor B, which blocked the killing of *Bacillus cereus*, was soluble in trichloroacetic acid and resistant to 90°C for 5 min. Both inhibitors are believed to contribute to the insecticidal nature of *B. thuringiensis*.

In microbial control of insect pests, *Bacillus thuringiensis* has so far been the organism most widely used (4, 5, 20). Its insecticidal properties are generally believed to stem from two toxins with different specificities. The β-exotoxin is a thermostable adenine nucleotide that is highly toxic for the larvae of certain flies (4, 20). The γ-endotoxin, which is both the main part of the crystalline inclusion and a normal constituent of the spore coat (7, 18, 21), is highly toxic for larvae of many *Lepidoptera* (5, 20). Besides these two rather well-characterized toxins, *B. thuringiensis* is also known to produce two hemolysins (14) and a metalloprotease (13).

The virulence of a bacterium, however, does not only depend on the production of toxins but also on the invasiveness of the strain and its ability to withstand the defense reactions of the host. The latter property can be due to passive resistance, as well as to the production of substances that block the immune system of the host. However, for vertebrates, only a limited number of microbial immune inhibitors are yet known (9, 11, 13, 15, 24); for invertebrate systems, lipid A seems to be the only such inhibiror reported (3, 8).

We have previously shown that pupae of giant silk moths (family *Saturniidae*) have an inducible, multicomponent defense system that requires both ribonucleic acid (RNA) and protein synthesis to be expressed (2, 8). We here report experiments which indicate that *B. thuringiensis* produces at least two substances that interfere with different parts of this immune system. To exclude as experimental factors the invasiveness of the toxins already known, we performed in vivo experiments with injected doses of mutants deficient in one or more toxins. We also report in vitro experiments with sterile culture filtrates and hemolymph from immunized pupae.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. We have used two parental strains of *B. thuringiensis*. One of these, Bt7, is identical to strain HL2 of Somerville (19), a crystal-negative mutant isolated from a streptomycin- and penicillin-resistant derivative of *B. thuringiensis* subsp. *alesti*. Strain Bt7 belongs to serotype 3, which does not produce any exotoxin (G. Carlberg, thesis, Univ. of Helsinki, Helsinki, Finland, 1973). The other parental strain, Bt11, is a streptomycin-resistant mutant (2), derived from an exotoxin-producing, crystal-negative line of *B. thuringiensis* subsp. *gelechiae* used by Sebesta et al. (16). Our sporulation-deficient mutants, strains Bt72 and Bt12, were selected as rifampin resistant (23) and subsequently tested for heat sensitivity after growth in a sporulation medium (7). Strain Bt74 is a spontaneous sporeforming revertant of strain Bt72.

Bacillus cereus is considered to be very closely related to *B. thuringiensis* (10, 21). Since the former organism is more convenient to work with, we used

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in our inhibitory assay strain Bc11, a streptomycin-resistant derivative of *B. cereus* ATCC 11778.

Normally, both *B. cereus* and *B. thuringiensis* were grown at 37°C in a rich medium. Viable counts were performed with double layers of soft agar, a procedure that gives small but distinct colonies. All plates contained streptomycin(100 μg/ml). We diluted *B. cereus* and *B. thuringiensis* in their growth medium, because dilutions in physiological salt solutions were found to increase the susceptibility to the defense reactions of the insects. For preparation of optimal amounts of inhibitors from *B. thuringiensis*, the strains were grown for 22 h in sporulation medium at 30°C (7). After centrifugation, remaining bacteria and spores were removed by sterile filtration using 0.20-μm plain membranes (Nalge/Sybron Corp., Rochester, N.Y.).

Insects used and their immunization. For experimental infections in vivo, we used pupae of *Callosamia promethea*, weighing about 1 g. For in vitro experiments with the immune system, we used hemolymph from diapausing pupae of *Hyalophora cecropia*, weighing about 5 g. All insects were obtained from an American dealer. After shipment to Umeå the pupae were stored at 8°C. During experiments they were maintained in a climate chamber (Sherer CEL44) at 25°C, about 75% humidity, and long-day conditions (15 h of light to 9 h of darkness). Immunization was performed by injecting viable cells of *Enterobacter cloacae* β12 into the thorax of the pupa (2). The doses given were, for *C. promethea*, 4×10^5, and for *H. cecropia*, about 10^6 viable cells. Immune hemolymph was collected 3 to 4 days later as described (2).

