Comparative Toxicity of Spores and Crystals from the NRD-12 and HD-1 Strains of *Bacillus thuringiensis* subsp. *kurstaki* to Neonate Beet Armyworm (Lepidoptera: Noctuidae)

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ABSTRACT The toxicities of different components of the spore–parasporal body complex of the NRD-12 and HD-1 strains of *Bacillus thuringiensis* subsp. *kurstaki* (Berliner) to neonate *Spodoptera exigua* (Hübner) were determined using diet incorporation bioassays. The LC_{50}'s of NRD-12 and HD-1 from lyophilized powders obtained from sporulated liquid cultures were 20.8 μg/ml diet and 49.3 μg/ml diet, respectively. Both strains produced a bipyramidal crystal containing a 135-kDa (kilodalton) protein (P1) and a cuboidal crystal containing a 65-kDa protein (P2). Preparations of the NRD-12 P1 crystal (LC_{50} = 63.0 μg/ml diet) were more toxic than similar preparations of HD-1 (LC_{50} = 153 μg/ml diet). Alternatively, the P2 crystal from HD-1 (LC_{50} = 34.2 μg/ml diet) was more toxic than preparations of the NRD-12 P2 crystal (LC_{50} = 72.4 μg/ml diet). Combinations of the P1 and P2 crystals of NRD-12 were more toxic (LC_{50} = 82.0 μg/ml diet) than HD-1 P1/P2 preparations (LC_{50} = 157 μg/ml diet). Spore preparations from HD-1 (LC_{50} = 117 μg/ml diet) were slightly more toxic than those from NRD-12 (LC_{50} = 166 μg/ml diet). Significant ($P \leq 0.005$) potentiation resulted when the LC_{25} of spores was added (1:1 wt/wt) to the LC_{25} of the 65-kDa/135-kDa protein combination from the same isolate. Significant ($P \leq 0.005$) potentiation also was observed when NRD-12 spores were added to HD-1 crystal protein and when HD-1 spores were added to the NRD-12 crystal protein. However, there was no difference in toxicity between these two combinations. These data suggest that the comparatively high toxicity of NRD-12 to *S. exigua* is due at least in part to the 135-kDa proteins of this strain.

KEY WORDS Insecta, beet armyworms, *Spodoptera exigua, Bacillus thuringiensis*

INTEREST IN MICROBIAL INSECTICIDES based upon the insecticidal bacterium, *Bacillus thuringiensis* subsp. *kurstaki* (Berliner) (serotype H 3a:3b) has been increasing in recent years because of the need for alternatives to chemical insecticides (Morris et al. 1986, as cited in Hefford et al. 1987). Development of transgenic plants expressing *B. thuringiensis* toxins (Shah et al. 1987) and increased use of *B. thuringiensis* in integrated pest management programs (Moar & Trumble 1987) have stimulated the search for strains of this bacterium that are more active against certain lepidopterous pests. Dubois (1985) reported that the NRD-12 strain of *B. thuringiensis* subsp. *kurstaki*, which was isolated from diseased spruce budworm, *Choristoneura fumiferana* (Clemens), larvae, was more toxic to spruce budworm, gypsy moth, *Lymantria dispar* (L.), and tobacco budworm, *Heliothis virescens* (F.), but not to the cabbage looper, *Trichoplusia ni* (Hübner), when compared with the HD-1 strain of *B. thuringiensis* subsp. *kurstaki*. This new strain was the active ingredient of the commercial product Javelin, introduced in 1985 by Sandoz Corporation (Wasco, Calif.), which Moar et al. (1986) showed to be 3–4 times more toxic to the beet armyworm, *Spodoptera exigua* (Hübner), than Dipel 2X (Abbott Laboratories, North Chicago, Ill.), which contains the HD-1 strain.

Most of the insecticidal activity of *B. thuringiensis* subsp. *kurstaki* is due to a protein or group of proteins (referred to frequently as P1) with a molecular mass of approximately 135 kDa that make up the characteristic bipyramidal crystal. Additionally, a second crystal type, cuboidal in shape, and containing a protein of approximately 65 kDa (P2) was discovered in HD-1 by Yamamoto & McLaughlin (1981). This protein not only killed lepidopteran larvae but also dipteran larvae such as mosquitoes. In addition to toxic protein crystals, there have been many reports of *B. thuringiensis* spores being insecticidal, and in several of these it has been shown that the *B. thuringiensis* preparations with the highest toxicity are those that contain spores and crystals (Heimpel & Angus 1959, Schesser & Bulla 1978, Mohd-Salleh & Lewis 1982, Li et al. 1987).

The high level of toxicity of the NRD-12 strain to larvae of *S. exigua* suggested that one or more components of the spore–parasporal inclusion body complex of this strain were more toxic to this species

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than the corresponding components of HD-1. The purpose of this study, therefore, was to determine which components of NRD-12 accounted for its higher toxicity to *S. exigua*.

Materials and Methods

Bacterial Isolates. HD-1 was isolated from Dipel 2X and NRD-12 from Javelin as described by Moar et al. (1986). Isolates were grown on nutrient agar, and single colonies containing bipyramidal inclusion bodies were isolated and grown for further study.

Growth Conditions and Parasporal Inclusion Body Isolation. Cells were grown in 500 ml of modified glucose–yeast–salts (GYS) medium (1 g glucose, 2 g $(NH_4)_2SO_4$, 3 g K_2HPO_4, 5 g yeast extract, 0.2 g $MgSO_4$, 80 mg $CaCl_2 \cdot 2H_2O$, and 50 mg $MnSO_4 \cdot H_2O$ per liter of medium) in 2-liter flasks at 260 revolutions per min at 30°C for about 72 h or until approximately complete autolysis had occurred. To prepare lyophilized powders from sporulated liquid cultures, the cultures were centrifuged at 20°C for 20 min at 12,210 × *g* after autolysis. The supernatant was discarded and the sediment was rinsed with water (double distilled) at least 3–4 times. The resulting pellet was resuspended and lyophilized.

To prepare lyophilized powders from spores, the spores were isolated by extraction and rinsing of the culture medium foam created by shaking the centrifuge bottles (modifications of Gingrich 1968). This procedure was repeated until the spore suspension was at least 95% pure when examined under the light microscope. Spores then were resuspended in water and lyophilized.

