REPORT

Broadening the target host range of the insecticidal Cry1Ac1 toxin from Bacillus thuringiensis by biotechnological means

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ABSTRACT

This work presents the obtention and characterization of mutant hybrid toxin CryAAC-$R_{423}S$, which displays an increased insecticidal activity in comparison to the wild type pore-forming Cry1Ac1 toxin from *Bacillus thuringiensis* var. *kurstaki* HD73. CryAAC-$R^{423}S$ contains domains I and II of Cry1Ac1, and domain III of Cry1Ca1 from *B. thuringiensis* var. aizawai HD133. Additionally, this toxin contains an arginine-to-serine mutation at position 423 ($R_{423}S$). Bioinformatic methods were used to obtain a 3D model of Cry1Ac1 structure in order to predict the structural consequences of R423S mutation. Normal Mode Analysis of wild-type and mutant variants revealed an increased flexibility for the exposed loop β7/β8 from domain II of Cry1Ac1-$R_{423}S$, probably as a result of $R_{423}S$ replacement. CryAAC-$R_{423}S$ was purified by FPLC and functionally characterized. In vitro oligomerization assays showed a higher formation rate of the pre-pore intermediate (~240 kDa) for $R_{423}S$ variant. This reaction is considered to be a critical step in the toxic pathway of Cry toxins. In dose-response bioassays, CryAAC-$R_{423}S$ was active against five lepidopteran species that constitute important agricultural pests in Cuba and the Caribbean. The new toxin may be incorporated as a new insecticide, either as Bt-sprays or as Bt-plants, to support the current agricultural practices for integrated pest management.

Keywords: *Bacillus thuringiensis*, CryAAC-$R_{423}S$, oligomerization, Normal Mode analysis, *Spodoptera frugiperda*

Introduction

The pore-forming Cry1Ac1 toxin from *Bacillus thuringiensis* (Bt) var. *kurstaki* HD-73 is an important component in several commercially available bio-insecticides (Dipel®, Thuricide®) and transgenic plants, such as BollGard I® and Bt Xtra®, from cotton (*Gossypium hirsutum* L.) and maize (*Zea mays* L.), respectively [1, 2]. Cry1Ac1 has been intensively studied, due to its high insecticidal activity against several polyphagous pests of the order Lepidoptera. It belongs to the Cry protein family, and as such is composed of 3 structural domains that play different roles in the events leading to insect toxicity [3].

Domain I is formed by a group of 7 amphipathic α-helixes centered on helix 5. This domain is involved in the processes of pore formation in vesicles and lipid rafts. Domain II, on the other hand, is formed by three anti-parallel β-sheets following a Greek key topology, and is known to be associated to the process of receptor binding at the intestinal microvilli; additionally, it has recently been shown to participate in the process of oligomerization prior to pore formation [4].

Domain III is formed by two pleated β-sheets in an interspersed arrangement and contains the determinants for target specificity. The properties of domain III have been exploited for the obtainment of chimeric toxins with broader target specificities by exchanging domain III regions between different wild-type Cry proteins [5-7]. In the case of Cry1Ac1, a chimeric CryAAC (1Ac/1Ac/1Ca) with a wider target range has been obtained by substituting its domain III with that from Cry1Ca1 of *B. thuringiensis* var. *aizawai* HD-133 [6].

In addition to domain swapping, site-directed mutagenesis has also been used for the improvement of Cry toxins. For Cry1Ac1, the mutation of arginine 423 to serine ($R_{423}S$) at domain II has resulted in higher toxicity towards *Mamestra brassicae* larvae [8].

The objectives of the present work are to obtain and functionally characterize a chimeric CryAAC-$R_{423}S$ toxin that combines the two modifications described above and to study the $R_{423}S$ mutation by Normal Mode Analysis (NMA). Additionally, we characterized the insecticidal activity of the new chimeric toxin against 5 lepidopteron agricultural pests by using bioassays.

