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Specificity of Activated CryIA Proteins from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 for Defoliating Forest Lepidoptera

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The insecticidal activity of the CryIA(a), CryIA(b), and CryIA(c) toxins from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 was determined in force-feeding experiments with larvae of *Choristoneura fumiferana*, *C. occidentalis*, *C. pinus*, *Lymantria dispar*, *Orgyia leucostigma*, *Malacosoma disstria*, and *Actebia fennica*. The toxins were obtained from cloned protoxin genes expressed in *Escherichia coli*. The protoxins were activated with gut juice from *Bombyx mori* larvae. Biological activity of the individual gene products as well as the native HD-1 toxin was assessed as the dose which prevented 50% of the insects from producing frass within 3 days (frass failure dose [FFD_{50}]). The three toxins were about equally active against *M. disstria*. In the *Choristoneura* species, CryIA(a) and CryIA(b) were up to fivefold more toxic than CryIA(c). In the lymantriid species, CryIA(a) and CryIA(b) were up to 100-fold more toxic than CryIA(c). The toxicity of HD-1 was similar to that of the individual CryIA(a) or CryIA(b) toxins in all of these species. None of the CryIA toxins or HD-1 exhibited any toxicity towards *A. fennica*. Comparison of the observed FFD_{50} of HD-1 with the FFD_{50} expected on the basis of its crystal composition suggested a possible synergistic effect of the toxins in the two lymantriid species. Our results further illustrate the diversity of activity spectra of these highly related proteins and provide a data base for studies with forest insects to elucidate the molecular basis of toxin specificity.

The HD-1 strain of *Bacillus thuringiensis* subsp. *kurstaki* is used in several commercial insecticides for the control of leaf-eating larval Lepidoptera in both agriculture and forestry. Most of the insecticidal activity of the *kurstaki* subspecies is attributed to one or more proteins that make up the characteristic bipyramidal crystal. The production of these proteins is coded by three types of genes classified as *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* (11), also known as the 4.5-, 5.3-, and 6.6-kb class genes (14). Little is known about the role of the corresponding gene products in determining the specificity of the crystal protein. Considerable differences in specificity of individual CryIA proteins have been reported for various Lepidoptera of agricultural importance (10, 11, 20, 21). Determination of specificity to a wider range of Lepidoptera is becoming increasingly relevant for a variety of reasons. Specificity data are needed to select toxin genes for incorporation into crops (27) or into other microorganisms to enhance their efficacy (19) and to design use strategies that will prevent or delay the development of pest resistance to *B. thuringiensis* (33). More importantly, specificity data can be coupled with existing knowledge of toxin structure to enhance our understanding of the molecular basis of toxin specificity and mode of action (e.g., see references 5 and 35). This in turn may be useful for tailoring toxin specificity through genetic engineering. Within the latter context, we are in the process of determining the specificity of CryI and CryII toxins to Lepidoptera that are of potential economic importance in North American forestry. In this paper we report the specificity of *cryIA* toxin gene products from HD-1 against seven defoliating species. Because our main objective is to obtain a data base for elucidating the importance of toxin-receptor interactions in determining specificity to these insects, we used toxins that were solubilized and activated prior to bioassay. The toxins were derived from cloned protoxin genes expressed in *Escherichia coli*.

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MATERIALS AND METHODS

HD-1 preparation. Cells of HD-1 (isolate 62-732-BD-Abbott) were grown in half-strength Trypticase soy broth under conditions described previously (28). Spores and crystals were harvested by centrifugation of the lysed culture and washed three times in 1 M NaCl containing 0.01% Triton X-100, followed by three washes in distilled water. The pellet was resuspended and stored frozen (−20°C) in distilled water.

Gene cloning and protein production. The cloning and expression of the three *cryIA* crystal protein genes from HD-1 were conducted by Masson et al. (17). To obtain toxin protein for the insect bioassays, transformed *E. coli* cells were grown for 2 days at 37°C in LB broth containing ampicillin (100 μg/ml). Cells expressing the *cryIA(a)* and *cryIA(b)* genes were cultured at the Forest Pest Management Institute. The cells were harvested by centrifugation and washed three times in 1 M NaCl with 0.01% Triton X-100, followed by six washes in distilled water. The final pellet was resuspended in distilled water and was incubated with lysozyme (1 g/50 ml), followed by incubation with Nonidet P-40 detergent (1.6%) (both steps involved overnight shaker incubation at room temperature). The insoluble protoxin protein was collected by centrifugation and washed three times in distilled water. Cells expressing the *cryIA(c)* gene were cultured at the Biotechnology Research Institute. Cells were lysed in a French pressure cell, and protoxin protein was purified as described previously (17). All pellets were resuspended in distilled water and stored refrigerated at 6°C.