Assay methods. Determination of the antibacterial activity against *Escherichia coli* K-12 D31 was as described before (2). For assay of the killing activity against *B. thuringiensis* or *B. cereus*, the reactions mixture contained 100 μl of hemolymph from immunized pupae of *H. cecropia* and 5 μl with about 2×10^3 viable cells of the appropriate strain. At different times during 2 h, portions of 10 μl were withdrawn and added to 3 ml of soft agar, which was spread on a plate. For assay of the inhibitory activity, 100 μl of hemolymph and 10 μl of sample were preincubated at 25°C for 30 min. Test bacteria, strain Bc11, were then added (2×10^3 cells in 5 μl), and incubation was continued for an additional 90 to 120 min. During this period, 10-μl portions were withdrawn at different times and plated for surviving test bacteria. When the number of assays was large, the reaction mixture contained 25 μl of hemolymph, 5 μl of sample, and 2 μl of test bacteria. Only two aliquots were withdrawn at 10 and 90 min.

Inhibition of lysis of *E. coli* D31 was performed as follows. Hemolymph (25 μl) from immunized pupae of *H. cecropia* and an equal volume of sample were incubated at 37°C for 30 min. After this time a portion of 25 μl was withdrawn and added to 1 ml of 0.1 M potassium phosphate buffer, pH 6.4, with 2×10^{-3} M dithiothreitol and about 10^8 viable cells of strain D31. The lysis of the bacteria was measured at room temperature in a spectrophotometer (Zeiss PQII) as the decrease in absorbance at 450 nm during 30 s or 1 min. All dilutions and controls were performed with sterile medium. Results are given as percentage of lysis inhibited.

RESULTS

Virulence of different strains of *B. thuringiensis*. We first compared a set of different mutants for their ability to grow in untreated and immunized pupae of *C. promethea*. A dose of about 10^3 viable cells of the respective strains was injected into the thorax of the pupa. Three days later, aliquots of hemolymph were removed and assayed for the number of viable bacteria. Table 1 shows that an immunization can delay or prevent an infection with *B. thuringiensis*. The fact that strain differences were more obvious in immunized pupae indicates that the mutants could differ in their response to the immune system. In further experiments we have therefore only used immunized pupae.

To substantiate the strain differences seen in Table 1, we performed dose-response curves with immunized pupae of *C. promethea*. The results in Fig. 1 show that the sporulation-deficient mutant Bt72 (*spo*$^-$) was less virulent than its parental strain Bt7 (*spo*$^+$). The revertant, strain Bt74, regained most of the virulence of strain Bt7. However, no difference was observed between strain Bt11 (*spo*$^+$) and strain Bt12 (*spo*$^-$). Thus, in this system sporulation as such does not always contribute to virulence (cf. references 6, 21). Figure 1 also shows that *B. thuringiensis* subsp. *gelechiae* (Bt11 and Bt12) was significantly less virulent than *B. thuringiensis* subsp. *alesti* (strain Bt7 and its derivatives).

One factor contributing to the virulence could be resistance to the immune system. To

TABLE 1. *Response of C. promethea pupae to injection with different mutants of B. thuringiensis*[a]

Mutant strain	Parent strain	Phenotype		Response at day 3	
		Spo	Exo	Untreated	Vaccinated
Bt7	PSR24	+	−	5×10^6	10^5
Bt72	Bt7	−	−	5×10^6	20
Bt74	Bt72	+	−	6×10^6	4×10^6
Bt11	Bt1	+	+	5×10^6	0
Bt12	Bt11	−	+	0	7

[a] All strains are crystal negative (see Materials and Methods). Sporulation-negative mutants were isolated as described (23). Strain Bt7 and its derivatives (serotype 3) do not produce the β-exotoxin (G. Carlberg, thesis, Univ. of Helsinki, Helsinki, Finland, 1973). Vaccination was performed by injecting each pupa with 2×10^5 viable cells of *Enterobacter cloacae* (2). At day 3, all pupae were challenged with 10^3 viable cells of the respective strain listed. Response is expressed as the number of viable bacteria/10 μl of hemolymph on day 3 after the challenge.