To isolate parasporal inclusion bodies, the medium was centrifuged (as described above) after removal of the spores. The supernatant was discarded, and the pellet rinsed with water. The pellet obtained from 500 ml of culture medium was resuspended in 60 ml water. Thirty ml of this suspension was sonicated for 1–2 min and then layered onto six SW 27 tubes (Beckman Instruments, Palo Alto, Calif.) (5 ml/tube) containing a 30–36% (30, 32, 33, 34, 35, and 36%) discontinuous sodium bromide (NaBr) density gradient (modified from Ibarra & Federici 1986). After centrifugation for 1 h at 52,000 × *g*, parasporal inclusion bodies, which typically separated in the gradient as two distinct bands (upper band, P2; lower band, P1), were extracted with a Pasteur pipette and two volumes of water added. This suspension then was centrifuged at 20°C for 20 min at 12,210 × *g* and rinsed with water. This procedure was repeated at least three times. The parasporal inclusion bodies were resuspended in water. If the resulting suspension was at least 99% devoid of spores and vegetative cells when examined under the light microscope, the suspension was either lyophilized or used for further purification of the P2. If the suspension was contaminated, the NaBr procedure was repeated. To increase the yield of the P2 cuboidal crystal, the pellets from the bottom of the centrifuge tubes from the NaBr gradients were collected, rinsed with water and run again on NaBr gradients. The P2 band was extracted, and this procedure was repeated until only a negligible amount of P2 band was observed. The extracted P2 then was rinsed and stored as previously described.

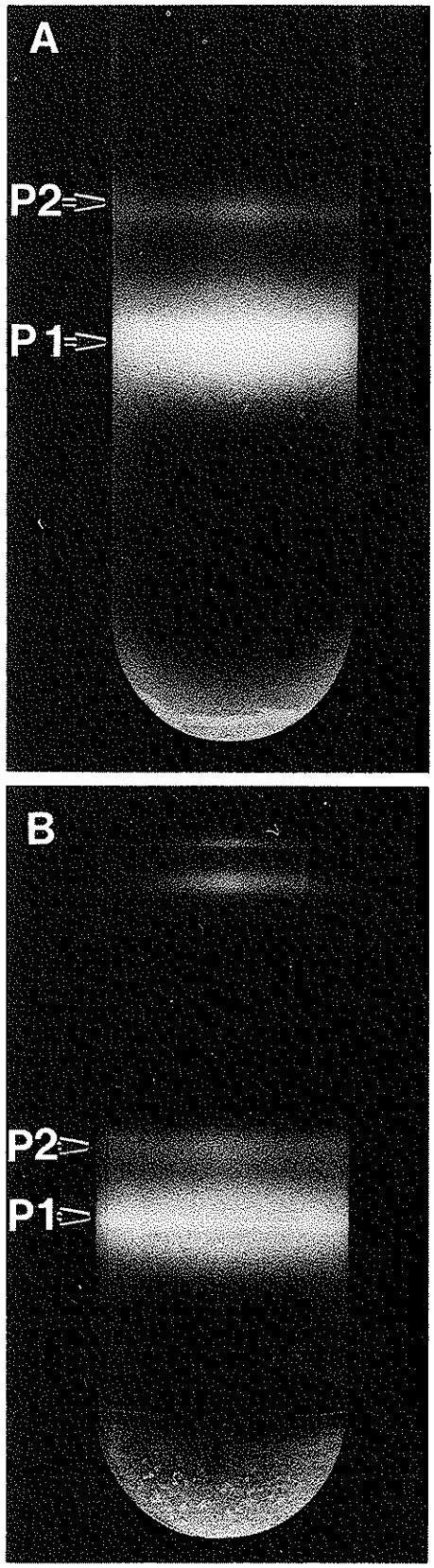
β-Exotoxin Assay. Assay of culture media supernatant from NRD-12 against third-instar *Musca domestica* L., a standard assay to determine the presence of the β-exotoxin (Fujiyoshi 1973, as cited in Padua et al. 1980) resulted in significant ($P \leq 0.05$) pupal mortality in comparison to HD-1 and water controls (unpublished data). The washing procedure described above eliminated most of the β-exotoxin detectable by the *M. domestica* bioassay of the supernatant from resuspended and centrifuged lyophilized powders from sporulated liquid cultures.

Crystal Morphology and Electron Microscopy. *Scanning Electron Microscopy.* NRD-12 P1 suspensions were placed on aluminum mounts and air dried. Samples then were sputter coated with gold and platinum. Samples were examined and photographed with a JEOL scanning electron microscope at a voltage of 15 kV.

Transmission Electron Microscopy. Diluted samples of P1 from HD-1 were pipetted onto grids covered with formvar film to obtain carbon replicas. The preshadow cast with carbon and platinum, the carbon replication, and dissolution of formvar and specimen were as described in Desjardins et al. (1973). For the plastic sections, P1 samples from both strains were initially embedded in agar. Fixation, dehydration, and Epon-Araldite embedding were as described by Ibarra & Federici (1986). Sections were cut with a Sorvall MT-2B microtome and stained first with lead citrate and then with uranyl acetate. For both procedures, the grids were examined and photographed with a Hitachi H-600 electron microscope with a 50μm objective aperture at an accelerating voltage of 75 kV.

Purification of P2 Crystals. The P2 suspension (containing some P1) and the P1 suspension (containing substantial amounts of P2) were separately resuspended in 1 ml water and treated with 1 m*M* dithiothreitol, 10 m*M* EDTA, and 25 m*M* Na_2CO_3 adjusted to pH 10 (modified from Yamamoto & Iizuka 1983) which solubilizes the P1 without solubilizing the P2. Both suspensions were incubated for 1 h at 37°C and then centrifuged at 17,400 × *g* for 20 min. The supernatant was discarded, the pellet was resuspended in water (sonicated for 1–2 min if necessary) and the procedure repeated three or four times. The final pellet was resuspended in water and lyophilized.