Toxin preparation. Activation of the HD-1 crystal-spore mixture and protoxin protein from recombinant *E. coli* was achieved by using gut juice extracted from fifth-instar larvae of the Chinese silkworm, *Bombyx mori* L. All preparations were activated with gut juice from the same batch. The gut juice was diluted to 1% in 0.2 M 3-cyclohexylamino-1-propanesulfonic acid-KOH buffer (pH 10.5; hereafter referred to as CAPS-KOH buffer) containing 0.2% dithiothreitol reducing agent and was filter sterilized. The activation procedure is described in detail by Gringorten et al. (7). Toxin solutions were centrifuged, dialyzed in 50-kDa molecular weight cutoff tubing against 0.1 M CAPS-KOH buffer, and passed through a 0.2-μm low-protein-binding Acrodisc filter (Gelman Sciences, Ann Arbor, Mich.). Gut juice solutions without toxin were processed in the same manner (gut juice control). Total protein concentration of the activated toxin solutions was determined by spectrophotometry, using the Bio-Rad dye-binding method with a crystal protein standard for reference and gut juice control for a blank. Toxin concentration was estimated by laser densitometry of Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), using purified CryIA(b) as a reference standard. The toxin samples and gut juice controls were refrigerated at 6°C in 1-ml aliquots during the 3 to 4 months required for completion of the insect bioassays.

Because the toxins were stored in the presence of gut enzymes (and at alkaline pH), their stability was examined by periodically monitoring changes in the intensity of toxin bands and the appearance of degradation products on 12% SDS-PAGE gels. The stability of toxicity was assessed in vitro (7) by using cell lines derived from neonate larvae of spruce budworm (IPRI-CF-1) and *Manduca sexta* (FPMI-MS-5). In vivo toxicity was monitored by force-feeding fourth-instar larvae of *Bombyx mori*.

Insect bioassays. Activity of toxin preparations derived from each *cryIA* gene and the native HD-1 crystal protein was determined by force-feeding larvae of spruce budworm, *Choristoneura fumiferana* Clemens, western spruce budworm, *C. occidentalis* Freeman, and jack pine budworm, *C. pinus* Freeman (*Tortricidae*); white marked tussock moth, *Orgyia leucostigma* (J. E. Smith), and gypsy moth, *Lymantria dispar* L. (*Lymantriidae*); forest tent caterpillar, *Malacosoma disstria* Huebner (*Lasiocampidae*); and black army cutworm, *Actebia fennica* (Tauscher) (*Noctuidae*). The *Choristoneura* species, *O. leucostigma*, *A. fennica*, and *M. disstria* came from laboratory stock maintained at the Forest Pest Management Institute. Larvae of *L. dispar* were obtained from egg masses collected the previous fall in southern Ontario. Age of the larvae was standardized as much as synchronization of cohort development permitted. We used 1-day-old sixth-instar larvae of *Choristoneura* spp., 1- to 3-day-old fourth-instar larvae of *L. dispar* and *O. leucostigma*, and 1- to 4-day-old fifth-instar larvae of *A. fennica* and *M. disstria*.

Twofold serial dilutions of toxin samples were prepared in 0.1 M CAPS-KOH buffer. A 4-μl drop of each dilution was delivered into the larval midgut region with a 0.25-ml syringe (Yale-BD) fitted with a blunted 30-gauge needle, using a model 1003 Microjector syringe drive and a model 1010 Microdoser (Houston Atlas, Houston, Tex.). Each assay included larvae dosed with 4 μl of a gut juice control solution. Dosed larvae were placed individually in 20-ml creamer cups with artificial diet at 25°C, 60% relative humidity, and a 16-h light:8-h dark photoperiod.