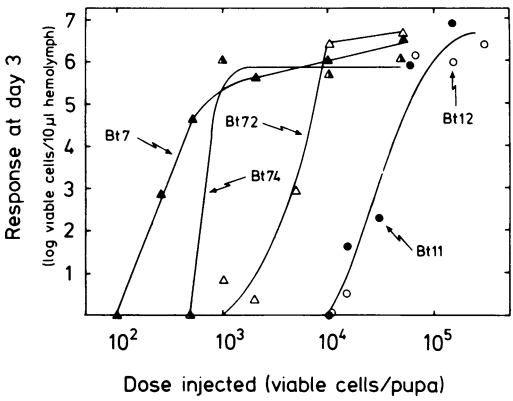

FIG. 1. *Dose-response curves for different mutants of B. thuringiensis subsp. alesti (triangles) and B. thuringiensis subsp. gelechiae (circles). Both parental strains, Bt7 and Bt11 (filled symbols), are crystal negative and sporeforming; strains Bt72 and Bt12 are sporulation-negative mutants (open symbols); and strain Bt74 (half-filled triangles) is a sporeforming revertant from Bt72. At day 0, doses of exponentially growing cells were injected into the thorax of immunized pupae of C. promethea. At day 3, hemolymph samples were assayed for the number of viable bacteria.*

investigate this possibility by in vitro experiments, large amounts of hemolymph were required. We therefore used hemolymph from immunized pupae of *H. cecropia* for a comparison of our two parental strains. Figure 2 shows that strain Bt11 was more resistant to the antibacterial activity than strain Bt7. However, this difference is small compared with those recorded in Fig. 1. It should also be emphasized that compared with the killing of *E. coli* or *Bacillus subtilis* (2, 3, 8) the killing of *B. thuringiensis* is a very slow and inefficient reaction. The curves in Fig. 2 also indicate that *B. thuringiensis* was killed by a multi-hit mechanism, contrary to the single-hit kinetics observed with *E. coli* (2). In the controls with normal hemolymph, both strains grew slowly.

Some of our results in Table 1 indicate that during an infection *B. thuringiensis* could interfere with the defense system of the host. We therefore followed the antibacterial activity of two immunized pupae. Two days after vaccination, one of the pupae received an injection with *B. thuringiensis*, and the other was given an equal volume of sterile W-saline, a salt solution physiological to *Lepidoptera* (25). Table 2 shows that the antibacterial activity was gradually suppressed simultaneously with the progression of the infection with *B. thuringiensis*. In the control, the antibacterial activity continued to rise. These results are consistent with the assumption that *B. thuringiensis* produces one or more substances that either inhibit the antibacterial activity as such or interfere with the formation of one or more of the components needed for this activity.

Evidence for a production of two immune inhibitors by *B. thuringiensis*. Preliminary experiments showed that a sterile culture filtrate obtained after growth of *B. thuringiensis* Bt7 exerted a marked inhibitory effect on the antibacterial activity in hemolymph from im-

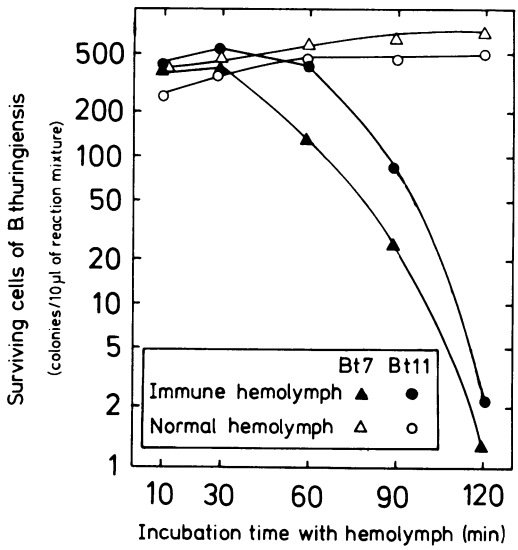

FIG. 2. *Surviving cells of B. thuringiensis after incubation with hemolymph from an untreated (open symbols) and from an immunized pupa (filled symbols) of H. cecropia. Strain Bt7 (triangles); strain Bt11 (circles). The reaction mixture contained 100 µl of hemolymph and 5 µl containing about 2 × 10³ viable cells. Aliquots were withdrawn at the times indicated and assayed for surviving bacteria.*

TABLE 2. *In vivo effects of B. thuringiensis on the antibacterial activity in immunized pupae of C. promethea*[a]