Parasporal Inclusion Body Composition. Protein composition of parasporal inclusion bodies was determined by modifying the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Ibarra & Federici (1986). A



3% stacking gel and an 11% running gel were run on a Hoefer SE 600 (Hoefer Scientific Instruments, San Francisco) vertical slab gel apparatus for 2 h at 90 V and 6–8 h at 180 V, respectively. Protein standards used for molecular masses (kDa) were lysozyme (14.4), soybean trypsin inhibitor (21.5), carbonic anhydrase (31), ovalbumin (45), bovine serum albumin (66.2), phosphorylase B (92.5), β-galactosidase (116.25), and myosin (200). In a related experiment designed to verify the purity of P2, SDS-PAGE was carried out as described above with the following modifications: An 8.75% running gel was cast on a Hoefer SE 400 "Tall Boy" vertical slab gel apparatus (32 cm × 18 cm) and run for 9 h at 90 V and 19.5 h at 180 V.

Determination of Toxicity. The toxicities of various *B. thuringiensis* subsp. *kurstaki* components were determined by bioassays using neonate *S. exigua* larvae. Larvae used in all tests were obtained from a laboratory colony established in 1982 from insects collected in Orange County, Calif., and maintained on artificial diet (Patana 1969) at 27 ± 1°C with a photoperiod of 16:8 (L:D). Artificial diet was used in all bioassays.

Seven to nine concentrations were tested per powder. Each concentration was added to 2–8 ml artificial diet. The mixture then was mixed with a vortex mixer for 1 min; approximately 200 μl then was poured into each well of a 24-well microtiter plate. Diet was allowed to solidify before one neonate (0–8 h old) *S. exigua* larva was placed in each well. Microtiter plates were covered with parafilm, and the lids replaced. Plates were placed in a growth chamber at 27 ± 1°C and a photoperiod of 16:8. Twenty-four insects were evaluated per concentration and each treatment was replicated at least three times. Larval mortality was assessed at day 7 for all treatments.

Data were analyzed using the Proc Probit procedure (SAS Institute 1985, 639–645) after correction for control mortality with Abbott's (1925) formula. Values from individual replicates were pooled. Control mortality was ≤10%.

Effect of Combining Components. Five concentrations of the 65-kDa/135-kDa combination and spores from each strain plus controls were prepared as described previously and evaluated concurrently, both independently and in combination. Treatments were replicated four times with 24 larvae per replicate. Joint effects were analyzed with a χ^2 test made in accordance with Finney (1971) and Salama et al. (1984). Data were only analyzed from experiments where control mortality was ≤10%.

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Fig. 1. Separation of the bipyramidal (135-kDa P1 protein) and cuboidal (65-kDa P2 protein) crystals of the HD-1 (A) and NRD-12 (B) strains of *B. thuringiensis* subsp. *kurstaki* in sodium bromide density gradients. P1, bipyramidal crystals; P2, cuboidal crystals.

Results and Discussion

Parasporal Inclusion Body Isolation. Both strains produced two distinct bands when centrifuged on 30–36% discontinuous NaBr gradients (Fig. 1). The lower band consisted predominantly of bipyramidal crystals (P1 protein) along with small quantities of P2, whereas the upper band contained cuboidal crystals (P2 protein) almost exclusively (Fig. 2 and 3). The pellet at the bottom of the tube contained spores, debris, and substantial amounts of the P1 and P2 crystals. The purity and separation of the two bands was dependent on the concentration and volume of the NaBr steps used. By increasing the volumes of the 32, 33, and 34% NaBr concentrations, greater separation was obtained between the two bands. The concentration of the culture slurry added to the NaBr gradients also was critical, because too high a concentration resulted in overloading of the gradient with much of the material ending up in the pellet. The slurry concentration was especially critical in separating out the P2 crystals, which, if the concentration was too high, formed aggregates easily that sedimented with the P1 crystals or to the pellet. Sonication immediately before each centrifugation using NaBr gradients reduced this problem considerably.

Purification of P2 Crystals. Because substantial amounts of P2 were required for bioassays against *S. exigua*, even greater amounts of the P1 and P2 bands were required for treatment to recover sufficient amounts of P2. When large amounts of P1 were treated and centrifuged, a gelatinous pellet resulted with a similar residue lining the tube. This pellet often was resistant to further solubilization and disruption. The gelatinous material may be the result of the solubilization process on the envelope surrounding the P1 (Fig. 4). Envelopes surrounding inclusions and parasporal bodies have been reported, although no correlation has been made between these envelopes and toxicity (Ibarra & Federici 1986). Resuspension in water, sonication, and subsequent centrifugation (repeated at least once) were required to resuspend these pellets.

Parasporal Inclusion Body Composition. Both strains produced proteins of approximately 135 kDa and 65 kDa as determined by SDS-PAGE (Fig. 5). The HD-1 results were consistent with reports in the literature documenting a molecular mass of 134 kDa for the P1 protoxin (Bulla et al. 1981), and 65–66 kDa for the P2 (Yamamoto & McLaughlin 1981, Donovan et al. 1988). Because the NRD-12 P1 5.3-kb (kilobase) gene product differs from the HD-1 5.3-kb gene product by only eight amino acids, and NRD-12 contains all three Hind III fragments corresponding in size to those present in HD-1 (Hefford et al. 1987), a similar molecular mass for the P1 proteins would be expected.

When NaBr gradients were run within 24 h of cell autolysis and the P1 band was analyzed immediately on SDS-PAGE, large amounts of 135 kDa appeared, and only small quantities of 65-kDa protein were present. If this same procedure was applied to the P2, the predominant band observed on SDS-PAGE was the 65-kDa band. This suggests that the 65-kDa band was primarily due to the P2