Toxicity was assessed 72 h after dosing by recording the number of larvae that had failed to produce fresh frass pellets, an indication of complete feeding inhibition. Feeding inhibition is a typical and well-known manifestation of cytotoxicity induced by *B. thuringiensis*. At low doses larvae can recover from feeding inhibition (24, 30), presumably by replacing damaged gut cells. Preliminary assays with spore-free toxins demonstrated that feeding inhibition is highly dose dependent and that larvae that recover do so within 3 days at 25°C. In contrast, mortality requires much higher doses and often does not exhibit a consistent dose-response relationship over a 7- to 18-day period (29). The 50% response dose, designated as the frass failure dose or FFD_{50} (29), and 95% confidence limits were calculated from pooled replicate assays, using the POLO computer program for probit analysis (25). *G* values (4) of replicate assays were usually <0.2 and never >0.3. The response in the control group was always <3% (n = 60 to 80). Significance of observed differences between toxicity values was based on nonoverlap of the 95% confidence limits ($P < 0.05$).

RESULTS

Toxin stability. Storing the activated toxins for several months at high alkaline pH and in the presence of gut enzymes did not affect their stability, as indicated by SDS-PAGE (Fig. 1) and toxicity assays (Table 1). Toxin protein appeared as a strong band at approximately 60 kDa on the gel (3), which was composed of most of the observed protein and appeared as a doublet. No obvious changes were apparent in the intensity or number of major bands between consecutive gels. Changes in some of the minor bands were detectable but did not affect toxicity of the preparations. Assays with CF-1 and MS-5 cells showed no change in toxicity. A twofold variation in threshold dose is sometimes observed between replicate assays and is not considered significant (7). Stability of toxicity was also supported by the silkworm bioassays, which indicated no substantial change in in vivo toxicity.

Toxin purity. Laser densitometry of the SDS gels permitted quantitative estimation of the activated toxin component of the protein solutions. Peak integrator values for the toxin bands (60 to 70 kDa) were referenced to a purified CryIA(b) standard and normalized to eliminate differences in the total amount of protein (from Bio-Rad determinations) placed on the gel. This resulted in the following estimates of toxin levels as a percentage of total protein: 84% for HD-1, 65% for CryIA(a), 53% for CryIA(b), and 73% for CryIA(c). These values were confirmed independently by high-pressure liquid chromatography (23). Correction for the presence of nontoxin protein permitted expression of toxicity values on the basis of the amount of toxin protein present.

Insect bioassays. Considerable differences in toxicity among the three CryIA toxins from HD-1 are demonstrated by the FFD_{50} values in Table 2. Differences were particularly evident between species representing the various families. In the *Choristoneura* species, CryIA(a) and CryIA(b) were equally toxic and somewhat more toxic than CryIA(c). In the lymantriid species, CryIA(b) tended to be slightly more toxic than CryIA(a) and was 20- to 100-fold more toxic than CryIA(c). All three were equally toxic to *M. disstria*. None of the toxins elicited a response in *A. fennica* at 3,500 ng/larva. When the dose was increased by administering a higher volume (up to 12 μl), no response was observed at maximum doses of 4,300 ng of CryIA(a), 12,500 ng of CryI(c), or 14,600 ng of HD-1. The toxicity of HD-1 was similar to the toxicity of the individual CryIA(a) or CryIA(b) toxins (or both) in all species. HD-1 appeared to be more

