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Redesigning *Bacillus thuringiensis* Cry1Aa Toxin into a Mosquito Toxin

Xinyan Sylvia Liu, and Donald H. Dean*

Department of Biochemistry, The Ohio State University,Columbus, OH, U.S.A., 43210-1292

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The *Bacillus thuringiensis* crystal protein Cry1Aa is normally selectively active against caterpillar larvae. Through rational design, toxicity (LC_{50} 45 μg/ml) to the mosquito *Culex pipiens* was introduced by selected deletions and substitutions of loop residues of domain II. Toxicity to its natural target *Manduca sexta* was concomitantly abolished. The successful grafting of the alternate mosquito toxicity onto the original lepidopteran Cry1Aa toxin demonstrates the possibility of designing and engineering a desired toxicity into any toxin of a common scaffold by reshaping the receptor binding region with desired specificities.

*Bacillus thuringensis (*Bt*)*, an aerobic, gram-positive spore-forming bacterium commonly found in soil, produces parasporal crystal (Cry) proteins with insecticidal activity against a wide range of pests. The structure and function of these toxins is well reviewed (4, 11, 17, 27). The N-terminal domain I is a bundle of eight α-helices with the central, relatively hydrophobic helix surrounded by amphipathic helices. Domain I reportedly functions in the formation and operation of ion channels (27). Domain II, consisting of three anti-parallel β-sheets connected by loops, has been linked to specific receptor binding (27). The C-terminal domain III adopts a lectin-like β-sandwich topology. A number of functional roles have been suggested for this domain, including receptor binding (7, 18, 20) and ion channel formation (8, 28, 32).

Due to the enormous selective pressure imposed by widespread use of Bt Cry proteins in agriculture worldwide, the development of better Cry toxins is of ever increasing importance. The ultimate goal of protein engineering of the insecticidal Cry proteins is to be able to design any Cry toxin to possess toxic activity against any insect. A more immediate goal is to introduce a specific activity into a toxin that does not possess it.

Several examples of protein engineering of Cry toxins have demonstrated enhancements of activity in toxins that already expressed some level of activity. *In vivo* domain substitutions of Cry1Ab resulted in a 4-fold enhancement of activity against *Spodoptera* (12). Site-directed mutations of individual residues in domain II loop regions of Cry3Aa led to a 10-fold increase of activity against *Tenebrio molitor* (33) and mutations in domain II loop regions of Cry1Ab resulted in a 34-fold increase in activity against the gypsy moth, *Lymantria dispar* (25). More extensive deletions and substitutions of domain II loop regions of a mosquitocidal toxin, Cry4Ba, gained toxicity to *Culex*, while its toxicity to the natural target species, *Anopheles* and *Aedes*, was not negatively affected (1). However, to date no manipulation of Cry proteins has completely changed the specificity of a toxin to a different order of insect. This project was a test of the ability of rational design, based on current knowledge of receptor binding epitopes, to synthesize a completely new activity into a Cry protein.

Cry1Aa and Cry4Ba are presumed to share a similar mode of action, but target distinct insect species. With its known tertiary structure and relatively well characterized receptor binding regions, Cry1Aa is an ideal candidate for the design of alternate specificity. Cry1Aa is a lepidopteran toxin with no natural activity toward mosquitoes. In this study, we have altered domain II loops of Cry1Aa to introduce mosquito toxicity.

Loop regions are excellent targets for genetic re-designing of novel toxins with diverse specificity by exchanging residues or chain lengths of the active sites without major disruption of the overall integrity of the toxin. Previously, we predicted the loop sequences of Cry4Ba (1). This prediction was recently verified with the elucidation of the toxin's 3-D structure (6).

Based on secondary sequence alignment (done by Clustal W) and structural analysis (done by SWISS-MODEL) of lepidopteran-specific Cry1Aa and dipteran-specific Cry4Ba, significant differences in length and composition were found in the first two of three

* Corresponding author: Department of Biochemistry, The Ohio State University, 484 W. 12th Ave., Columbus, Ohio, USA, 43210. Tel: (614) 292-8829. Fax: (614) 292-3206. E-mail: dean.10@osu.edu

loops in domain II of Cry1Aa and Cry4Ba (4BL3PAT). Interestingly, it was reported by Abdullah *et al.* (1) that when loop 3 (PAT) of 4BL3PAT was replaced by GAV, a Cry1Aa homologous loop sequence, its toxicity toward mosquito *Culex* was further enhanced. For this reason the third loop of Cry1Aa was left unchanged in the subsequent protein engineering work.