Treatment of pupae	Viable cells of Bt7/10 µl of hemolymph		Antibacterial activity (Δlog viable *E. coli*/1 min)		
	24 h	48 h	−1 h	24 h	48 h
W-saline			2.4	3.1	3.7
Strain Bt7	8	10^6	1.7	2.5	0

[a] Two pupae of *C. promethea* were vaccinated with *E. cloacae*. When the antibacterial activity was expressed (zero time), one pupa was given an injection of 25 µl, containing 5 × 10³ viable cells of *B. thuringiensis* Bt7, and the other was given an equal volume of a physiological salt solution, W-saline (25). Hemolymph samples were removed at the times indicated and assayed for their antibacterial activity (2) and for the number of viable cells of strain Bt7.

munized pupae of *H. cecropia*. A sonicated extract of the same strain did not show much inhibitor effect. We therefore performed a series of experiments with sterile culture filtrate which aimed towards a preliminary characterization of this immune inhibitory activity. We have previously shown that *E. coli* lipopolysaccharide inhibited lysis of *E. coli* without affecting the killing of *B. subtilis* (2, 8). As assay methods we therefore used the inhibition of lysis of *E. coli* D31 and the inhibition of killing of *B. cereus* Bc11. Table 3 shows that these assay methods gave different results for solubility in trichloroacetic acid and sensitivity to heat. The results imply that at least two inhibitory substances are present in the culture filtrate from strain Bt7. No effect was obtained with Trasylol, an inhibitor of proteolytic enzymes (cf. reference 13). The substance monitored by inhibition of strain D31 lysis will in the following be referred to as inhibitor A, whereas the substance assayed by blocking of strain Bc11 killing will be called inhibitor B.

In an attempt to characterize these inhibitory assays, we compared the effects obtained by different amounts of culture filtrate. Figure 3 shows that for inhibitor A a sigmoid curve was obtained with a undiluted culture filtrate. After a threefold dilution, the inhibition was approximately proportional to the amount of culture filtrate added. This method can therefore be used as a quantitative assay for inhibitor A.

Figure 4 shows that different amounts of culture filtrate to a varying degree inhibited the killing of strain Bc11. With undiluted culture filtrate, strain Bc11 grew in the hemolymph. We previously used the killing of *E. coli* as our main assay for the characterization of the im-

TABLE 3. *Characterization of inhibitors from B. thuringiensis*[a]

Addition to hemolymph	*E. coli* D31 lysis (%)	*B. cereus* Bc11 survival (%)
Control with unused medium	100	<1
Untreated filtrate	<5	100
Filtrate after trichloroacetic acid precipitation	84	100
Filtrate after 90°C/5 min	92	100
Filtrate after Trasylol treatment	12	100

[a] Assays and preparation of culture filtrate from strain Bt7 were as described in Materials and Methods. Trichloroacetic acid was used at a final concentration of 5%; excess trichloroacetic acid was removed by ether extractions. Trasylol treatment was as before (8).

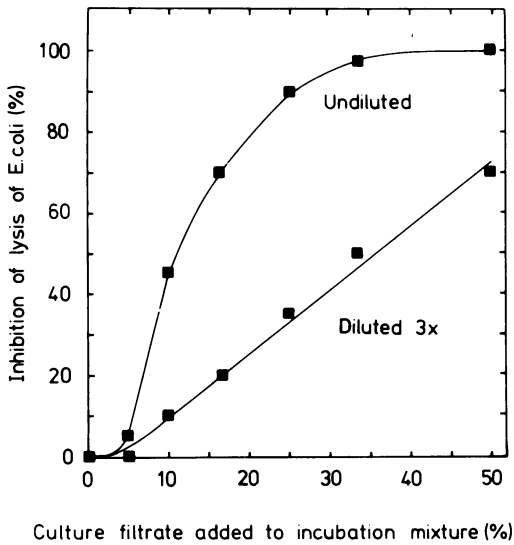

FIG. 3. *Characterization of the assay for inhibitor A. Hemolymph (25 μl) from an immunized pupa of H. cecropia was incubated with 25 μl of different dilutions of sterile culture filtrate from B. thuringiensis Bt7. After 30 min of incubation at 37°C, a portion of 25 μl was withdrawn and added to 1 ml of buffer containing about 10^8 viable cells of E. coli D31. The lysis of this bacterial suspension was followed by reading absorbance at 450 nm after 1 min of further incubation. For details see Materials and Methods.*