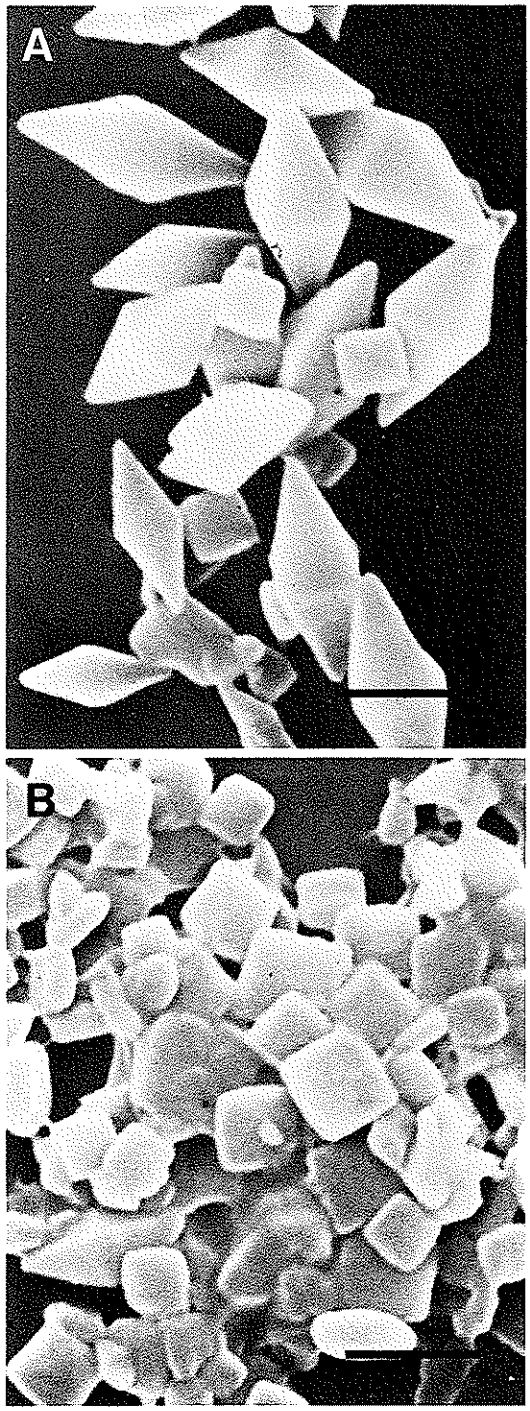

Fig. 2. Crystal morphology of *B. thuringiensis* subsp. *kurstaki* strain NRD-12. Scanning electron micrographs of P1 (A) and P2 (B) after purification. Bar = 1 micron.

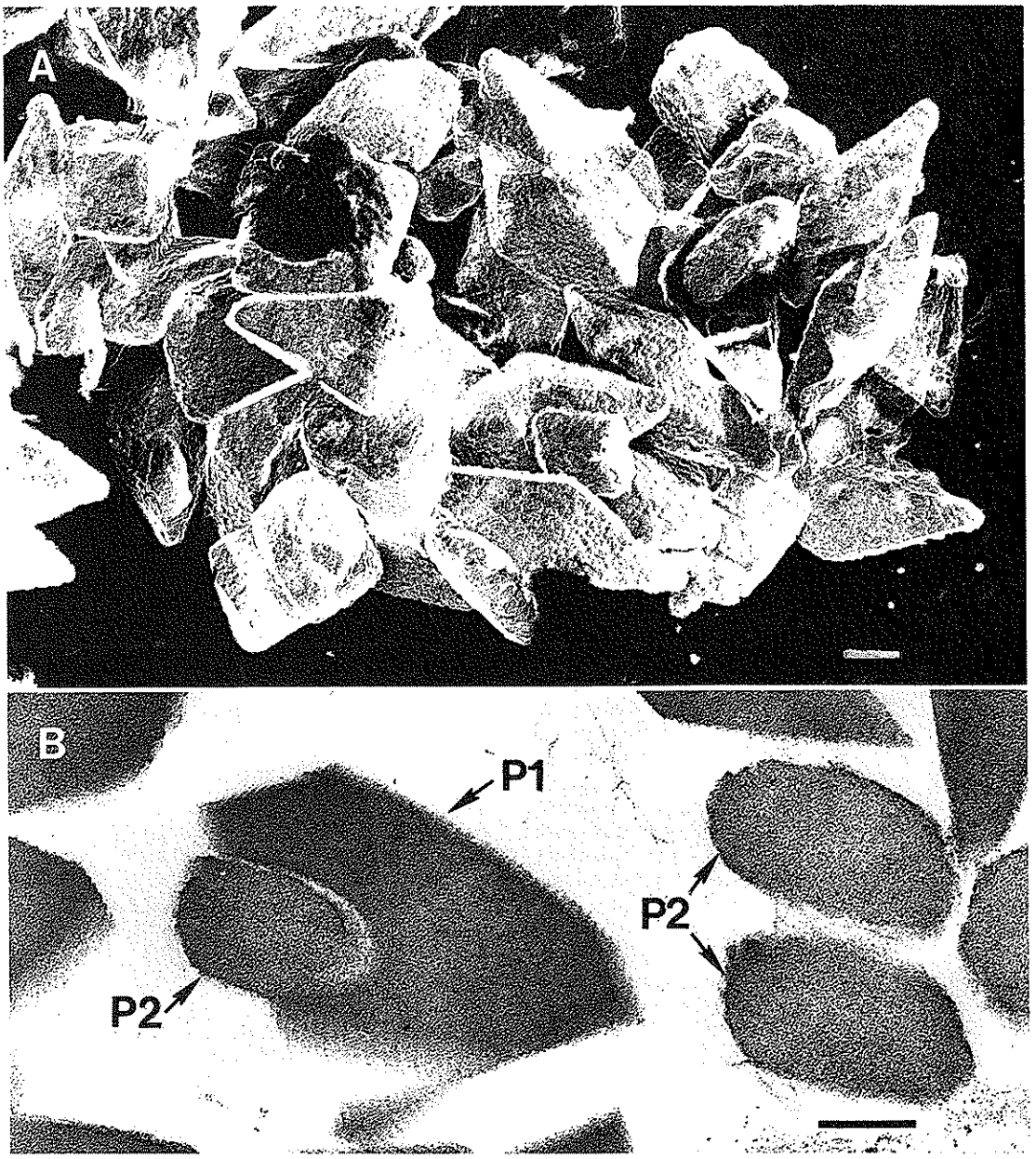

Fig. 3. Crystal morphology of *B. thuringiensis* subsp. *kurstaki* strain HD-1. Transmission electron micrographs of carbon replica (A) and plastic section (B) of P1 after purification. Bar = 200 nm.

and not to an approximately 65-kDa peptide resulting from cleavage of the P1. These results also demonstrate that isolation of relatively pure P1 and P2 crystals is possible using NaBr gradients.