The first two loop regions of Cry1Aa were changed by site-directed mutagenesis, using 4BL3PAT as a template. Loop 1 (residues ^{311}RG^{312}) in Cry1Aa was replaced by YQDL, the loop 1 sequence in Cry4Ba, to extend its length. This mutant was named L1. Cry1Aa loop 2, LY^{367}RRIILGSGPNNQ^{378}, was altered in two separate steps. LYRRIIL was first deleted to produce an intermediate mutant called D3. The loop 1 mutation in L1 was then introduced to D3 creating a reference mutant called L1D3. When changed individually or in combination, none of L1, D3 and L1D3 gave rise to a mosquitocidal toxin. Under the guidance of molecular modeling, a third mutant named 1AaMosq with an additional substitution of NNQ by G was built into L1D3 to mimic the shorter second loop in Cry4Ba but maintain the turn between two β-sheets.

Shown in Fig. 1 are the solved structure of Cry1Aa wild-type toxin and the modeled structure of 1AaMosq mutant. The loops at the bottom of the molecules are loops 1, 2, and 3 from the right to left. Loop 1 is elongated when RG was mutated to YQDL, the relatively long loop 2 is shortened to merely a turn by two rounds of deletion, while loop 3 was left unchanged in 1AaMosq.

Expression and purification of the crystal toxins was essentially as described elsewhere (14). The near UV spectral region of wild-type and mutant Cry1Aa showed no significant variation, indicating that the defined tertiary structure was not disturbed (data not shown). The gradual differences in far UV region agree with the changing ratio of loop components. The results of bioassays on *C. pipiens* larvae (shown in Table 1) indicate that Cry1Aa wild-type and intermediate mutants (L1, D3, L1D3) have no apparent toxicity, while 1AaMosq with triple mutations in both loops 1 and 2 has enhanced activity against *C. pipiens* at μg/ml levels. Concomitant with the gain in mosquito toxicity, toxicity toward *Manduca sexta* determined by surface contamination bioassay was abolished during several rounds of changes in loop residues, confirming the importance of domain II loops in specificity and activity.

The idea of using a protein of known three-dimensional structure to present motifs of various functions or specificity has been a primary goal of protein engineering (16). The use of so-called protein scaffolds for generation of novel binding proteins via combinatorial engineering has emerged as a powerful alternative to natural or recombinant antibodies (22).

The results of this study are an example of enhancing Cry toxicity through an approach that integrates sequence comparison, computational prediction and rational design by mutagenesis. Table 2 shows the toxicity of known mosquitocidal toxins from Bt and *B. sphaericus*. Toxicity of engineered Cry1AaMosq is greater than that