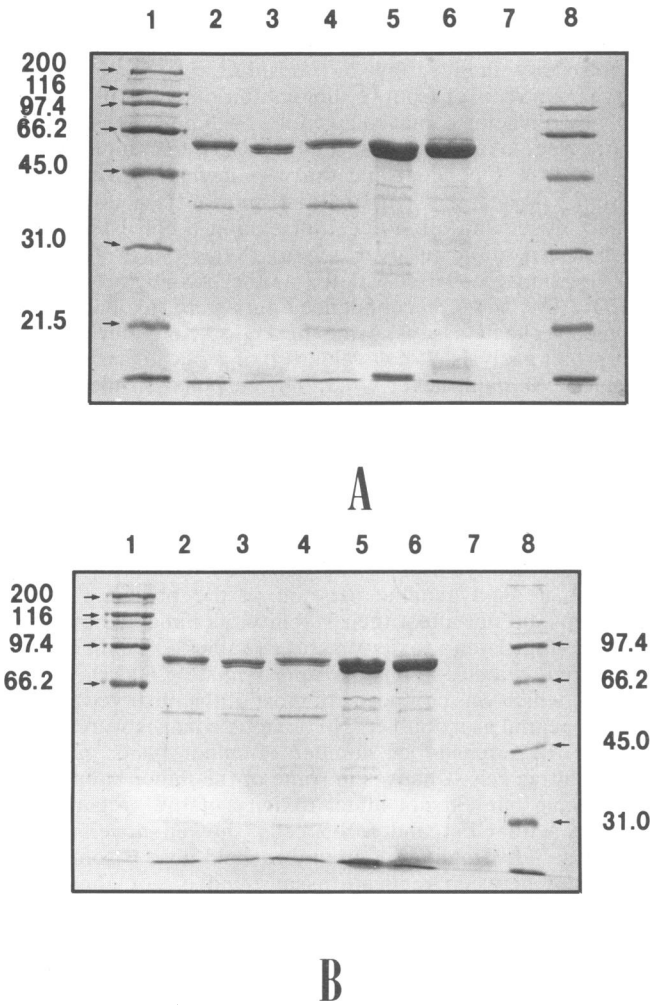

FIG. 1. Coomassie blue-stained SDS-PAGE gels of activated and dialyzed toxin solutions before (A) and after (B) 3 months of storage (6°C). Lanes 1 and 8, Molecular weight markers (10^3); lane 2, CryIA(a); lane 3, CryIA(b); lane 4, not applicable to this study; lane 5, CryIA(c); lane 6, HD-1; lane 7, gut juice control.

toxic than CryIA(c) to the *Choristoneura* spp. and was markedly more toxic than CryIA(c) to the lymantriid species.

Comparisons at the 50% response level can be misleading when probit regression lines do not have equal slopes (4). Unequal slopes occurred in the assays with *C. fumiferana*, *C. pinus*, and *L. dispar* (likelihood ratio test [26]). However, FFD_{95} values (Table 2) and FFD_{50} values revealed similar patterns, indicating that departure from parallelism was not a serious problem.

DISCUSSION

In this study we have demonstrated considerable differences in the specificity of activated CryIA toxins from HD-1 to seven species of defoliating forest Lepidoptera. The protoxin genes were cloned and expressed in *E. coli*, and the insoluble protoxin protein was solubilized and subjected to proteolytic digestion prior to bioassay. Activated toxins are frequently used in specificity studies (9, 16, 31) and are usually obtained by incubation with trypsin. We used silkworm gut juice instead because, unlike trypsin, the high pH of the activation mixture (pH 10.5) was optimum for proteolytic gut enzymes, as well as for solubilization of protoxin protein (3). The choice of activation enzyme does not appear to have a large effect on specificity of lepidopteran-active toxins, because the same specificity was obtained after activation with various lepidopteran gut juices or trypsin in several studies (8, 9, 12, 15). The only known case in which the source of gut juice did play a role involves a dual specificity toxin activated with gut juice from different insect orders (lepidopteran versus dipteran toxicity [8]).

TABLE 1. Stability of activated toxins derived from HD-1 and CryIA proteins during refrigerator storage as measured by the threshold dose in lawn assays against cell lines from *C. fumiferana* (CF-1) and *M. sexta* (MS-5) and by 50% lethal dose in force-feeding assays with larvae of *Bombyx mori* (Bm)

Target	No. of days at 6°C	Effective dose (ng of total protein)			
		HD-1	CryIA(a)	CryIA(b)	CryIA(c)
CF-1	7	0.39	—[a]	6.25	0.78
	43	0.19	—	3.13	0.19
	70	0.39	—	3.13	0.39
MS-5	7	6.25	6.25	—	12.5
	43	12.5	6.25	—	6.25
	70	6.25	6.25	—	12.5
	93	6.25	6.25	—	12.5
Bm	22	2.8	2.0	—	—
	43	4.4 (3.2–5.8)[b]	2.9 (2.4–3.6)	—	—
	90	4.3 (3.7–4.9)	1.6 (1.4–1.8)	—	—

[a] —, Not susceptible.
[b] 95% confidence limits are given in parentheses.