To determine the purity of P2 before toxicity bioassays, the partially purified P2 crystals (after solubilization of P1) were analyzed by SDS-PAGE. Most of the protein observed had a molecular mass of 60 kDa or 65 kDa (Fig. 6). Minor quantities of other peptides were also apparent, especially in the HD-1 preparations. This indicates that the solubilization and rinsing procedure did not entirely remove all of the P1 or P1 degradative products. Yamamoto & Iizuka (1983) also noted several intermediate peptides when proteins were digested with insect gut proteases and suggested that these resulted from random attack of the protease-sensitive portions of the P1. The amino acid composition of the 60-kDa protein from NRD-12 (unpublished data) is very similar to the amino acid composition of the *B. thuringiensis* subsp. *kurstaki* HD-263 P2 (Donovan et al. 1988), suggesting that

at least the 60-kDa and probably the 65-kDa proteins are P2 in origin and not from P1. Therefore, we conclude that most of the toxicity obtained in our "P2 toxicity bioassays" is the result of ingestion of P2 crystals.

Toxicity. NRD-12 lyophilized powders prepared from sporulated liquid cultures were considerably (approximately 2.5 times) more toxic to *S. exigua* than HD-1 (Table 1). These results are in agreement with those of Moar et al. (1986), which demonstrated a three- to fourfold increase in toxicity for formulated materials containing NRD-12 versus HD-1. Comparisons between lyophilized powders from sporulated liquid cultures should be more reliable than comparisons between formulated materials because the amount of active ingredient can vary in the latter. Furthermore, formulated materials can contain compounds other than the listed active ingredient that also are insecticidal (Brattsten & Wilkinson 1977, Umetsu et al. 1981).

The NRD-12 P1 was approximately 2.5 times more toxic than the HD-1 P1 (Table 1). The majority of insecticidal activity of *B. thuringiensis* subsp. *kurstaki* strains is attributed to the P1, so it is not surprising to find that the increase in activity of NRD-12 to *S. exigua* is also due to the P1. Interestingly, Hefford et al. (1987) described the 5.3-kb gene of NRD-12 to be a hybrid between the HD-1 4.5-kb gene and the HD-73 6.6-kb gene and suggested this hybrid might be important to the toxicity of NRD-12 crystals. The NRD-12 P1 showed the same increase in activity compared with the HD-1 P1 as the lyophilized powders from sporulated liquid cultures. However, the toxicity of the P1 for both strains was only approximately one-third that of the lyophilized powders from sporulated liquid cultures, indicating that another *B. thuringiensis* subsp. *kurstaki* component was contributing to the insecticidal activity.

The P2 of HD-1 was approximately 2 times as toxic to *S. exigua* as the P2 of NRD-12 (Table 1). Some of this increase in activity of HD-1 compared with NRD-12 could be due to the increase in the amount of contamination from P1 degradation products (Fig. 5). However, the LC_{50}'s for both P2s are similar to the LC_{50} of HD-1 P2 against 1 d-old *H. virescens* larvae (LC_{50} = 54.4 μg/ml diet) (Yamamoto & Iizuka 1983). Because the active fraction of P1 is approximately 65 kDa (or about 50% of the protoxin) and approximately 60 kDa for P2 (Yamamoto & Iizuka 1983), the LC_{50}'s for P1 should be reduced by approximately 50% to be compared with P2 toxicity on a wt/wt basis. The HD-1 P2 therefore would be more toxic than the active fraction of P1, whereas the NRD-12 P2 would be less toxic than the active fraction of its P1.

In preliminary tests (unpublished data), both *B. thuringiensis* subsp. *kurstaki* strains also demonstrated toxicity against second-instar *Aedes aegypti* (L.) larvae. HD-1 P2 was as toxic against *A. aegypti* as Yamamoto & McLaughlin (1981) reported for second-instar *A. taeniorhynchus* (Wiedemann), whereas the NRD-12 P2 was slightly less toxic. These data indicate that the P2 is toxic to *S. exigua* and mosquitoes. Widner & Whiteley (1989) reported that a recombinant *Escherichia coli* containing the P2 gene from HD-1 (cryB1) was toxic to *A. aegypti* and the lepidopteran *Manduca sexta* (L.) although another recombinant *E. coli* containing a similar HD-1 gene (cryB2), which was 89% homologous to cryB1, was toxic only to *M. sexta*. Donovan et al. (1988) reported that a recombinant *B. megaterium* containing the P2 gene in pEG204 from HD-263 was toxic to *A. aegypti* and the lepidopterans *H. virescens* and *L. dispar*. Donovan et al. (1988) also stated that the P1 (from HD-1) and P2 (from HD-263) proteins from *B. thuringiensis* subsp. *kurstaki* shared a 37% homology over a sequence of 100 amino acids toward the NH_2 end of the molecule. The NH_2 terminus is the region responsible for the majority of lepidopteran activity (Whiteley et al. 1987). Additional research is needed to determine the domain of P2 responsible for activity against mosquitoes and to determine the amount of overlap of this domain with that domain responsible for lepidopteran activity. Because the P2 of both HD-1 and NRD-12 were shown to be toxic to lepidopterous insects, additional bioassays against different lepidopteran species should help determine if P1 and P2 have activity against the same range of insect species.

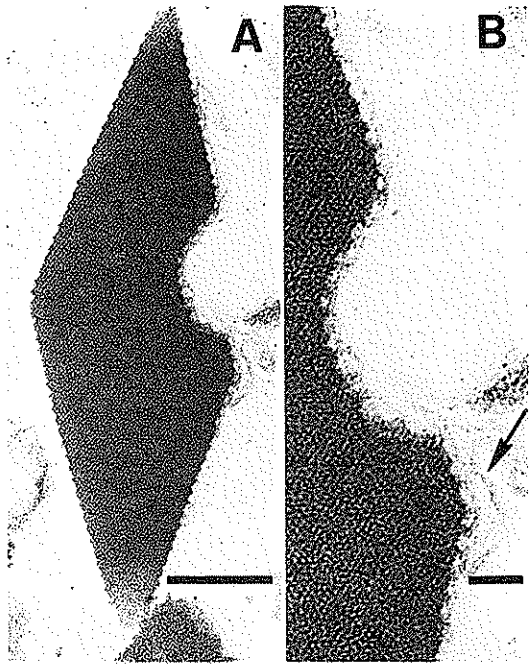

Fig. 4. Crystal morphology of *B. thuringiensis* subsp. *kurstaki* strain NRD-12. Transmission electron micrographs of a P1 plastic section (A) and an enlarged micrograph with the arrow pointing to the crystal envelope (B) after purification. Bar in A = 200 nm, bar in B = 50 nm.