Results and discussion

Bioinformatics and molecular biology techniques were used to design and introduce changes into the amino-acid sequence of Cry1Ac1 oriented towards the obtainment of a toxin with broader target specificity. The strategy followed for this purpose consisted in I) modeling the 3D-structure of Cry1Ac1, and performing NMA on Cry1Ac1 and its $R_{423}S$ variant; II) obtainment, by overlapping extension polymerase chain reaction (PCR) and site-specific mutagenesis, of the gene coding for the chimeric CryAAC-$R_{423}S$ protein; III) recombinant expression and purification by FPLC, as well as functional analysis of the obtained chimeric protein, and IV) study of its host range and insecticidal activity by bioassays and Probit analyses.

Structural analysis of the Cry1Ac1 toxin and its $R_{423}S$ variant

A 3D-model of the structure of Cry1Ac1 was obtained based on a structure-led sequence alignment between Cry1Aa1 (PDB: 1CIY) and Cry1Ac1, comprising residues 15 to 620 (Cry1Ac1 ordinates, figure 1A)

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and taking advantage of the high sequence similarity of both proteins (82%), which confers a high degree of confidence to the resulting model. A comparison between the structure of Cry1Aa1 (obtained from X-ray diffraction data) and the Cry1Ac1 model reveals a complete agreement with the general model described for the Cry protein family, that is, an α+β three-domain structure [3]. Arginine 423 (R_{423}) is located in loop β9/β10 from domain II, and its change to serine (S) has been shown to increase the biological activity of Cry1Ac1. According to our model, R_{423} interacts mainly with residues T_{392} and S_{394} of the solvent-exposed β7/β8 loop (Figure 1B). A $R_{423}S$ mutation would, therefore, result in the loss of 2 hydrogen bonds and in a relaxation of steric constraints, due to the smaller side chain of the new residue.

The flexibility of each residue of the wild-type Cry1Ac1 toxin and the Cry1Ac1-$R_{423}S$ mutant was also studied by molecular dynamics, using NMA (figure 2). As it can be seen in the figure, the $R_{423}S$ substitution results in a considerable increase in flexibility for loop β7/β8 of Cry1Ac1, yielding a peak centered on S_{394}. The mutation, in turn, had no effect on the flexibility of the remaining residues. This result constitutes the first case in which NMA has been used for predicting the effects of a mutation in the context of a Cry toxin, and the first report suggesting that loop β7/β8 may be relevant for the insecticidal activity of Cry proteins [9, 10].

Obtention of the CryAAC-$R_{423}S$ toxin and *in vitro* oligomerization assay

The determinants for host specificity of the Cry toxins reside within domain III, and exchanging this region among members of the family influences the target host range [5]. In order to broaden the insect specificity of Cry1Ac1, the gene region coding for domain III was replaced with the corresponding region from the gene for Cry1Ca1 using overlapping extension PCR. Additionally, and in order to increase the activity of the resulting CryAAC hybrid toxin, we introduced the $R_{423}S$ mutation by site-specific mutagenesis. The resulting gene was cloned into a binary expression vector and this construction was in turn electroporated into the acrystalliferous IPS-78/11 strain of *B. thuringiensis* var. *israelensis*, verifying the formation of parasporal inclusions in the resulting CryAAC-$R_{423}S$ transformants by phase contrast microscopy and transmission electron microscopy. CryAAC-$R_{423}S$ was

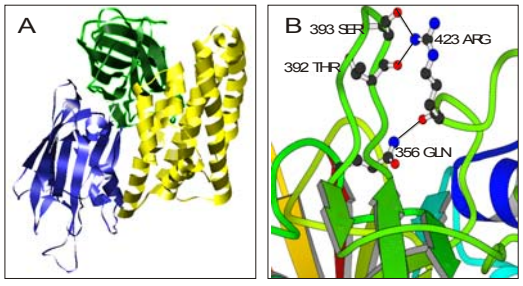

Figure 1. Three dimension model the structure of Cry1Ac1 (domain I: yellow, domain II: blue and domain III: (A), and green). B- Close-up of residue R_{423} and its interaction with neighboring residues in the 3D-structural model of Cry1Ac1 (B)