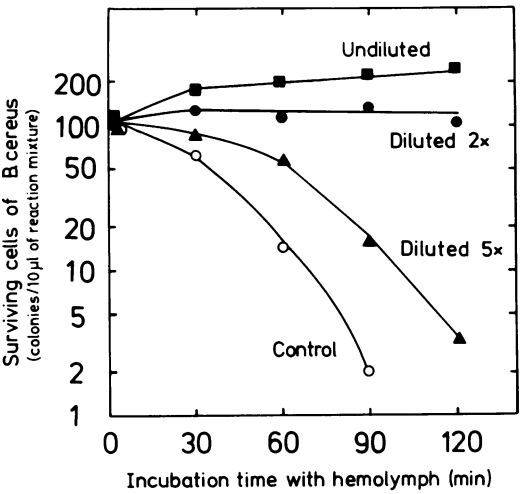

FIG. 4. *Inhibition of killing of B. cereus Bc11 by different amounts of sterile culture filtrate from B. thuringiensis Bt7, the procedure used for assay of inhibitor B. Culture filtrate undiluted (■), diluted twice (●), diluted five times (▲), and control with sporulation medium (○). Hemolymph from an immunized pupa of H. cecropia was preincubated with the culture filtrate at 25°C for 30 min. About 2 × 10^3 viable cells of strain Bc11 were then added, and aliquots were removed at different times and assayed for surviving bacteria.*

mune system in saturniid pupae (2, 3, 8). This reaction is about 10^3 times more efficient than the killing of *B. cereus* shown in Fig. 4. The assay of inhibitor B is therefore rather insensitive and at best a semiquantitative method for estimating this substance.

We next compared the amounts of inhibitor A produced by different strains during growth experiments. Figure 5 shows that the concentration of inhibitor A increased in the culture filtrate at the time when the bacteria were leaving the exponential phase of growth. Strains Bt7 and Bt72 produced about equal amounts of inhibitor A, whereas significantly lower levels were produced by strain Bt11. In a similar experiment, we also compared the amounts of inhibitor A produced by a hemolysis-negative double mutant, its wild-type ancestor strain Bt11, and the international reference strain E61. There were no significant differences among these three strains in the amounts of inhibitor A produced.

The results in Table 3 suggest that inhibitor A could be a macromolecule, whereas inhibitor B could be of a low-molecular-weight nature. We therefore subjected freeze-dried culture filtrate to a fractionation on a column with Sephadex G-25. The gel filtration in Fig. 6 shows that inhibitor A was eluted slightly after the void volume, whereas most of the material was retarded. Radioactive leucine was used as an internal standard. Because of the time-consuming assay, we only tested certain of the fractions for the presence of inhibitor B. The result indicates that inhibitor B was also eluted with the void volume. The first peak was therefore pooled and precipitated with trichloroacetic acid to a final concentration of 5%. The precipi-

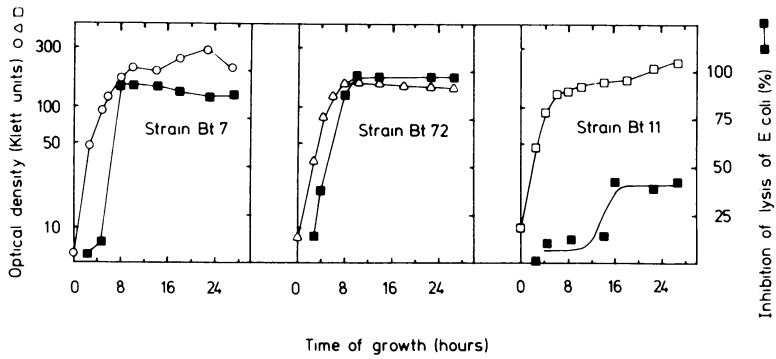

FIG. 5. *Comparison of the amounts of the inhibitor A produced by different strains of B. thuringiensis during growth in sporulation medium (7). The cultures were incubated on a rotary shaker at 30°C. At different times, aliquots were removed and plated for viable bacteria (open symbols). Other aliquots were centrifuged, sterile filtered and, after a threefold dilution, assayed for ability to inhibit lysis of E. coli D31* (■). *For assay details see Materials and Methods.*