By assaying toxins that were solubilized and activated in vitro in the same enzyme system, we could minimize differential processing of the ingested protein as a factor contributing to specificity. Differential solubilization has been shown to affect specificity of crystal proteins (13, 15) and can be expected to play an even greater role when *E. coli*-produced proteins are used. Not only are the latter less soluble than native crystal protein (34), but also differences in solubility were observed among the three *cryIA* cloned gene products under reducing alkaline conditions (pH 10.5; data not shown). Differential activation among lepidopterous hosts does not appear to play a major role, because several studies report that activated toxins have the same activity spectra as the corresponding protoxins (31, 32). Because such data are available for a limited number of species only, we standardized the activation step among the test species by uniform treatment of the protoxin protein with gut juice from one source. It is possible that the observed specificities were influenced by further proteolytic processing of the activated toxins after force-feeding. We do not expect this to be a major factor because *cryIA* gene products activated with gut juice from either silkworm or spruce budworm larvae displayed comparable toxicity to either species (6).

We thus interpret the specificity differences observed in this study to reflect predominantly differences at the receptor level. Recent experiments suggest that heterogeneity of midgut cell receptors among different species of Lepidoptera is a major determinant of toxin specificity (9, 31–33). Together those studies indicate that a species can possess more than one type of receptor and that receptors can differ in their concentration and affinity for different toxins. How-

TABLE 2. Toxicity of activated toxins derived from HD-1 and CryIA proteins to *C. fumiferana* (Cf), *C. occidentalis* (Co), *C. pinus* (Cp), *O. leucostigma* (O1), *L. dispar* (Ld), *M. disstria* (Md), and *A. fennica* (Af), expressed as the FFD_{50} and FFD_{95}

Species	Toxin	No. of larvae (no. of dilutions)	Slope ± SE	FFD_{50} (ng of toxin protein/larva)	Confidence limit 95%		FFD_{95} (ng of toxin protein/larva
					Lower	Upper	
Cf	HD-1	220 (10)	1.84 ± 0.21	16.8	12.5	22.2	131
	CryIA(a)	200 (8)	2.32 ± 0.29	12.6	8.4	18.6	64
	CryIA(b)	160 (8)	2.26 ± 0.29	13.2	10.0	17.2	70
	CryIA(c)	220 (9)	1.41 ± 0.17	27.9	19.2	40.2	409
Co	HD-1	170 (6)	1.83 ± 0.26	18.1	13.4	27.0	269
	CryIA(a)	250 (11)	1.28 ± 0.13	11.6	6.2	19.5	179
	CryIA(b)	190 (9)	1.56 ± 0.21	10.8	5.5	19.6	152
	CryIA(c)	210 (9)	1.53 ± 0.18	57.6	41.6	82.4	838
Cp	HD-1	160 (7)	2.19 ± 0.30	13.1	8.5	19.2	74
	CryIA(a)	150 (7)	1.67 ± 0.24	9.2	1.8	19.8	89
	CryIA(b)	160 (7)	2.63 ± 0.35	17.3	11.7	24.9	73
	CryIA(c)	160 (7)	2.28 ± 0.30	37.4	28.5	48.7	196
O1	HD-1	220 (9)	1.40 ± 0.18	93.8	44.3	162.9	1,518
	CryIA(a)	250 (9)	1.10 ± 0.14	52.6	36.2	76.3	871
	CryIA(b)	230 (10)	1.49 ± 0.16	25.0	15.1	40.1	407
	CryIA(c)	180 (8)	1.56 ± 0.28	560	270	986	9,433
Ld	HD-1	160 (8)	1.59 ± 0.24	28.6	19.2	40.0	307
	CryIA(a)	190 (8)	1.37 ± 0.18	56.2	27.9	102.9	883
	CryIA(b)	180 (9)	2.07 ± 0.25	22.0	16.6	28.9	407
	CryIA(c)	90 (6)	2.87 ± 0.71	2,484	1,873	3,240	9,358
Md	HD-1	230 (10)	1.39 ± 0.19	24.0	15.9	35.0	364
	CryIA(a)	160 (7)	1.08 ± 0.19	36.9	12.5	83.2	1,241
	CryIA(b)	160 (7)	1.66 ± 0.24	25.5	15.1	39.6	249
	CryIA(c)	210 (10)	1.25 ± 0.16	26.5	11.8	51.8	543
Af	HD-1	50 (1)		>3,500			
	CryIA(a)	50 (1)		>3,500			
	CryIA(b)	50 (1)		>3,500			
	CryIA(c)	50 (1)		>3,500			