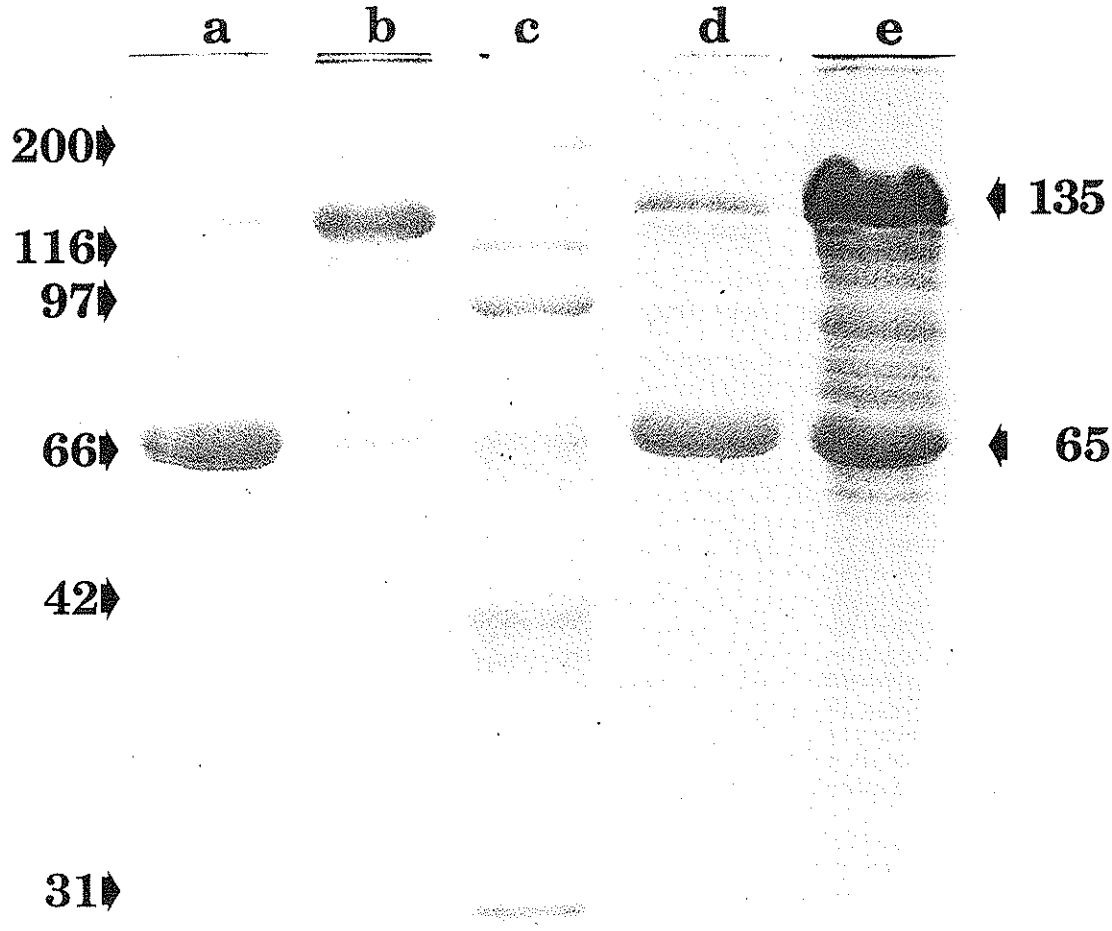

Fig. 5. Analysis of the parasporal body proteins of the HD-1 and NRD-12 strains of *B. thuringiensis* subsp. *kurstaki* by SDS-PAGE. a, NRD-12 P2; b, NRD-12 P1; c, protein standards; d, HD-1 P2; e, HD-1 P1. All proteins except the standard were isolated on NaBr gradients. Left margin, molecular masses (kDa) of the protein standards; right margin, *B. thuringiensis* subsp. *kurstaki* proteins.

Combinations of the P1 and P2 of NRD-12 were approximately 2 times more toxic to *S. exigua* than the P1/P2 combination of HD-1 (Table 1). These LC_{50}'s are very similar to the LC_{50}'s for the P1 tested alone, suggesting that no potentiation occurred between P1 and P2. However, the ratio of P1:P2 used was probably not the same ratio as in the lyophilized powders from sporulated liquid cultures, because the P2 forms aggregates that are more difficult to separate using NaBr gradients. Consequently, substantial amounts of P2 could always be obtained from the P1 band and the pellet when NaBr gradients were repeated.

When purified spores were assayed for toxicity, HD-1 spores were shown to be slightly more toxic than NRD-12 spores (Table 1). HD-1 spores were also shown to be slightly more toxic than HD-1 P1, whereas the NRD-12 spores were shown to be approximately 2.5 times less active than NRD-12 P1. Tyrell et al. (1981) reported that total protein extracted from the spores of *B. thuringiensis* subsp. *kurstaki* was only 20% less toxic than the crystal protein against *Manduca sexta* (L.), indicating that *B. thuringiensis* subsp. *kurstaki* spores can be insecticidal. However, Somerville et al. (1968) reported that this insecticidal protein composed only 12% of the spore dry weight in *B. thuringiensis* subsp. *alesti*. The insecticidal activity of *B. thuringiensis* subsp. *kurstaki* spores to *S. exigua* is not likely due exclusively to the crystal protein on the spore coat. Because spore preparations were between 95% and 100% crystal free, there is a chance that this small amount of P1 could have contributed to the toxicity, but cannot account for the level of toxicity observed.

Effects of Combining Components. Combining an equal amount of spores with the P1/P2 combination resulted in a substantial increase in activ-

ity compared with the lyophilized powders from the sporulated liquid cultures (Table 1). There were no differences in activity when the HD-1 P1/P2 combination was added to either HD-1 or NRD-12 spores in a 1:1 ratio. The NRD-12 P1/P2 combination mixed with HD-1 spores at a ratio of 1:1 was slightly more toxic than the mixture of NRD-12 P1/P2 combination and NRD-12 spores. This discrepancy can be explained partly by the increased activity of the HD-1 spores. The increase in activity of 1:1 combinations compared with the lyophilized powders from sporulated liquid cultures could be explained by the fact that the sporulated liquid cultures contained not only P1, P2, and spores but cell debris and vegetative cells as well. Additionally, for the purpose of these experiments, we used a 1:1 ratio (wt/wt) of spore to crystal that probably is not the ratio of the crystals to spores that occurs naturally in sporulated cultures. Burges et al. (1976) also reported that a 1:1 crystal:spore ratio of *B. thuringiensis* serotype 5 resulted in the highest degree of toxicity against *Galleria mellonella* (L.), whereas Mohd-Salleh & Lewis (1982) reported 4:6 and 2:8 spore/crystal ratios resulted in the highest levels of toxicity of *B. thuringiensis* subsp. *kurstaki* HD-263 against neonate *Ostrinia nubilalis* (Hübner) larvae. Differences in spore concentration in the crude culture preparations between the two strains could therefore also be partly responsible for the higher toxicity of NRD-12.