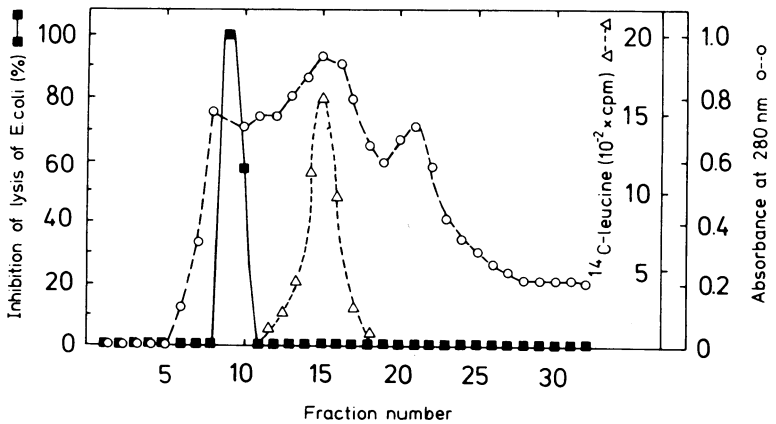

FIG. 6. *Gel filtration of freeze-dried culture filtrate from B. thuringiensis Bt7. The column (23 by 2 cm) contained Sephadex G-25, it was equilibrated with 0.1 M potassium phosphate buffer, pH 6.4, the flow rate was 60 ml/h, and the fraction volume was about 3 ml. Each fraction was assayed for ability to inhibit E. coli lysis* (■), *for adsorption in ultraviolet light* (○), *and for* ^{14}C (△) *from the labeled leucine used as internal standard.*

tate was removed and excess trichloroacetic acid was extracted by ether from the supernatant. Only inhibitor B could be demonstrated in this supernatant. We therefore extracted freeze-dried culture filtrate with 5% trichloroacetic acid and removed excess trichloroacetic acid by ether extractions. The preparation so obtained was passed through the same column of Sephadex G-25 as was used before. The elution pattern given in Fig. 7 shows that inhibitor B again appeared right after the void volume. However, other gel filtration experiments have indicated that lower-molecular-weight forms also could exist. It may therefore be possible that the main peak of inhibitor B in Fig. 7 represents an aggregated form, but further experimentation is required to clarify this point.

DISCUSSION

Can inhibitor A be identical to any of the known toxins? Since inhibitor A is heat labile and trichloroacetic acid insoluble it cannot be identical to the β-exotoxin, a heat-stable adenine nucleotide (20). Furthermore, we have shown that inhibitor A was produced by strains Bt7 and Bt72 (Fig. 5), derivatives of serotype 3, which is known to be devoid of the β-exotoxin (G. Carlberg, thesis, Univ. of Helsinki, Helsinki, Finland, 1973).

The γ-endotoxin is both the main part of the protein crystal and a constituent of the spore coat (7, 18, 21). Since the parental strains of *B. thuringiensis*, Bt7 and Bt11, are both crystal negative, our sporulation-deficient mutants, Bt72 and Bt12, should both be expected to produce minimal amounts of the γ-endotoxin. However, strains Bt12 and Bt72 both produced inhibitor A, and no difference was found in the amounts produced by the sporulation-negative mutant Bt72 and its parental strain Bt7 (Fig. 5). It is therefore highly unlikely that inhibitor A could be identical to the γ-endotoxin.

Our hemolysis-negative double mutant is presumably deficient in the hemolytic factors described (14). We therefore compared the amounts of inhibitor A produced by this hemolysis-negative strain and its wild-type ancestor. Since no difference was found, it is unlikely that inhibitor A is identical to any of the hemolysins.

We have also shown that an inhibitor of proteolytic enzymes (Trasylol) was without effect on inhibitors A and B. It is therefore unlikely that these substances are proteolytic enzymes (cf. reference 13).