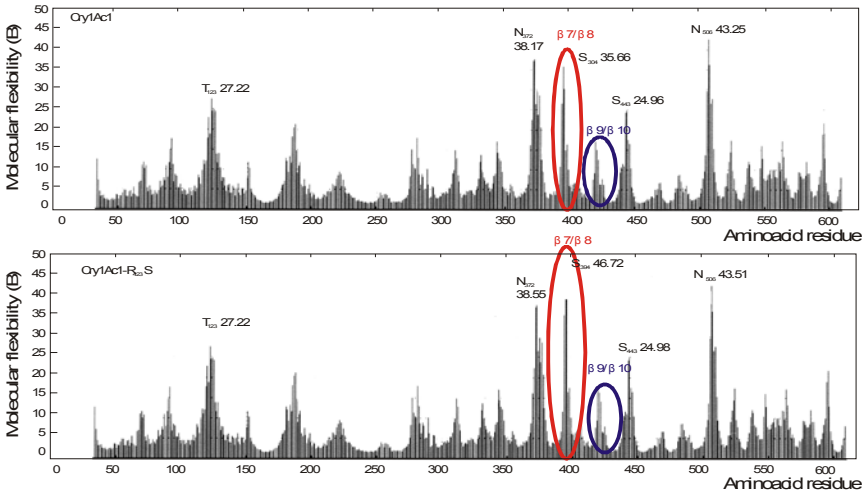

Figure 2. Molecular flexibility values (B) for each aminoacid residue from toxins Cry1Ac1 and Cry1Ac1-R423S. The B values for residues at the center of the most flexible regions are explicitly shown. Loops β7/β8 and β9/β10 are circled by red and blue ellipsoids, respectively.

purified by anion exchange chromatography (AEC) and characterized by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels (figure 3A). Purity of the final preparation was estimated by densitometric analysis to be around 92-95%.

This is the first report of the construction and recombinant expression of a chimeric CryAAC-$R_{423}S$ toxin, together with the procedure for its purification [9, 10]. Since Cry proteins are pore-forming toxins, pre-pore oligomerization is considered to be a critical step in the series of biochemical events leading to toxicity. Therefore, we decided to examine the kinetics of oligomerization of CryAAC-$R_{423}S$ by an *in vitro* assay. Figure 3B shows that the introduction of the $R_{423}S$ mutation into CryAAC increases the rate of formation of the pre-pore oligomeric intermediate, a result that for the first time reveals the molecular basis for the phenotypic effects of this mutation [10].

Insecticidal activity of CryAAC-$R_{423}S$ against neonate larvae from five species of lepidopteran pests

Dose-response bioassays were implemented for studying the toxicity of CryAAC-$R_{423}S$ against neonate larvae from five different species of lepidopteron pests, estimating the median lethal dose (LD_{50}) by probit analysis. The species included in the assays were *Manduca sexta*, *Pieris brassicae*, *Plutella xylostella*, *Mamestra brassicae* and *Spodoptera frugiperda*, which constitute important agricultural pests for economically important crops in the Caribbean region such as tobacco, tomato, cabbage, maize and rice, among others. Table I shows the results from these bioassays. Based on these results, it can be concluded that the substitution of domain III from Cry1Ca1 for the wild-type domain III of Cry1Ac1 broadens the insect specificity of the latter towards pests such as *M. brassicae* and *S. frugiperda* [11]. It is important to underline that *S. frugiperda* (common name: fall armyworm) is the most important pest affecting corn in Cuba, causing losses of up to 40% in annual yields.

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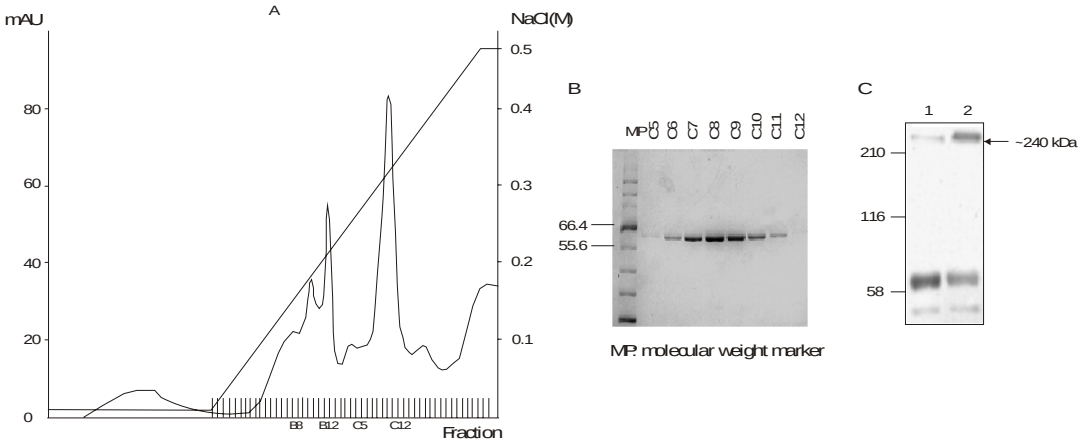