The 1:1 ratio of the P1/P2 combination LC_{25}'s: spore LC_{25}'s resulted in significant ($P \leq 0.005$) potentiation when compared with the LC_{25} of P1/P2 combination and spores tested individually (Fig. 7). Significant potentiation ($P \leq 0.05$) also occurred when other LC values were used, but was not consistent across all combinations. This trend agrees with the general theory of probit analysis, which states that there is more variability the greater the deviation from the LC_{50} (Finney 1971).

In this study, we have provided evidence that the comparatively high toxicity of NRD-12 to neonate *S. exigua* is due primarily to the bipyramidal crystal (P1). Though this suggests that the high toxicity of this strain is due to one or more of the 135-kDa proteins in the P1 crystal, there are other factors which could have contributed to, or account for this toxicity. For example, it is known that the β-exotoxin can potentiate the activity of formulated materials containing either NRD-12 or HD-1 (Moar et al. 1986). Though our *M. domestica* bioassays indicated that we had eliminated most of the β-exotoxin from our lyophilized powders prepared from sporulated liquid cultures by the washing procedure, any minor quantities present may have contributed to the high toxicity observed in preparations of this strain.

The HD-1 strain used in our tests contained only two P1 toxin genes based on Southern blot analysis of Hind III digests (L. Masson, National Research Council Canada, personal communication). Wilcox et al. (1986) reported that the "two gene" HD-1-S 1980-standard was more than one-third less toxic to third-instar *S. exigua* than the "three gene" HD-1-S-1971 standard. Still, this would not explain the greater than twofold increase in toxicity of the P1 crystal of NRD-12 in comparison with the P1 of our HD-1 strain. Further genetic analysis of NRD-12 combined with bioassays using a "three gene" HD-1, which was the exclusive type found in Dipel from 1975–1984 (Wilcox et al. 1986), will help determine if NRD-12 is still more toxic than HD-1. Additional studies aimed at testing products produced by clones of those genes responsible for the

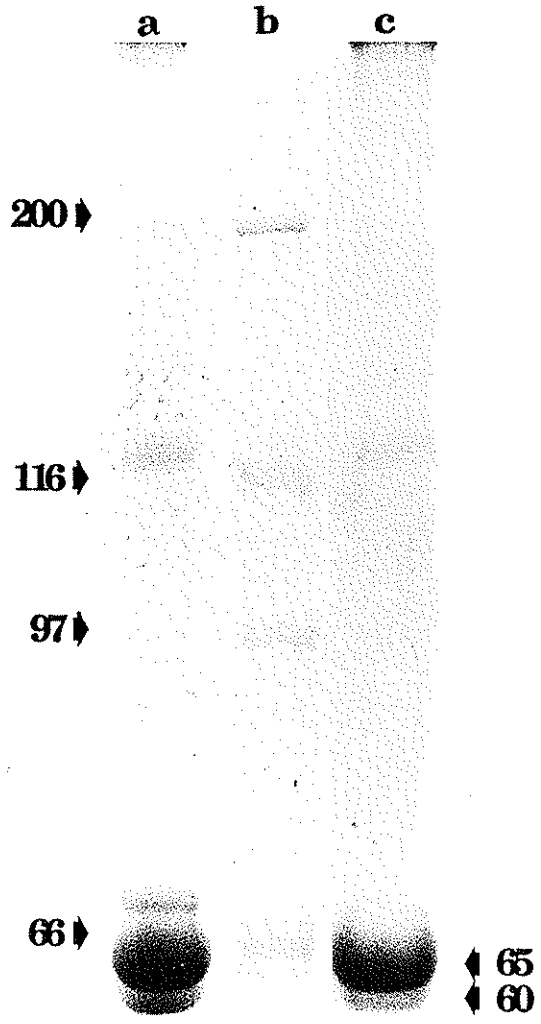

Fig. 6. Analysis of the partially purified P2 proteins of the HD-1 and NRD-12 strains of *B. thuringiensis* subsp. *kurstaki* by SDS-PAGE. a, HD-1 P2; b, protein standards; c, NRD-12 P2. The P1 and P2 bands from both strains were solubilized after isolation on NaBr gradients. Left margin, molecular masses (kDa) of the protein standards; right margin, *B. thuringiensis* subsp. *kurstaki* proteins.