Can inhibitor B be identical to any of the known toxins? In our preliminary characterization inhibitor B was found to be heat stable and soluble in trichloroacetic acid. Therefore, it can hardly be of a protein nature and thus not identical to either the γ-endotoxin, the hemolysins, or the metalloprotease. The β-exotoxin is known to be heat stable and to inhibit in vitro synthesis of RNA and polyphenylalanine (1, 17, 22). Since we have previously shown that induction of immunity in *Samia cynthia* required both RNA and protein synthesis, we expected the β-exotoxin to have an immunosuppressive effect on our insect system. However, our dose-response curves (Fig. 1) showed that strains

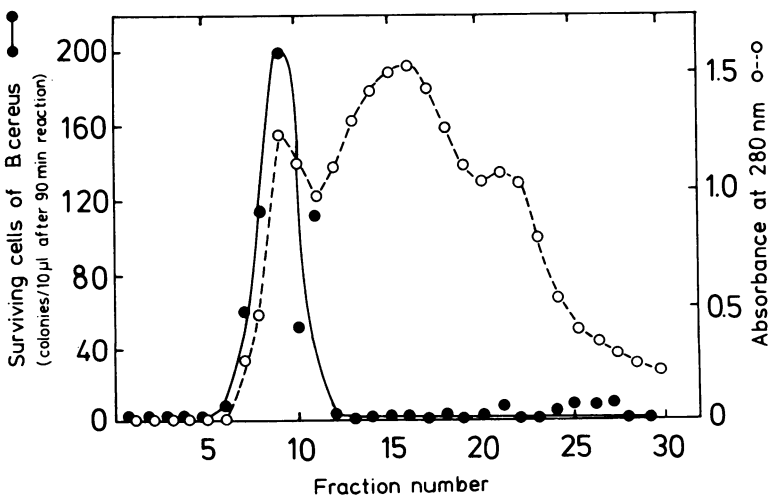

FIG. 7. *Gel filtration of culture filtrate from B. thuringiensis Bt7, extracted with 5% trichloroacetic acid. The column and the experimental conditions were the same as used in Fig. 6. Each fraction was assayed for ability to inhibit the killing of B. cereus Bc11 (●) and for adsorption in ultraviolet light* (○).

Bt11 and Bt12, which both produce significant amounts of the β-exotoxin, were much less virulent than Bt7 and its derivatives, which all are deficient in the β-exotoxin. Furthermore, since sterile culture filtrate of the latter strains contain inhibitor B, this substance cannot be identical to the β-exotoxin.

Staphylococcal protein A and lipid A from gram-negative bacteria are both known to block the activity of mammalian complement (9, 24). We have previously shown that the antibacterial activity in *H. cecropia* in several respects resembles complement and that lipid A in low concentrations is an effective inhibitor of the killing of *E. coli* (3, 8). We therefore investigated whether protein A and lipid A affected the killing of *B. cereus*. Since no evidence was obtained for such an activity (unpublished data), we conclude that inhibitor B must interfere with step(s) different from those of other known immune inhibitors. However, it should be emphasized that inhibitor B is only characterized as trichloroacetic acid soluble and heat resistant. Gel filtration on G-25 (Fig. 7) would indicate that inhibitor B is a macromolecule. However, further experiments are needed to clarify whether this material represents an aggregated form of a low-molecular-weight substance.

Virulence of different strains of *B. thuringiensis*. Many investigators have previously tried to compare different strains of *B. thuringiensis*, in most cases by feeding spore-containing preparations to living insects and recording either the mean lethal dose or the food consumed (4, 5, 6, 21). In our comparison of the virulence of different strains we always injected known doses of log-phase bacteria, and we recorded the number of viable cells in the hemolymph. This procedure eliminates the invasiveness of the bacteria as an experimental factor and makes it somewhat easier to discuss the remaining factors that contribute to the virulence. By this method of study, we have shown that in *C. promethea* there seems to be no significant contribution to the virulence from either the β-exotoxin or the γ-endotoxin. However, three other factors have been found that can contribute to the virulence, namely, the passive resistance toward the antibacterial activity and the production of the two inhibitors, A and B. Neither of these factors has been experimentally demonstrated before, and at the present stage we cannot safely estimate their respective contributions to the virulence.

It should be emphasized that the net result of an infection with injected *B. thuringiensis* must be a dynamic process in which bacterial synthesis of the immune inhibitors and the host synthesis of the immune proteins are competing with each other. In addition, the ability of the bacteria to repair the lésions caused by the defense reaction must be a factor of relevance. Since the interaction between the bacteria and the immune system, as well as between the inhibitor and the immune system, probably takes place with constant stoichiometric ratios, the ability of the different strains to produce the inhibitors, together with the growth rates, may become crucial factors that determine the final fate of an infection.

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