ever, toxin binding studies need to be conducted to verify the importance of binding-site characteristics as a possible explanation of the observed activity spectra. Specificity differences can also be related to the toxin's pore-forming ability following formation of the toxin-receptor complex, as suggested by the inverse correlation between binding and toxicity of CryIA(b) and CryIA(c) toxins in *L. dispar* (36).

Specificity data need to be considered within the constraints imposed by the procedures that were used for production and assay of the toxins, because different procedures can introduce several experimental artefacts, as demonstrated by Arvidson et al. (1). Those authors report that cloning of individual protoxin genes from multigene strains can result in protoxin instability and altered specificity depending on the organism used to express the gene product. The use of activated toxins in this study allowed expression of toxicity on the basis of actual toxin content rather than total protein and thus eliminated protein stability as a factor. However, the protoxin proteins were produced in *E. coli*, which in some cases can alter specificity. Arvidson et al. (1) observed that expression of a *cryIA(c)* toxin gene in *E. coli* altered toxicity towards *Heliothis virescens* but not to *Trichoplusia ni*. It is not known how widespread this phenomenon is because other studies report no differences in insecticidal activity between native and *E. coli*-produced CryIA(c) proteins (16), while in our experience CryIA(c) from recombinant *E. coli* is as toxic as CryIA(c) produced by the HD-73 strain. We also know that the low toxicity of CryIA(c) to *L. dispar* is not a cloning artefact because *B. thuringiensis* strains containing the CryIA(c) toxin singly have generally low or no toxicity to this species (2, 36).

Little is known about the contribution of each CryIA protein, individually or possibly through mutual interactions, to in vivo toxicity of the HD-1 crystal. We assessed possible interactive effects by comparing observed toxicity of the toxin mixture derived from HD-1 with toxicity expected on the basis of relative protein composition of HD-1 crystals and the observed toxicity of each individual component (Table 3). Crystals of HD-1 are composed of 13.6% CryIA(a), 54.2% CryIA(b), and 32.2% CryIA(c), as determined by cyanogen bromide cleavage of protoxin (17) derived from the same HD-1 isolate used in this study. Based on a weighted average toxicity of these components, expected toxicity was still within the 95% confidence limits of the observed toxicity in *C. fumiferana*, *C. occidentalis*, and *M. disstria*, suggesting an additive effect among the three toxins in those species. Observed toxicity was about two times greater than expected toxicity in *C. pinus* and *O. leucostigma* and greatly exceeded expected toxicity in *L. dispar*, suggesting a possible synergistic interaction.

A factor not considered in the calculation of expected HD-1 toxicity is the possible presence of P2 or CryII proteins in the HD-1 preparation. Although generally a minor component (22), those proteins have some lepi-

TABLE 3. Expected toxicity of activated HD-1 based on toxicity of individual gene products

Species	FFD_{50} (ng/larva)	
	Observed[a]	Expected[b]
C. fumiferana	16.8	17.9
C. occidentalis	18.1	25.9
C. pinus	13.1	22.7
O. leucostigma	93.8	200
L. dispar	28.6	819
M. disstria	24.0	27.3

[a] Data from Table 2.
[b] Weighted average of gene products in Table 2; weighting factors: 0.136 for CryIA(a), 0.542 for CryIA(b), and 0.322 for CryIA(c), from Masson et al. (17).

dopteran toxicity (35) and could have a synergistic effect on CryIA toxins (22) in some species and not in others. However, when the CryIA toxins were mixed in the ratio reported for HD-1 crystals, we observed the same FFD_{50} against gypsy moth larvae as the HD-1 toxin preparation (data not shown). This suggests that CryII toxins either were not present in the HD-1 preparation (possibly due to differential solubilization under the conditions used for toxin activation) or did not play a role in the observed toxicity of that preparation. Further studies are being conducted to confirm the importance of synergistic interactions in determining toxicity of the HD-1 crystal protein to *L. dispar*.

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