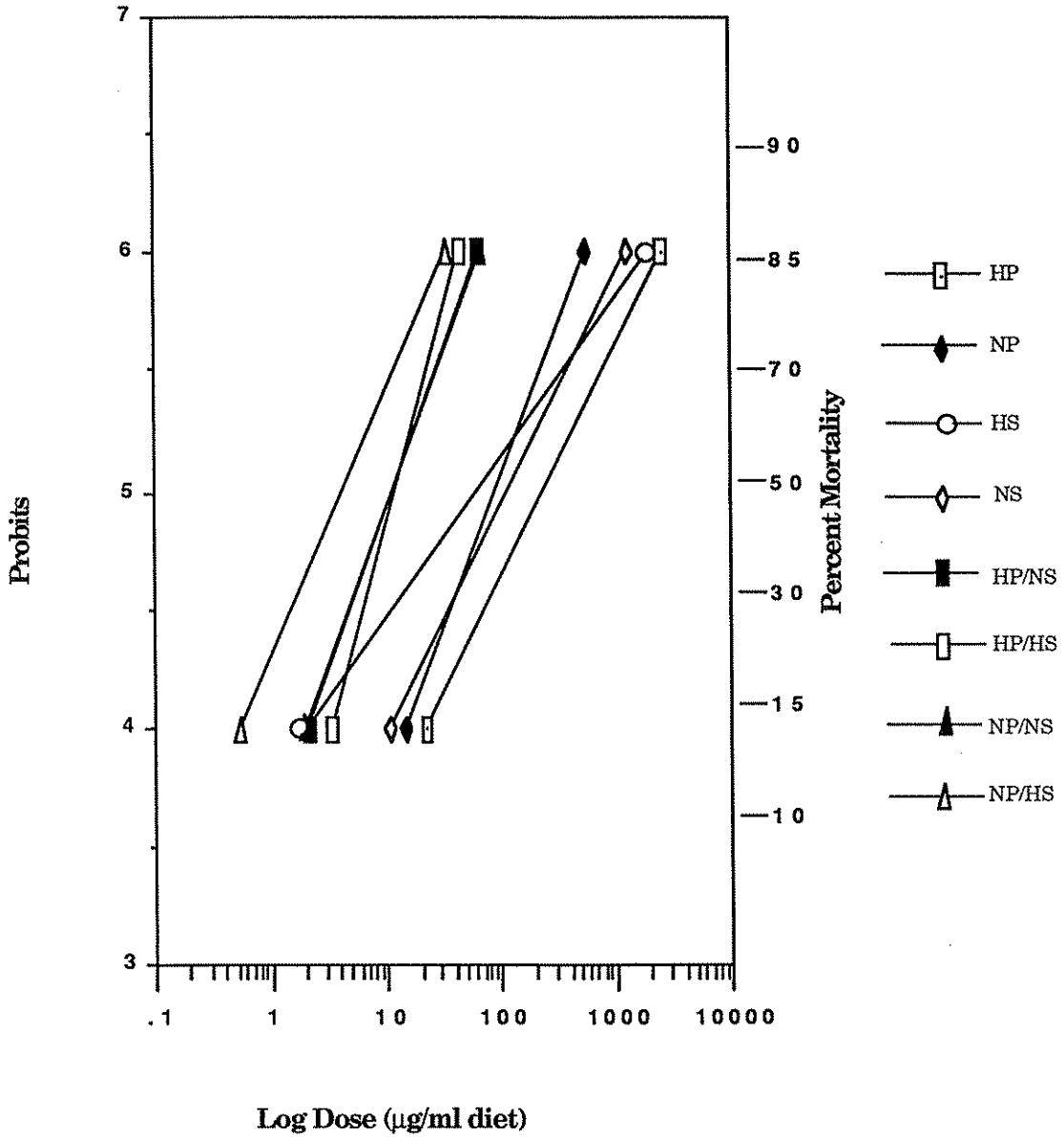

Fig. 7. Toxicity of the parasporal body components and spores of the HD-1 and NRD-12 strains of *B. thuringiensis* subsp. *kurstaki* to neonate *Spodoptera exigua*. The 65-kDa/135-kDa crystal proteins and spores were tested individually and in combination. Significant potentiation ($P \leq 0.005$) occurred when LC_{25}'s of either 65-kDa/135-kDa combination were added to the LC_{25} of either spore. H, HD-1; N, NRD-12; P, 65-kDa/135-kDa combination; S, spore, /, combination of the two components indicated.

HD-1 and NRD-12 bipyramidal crystal proteins also may help resolve some of these concerns.

We have also shown that the P2s from both strains were toxic to *S. exigua*. In a normal *B. thuringiensis* subsp. *kurstaki* cell, a single P1 crystal and a single P2 crystal are produced. On a wt/wt comparison, the P2 was as active or more active than the P1. However, because the P1 must be degraded by approximately 50% to become active (the P2 is degraded by only approximately 9%), the P1 (especially for NRD-12) activated toxin is more toxic.

Spores were shown to increase the toxicity of the crystals significantly and were probably partly responsible for the high toxicity seen in bioassays with formulated materials (Moar et al. 1986). The fact that the toxic action of the spore cannot be entirely explained by the amount of the crystal protein on the spore coat suggests an additional toxic factor. Accurate identification of toxic components of *B. thuringiensis* becomes extremely important when trying to incorporate *B. thuringiensis* toxin genes into plants to kill lepidopterous insects. In the case

Table 1. Comparative toxicity of spores and crystals from NRD-12 and HD-1 strains of *B. thuringiensis* subsp. *kurstaki* to *S. exigua*

Strain	*n* (no. concn, no. replicates)[a]	Slope ± SEM	LC_{50} (95% FL)[b]
	Sporulated liquid cultures		
HD-1	1,052 (9, 8)	1.53 ± 0.142	49.3 (38.8–62.2)
NRD-12	1,370 (10, 8)	1.48 ± 0.084	20.8 (17.9–24.1)
	135-kDa bipyramidal crystal (P1 protein)		
HD-1	781 (9, 5)	1.61 ± 0.193	153 (118–201)
NRD-12	950 (10, 5)	1.75 ± 0.196	63 (47–84)
	65-kDa cuboidal crystal (P2 protein)		
HD-1	429 (12, 3)	1.48 ± 0.151	34.2 (26.9–42.5)
NRD-12	405 (12, 3)	1.28 ± 0.246	72.4 (49.6–128.4)
	65-kDa + 135-kDa proteins[c]		
HD-1	844 (8, 7)	1.00 ± 0.124	157 (106–268)
NRD-12	1,080 (9, 7)	1.12 ± 0.074	82 (70–98)
	Spores		
HD-1	732 (7, 5)	1.32 ± 0.231	117 (66–209)
NRD-12	1,219 (8, 7)	0.98 ± 0.120	166 (124–233)
	65-kDa + 135-kDa proteins + spores[d]		
HD-1 P + HD-1 S	499 (5, 4)	1.85 ± 0.185	12.1 (8.68–15.6)
HD-1 P + NRD-12 S	422 (5, 4)	1.40 ± 0.161	11.8 (7.50–16.3)
NRD-12 P + HD-1 S	477 (5, 4)	1.15 ± 0.175	4.35 (1.80–7.27)
NRD-12 P + NRD-12 S	423 (5, 4)	1.36 ± 0.249	11.5 (5.10–18.1)

[a] Total number of insects assayed.
[b] LC_{50} values in μg/ml diet.
[c] Protein ratio, as they appeared as bands in NaBr gradients.
[d] P, proteins; S, spores; proteins/spores 1:1.

of *S. exigua*, some feature of the spore may be essential in obtaining the high toxicity characteristic of NRD-12.

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