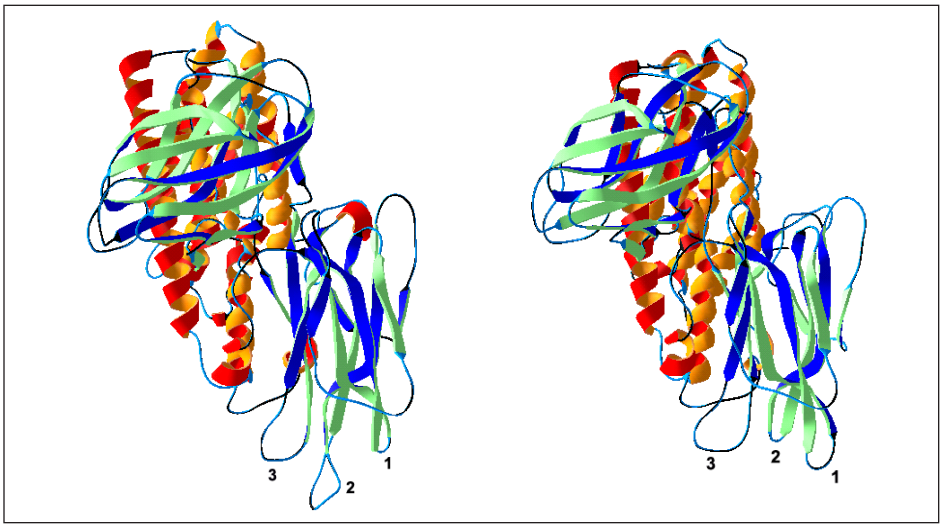

FIG. 1. Structures of Cry1Aa wild type toxin and 1Aa Mosq mutant toxin. On the left is the structure of Cry1Aa wild type toxin. On the right is the modelled structure of 1AaMosq mutant toxin. The loops at the bottom of the molecules are loops 1, 2 and 3 from the right to left. Loop 1 is elongated, loop 2 is shortened and loop 3 is left unchanged in 1AaMosq.

TABLE 1. Bioassay results of Cry4Ba and Cry1Aa toxins to *Manduca sexta* and *Culex pipiens*.

	LC_{50}	
Toxins	*Manduca sexta* [a] (ng/cm^2)	*Culex pipiens* [b]
4BL3PAT	*ND*[d]	*95 ng/ml (69-130)* [e]
4BL3GAV	*ND*[d]	*70 ng/ml (34-129)* [e]
1Aa	*3.37(1.92-7.67)*	*no mortality* [c]
1Aa L1	*6.29(4.48-7.98)*	*no mortality* [c]
1Aa D3	*10.67(6.39-43.30)*	*no mortality* [c]
1Aa L1D3	*1664(1302.15-2175.93)*	*no mortality* [c]
1AaMosq	*no mortality*	*45.73 μg/ml (32.18-59.76)*

a. Two-day old larvae of *M. sexta* were used for bioassays. Mortality was recorded after 5 days exposure to a serial dilution of the toxins. The 95% confidence limit is indicated in parentheses.b. Two-day old larvae of *C. pipiens* were used for bioassays. Mortality was recorded after 24 hours exposure to a serial dilution of the toxins.
c. No mortality at 100μg/ml.
d. ND: not determined.
e. Cited from Abdullah *et al*. (1)

TABLE 2. Toxicity of mosquitocidal proteins ranges of reported toxicity (ng/ml).

	A. aegypti	*A. quadrimaculatus*	*A. stephansi*	*A. gambiae*	*C. quinquefasciatus*	*C. pipiens*	Ref.
Cry1Aa	not active			not active		not active	this work
Cry1AaMosq						42,000	this work
Cry1C	141,000			283,000	126,000		(29)
Cry2Aa	500-1000	38	--	--	1630	>200,000	(5, 10, 21, 31)
Cry4Aa	563-1600		7400	1117	251-980	400	(3, 13)
Cry4Ba	61	25			>80,000	>20,000	(2, 13)
$4BRAL3_{PAT}$	53	44	3		65	95	(1)
$4BRAL3_{GAV}$	44	52			114	37	(2)
Cry10Aa	low toxicity	not active				not active	(14, 30)
Cry11Aa	20-287		455		39.7-64	268-37	(9, 14)
							(15, 23, 24)
Cry11Ba	18-30		42.7		6.5	10	(14, 23)
Cry11Bb	17.9	166.3 (*A. albimanus*)			34.1		(23)
Cry19Aa	1,400,000	3	1039		35	6-187	(2)
Cry19Ba	nd		not active			7520	(26)
Cry20Aa	648,000				700,000		(19)
Non-Cry Proteins							
BinA-B	not active	not active				15.4-487	(21a)

of several natural toxins (Cry1Ca, Cry2Aa, Cry4Ba and Cry20Aa). The successful grafting of the alternate mosquito toxicity onto the original lepidopteran Cry1Aa toxin demonstrates the possibility to design and engineer desired toxicity into any toxin of a common scaffold by reshaping the receptor binding region with desired specificities. By varying the specificity elements in loop regions on a general scaffold, a customized toxin can be selectively tuned to target different insect species.

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