Figure 3. Obtention of the CryAAC-R_{423}S toxin. A- FPLC chromatogram from the purification of CryAAC-R_{423}S; B- Electrophoretic analysis by 12% SDS-PAGE of fractions from the major peak; C- Oligomerization assay: 1- CryAAC, 2- CryAAC-R_{423}S.

The dose-response bioassays also highlight the phenotypic effects of the R_{423}S mutation at domain II of CryAAC. By comparing the LD_{50} values of CryAAC-R_{423}S to those of CryAAC, it can be seen that this mutation had a significant effect in the insecticidal activity of the former, incrementing its toxicity approximately two-fold against *M. sexta* and *S. frugiperda* (17 ng/cm^2 vs. 48 ng/cm^2 and 165 ng/cm^2 vs. 288 ng/cm^2, respectively). On the other hand, the introduction of the R_{423}S mutation had no significant statistical effect ($P>0.05$) on the insecticidal power of CryAAC (expressed in terms of its LD_{50}) against neonate larvae from *P. brassicae* and *P. xylostella*. Actually, in these two latter cases the insecticidal potency was even lower than that of the parental Cry1Ac1, suggesting a compromise between specialization and target host range. It has been shown that for some Cry toxins domains II and III are jointly involved in the stages of recognition and binding to the receptor in the plasmatic membrane of the target cell [3]. Therefore, the fact that the replacement of the original domain III of Cry1Ac1 affected the toxicity of CryAAC and CryAAC-R_{423}S against *P. brassicae* and *P. xylostella* suggests that this domain indeed participates in the events leading to the appearance of toxicity in these insects.

In summary, the high insecticidal activity of CryAAC-R_{423}S against the five assayed species demonstrates the practical importance and the usefulness of this new toxin as a broad range biological insecticide against agricultural pests [9-11].

Table 1. LD50 values (ng/cm2) for the chimeric toxins and their parentals in bioassays against neonate larvae from five different lepidopteran species

Insect	Cry1Ac1	Cry1Ca1	CryAAC	CryAAC-R_{423}S
M. sexta	5(3.2-7.8)[a]	19.1(9.4-33)	48(30.5-70.1)	17(11-25)
P. brassicae	9(5.57-14.2)	382(249-544)	50.2(34.9-72.9)	44(31.1-67.8)
P. xylostella	15(7.1-22)	112(88-170)	40.2(25-69)	38(19-63)
M. brassicae	N.T.[b]	581(480-748)	1280(952-2000)	1098(839-1609)
S. frugiperda	N.T.	N.T.	288(189-474)	165(117-198)

[a] confidence intervals (95%).
[b] N.T.:not toxic at concentrations > 3000 ng/cm^2.

Conclusions

The results obtained in this work show that it is possible to design and obtain new insecticidal toxins using tools from the accelerated molecular evolution arsenal (prediction by modeling and site-specific mutagenesis). This is the first report detailing the structural consequences of the mutation of residue R_{423} from domain II of Cry1Ac1 and its importance for the insecticidal activity of this protein [9, 10]. The new chimeric toxin exhibits a higher insecticidal activity than the parental molecule and is active against a wider number of pests, including the fall armyworm, which constitutes the main pest for maize crops in Cuba. CryAAC-R_{423}S might be incorporated as a Bt-spray or a Bt-plant into the national programs for the integrated control of pests, becoming an additional tool for the protection of crops such as corn, rice, tomato and cabbage against their main insect pests.