The mutation $R_{423}S$ in the Bacillus thuringiensis *hybrid toxin CryAAC increases* in vitro *oligomerisation and* in vivo *toxicity against* Spodoptera frugiperda

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RESEARCH

ABSTRACT

Bacillus thuringiensis hybrid toxin CryAAC (1Ac/1Ac/1Ca) is toxic to fall armyworm (*Spodoptera frugiperda*). It has been previously demonstrated that a $R_{423}S$ mutation that increases flexibility in the CryAAC loop β7/β8 (G_{391}-P_{397}) had a positive effect in the toxicity of the hybrid against *Mamestra brassicae*. According to above mentioned results, we have investigated the toxicity of the mutant hybrid CryAAC-$R_{423}S$ against *S. frugiperda* at two stages of larval development. Bioassays demonstrated that $R_{423}S$ substitution in CryAAC almost doubled the toxicity to *S. frugiperda* neonates (LC_{50} 165 against 288 ng/cm^2) and had the strongest inhibitory effect on the third-instar larvae growth [growth inhibition (G.I.) 137% against 112%]. Features of CryAAC in *S. frugiperda* such as, protoxin *in vitro* processing with gut juice and binding of FPLC-purified toxins to proteins from brush border membranes were not affected after $R_{423}S$ substitution. On the contrary, protoxin activation in the presence of *S. frugiperda* brush border membrane vesicles revealed differences in the concentration of generated oligomeric form between the hybrid and the mutant hybrid toxin that could be the reason for the increased *in vivo* toxicity observed for CryAAC-$R_{423}S$.

Keywords: Bacillus thuringiensis, Cry toxins, *Spodoptera frugiperda*, $R_{423}S$ mutation, toxin oligomerisation

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RESUMEN

La mutación R423S en la toxina híbrida CryAAC de *Bacillus thuringiensis* incrementa la oligomerización *in vitro* y la toxicidad *in vivo* contra *Spodoptera frugiperda*. La toxina híbrida CryAAC (1Ac/1Ac/1Ca) de *Bacillus thuringiensis* es toxica contra la palomilla del maíz (*Spodoptera frugiperda*). Previamente se demostró que la mutación $R_{423}S$ que incrementa la flexibilidad en el lazo β7/β8 (G_{391}-P_{397}) de CryAAC tuvo un efecto positivo en la toxicidad del híbrido contra *Mamestra brassicae*. De acuerdo a esto, nosotros hemos investigado la toxicidad del híbrido mutante CryAAC-$R_{423}S$ contra dos estados del desarrollo larval de *S. frugiperda*. Los bioensayos demostraron que la sustitución $R_{423}S$ en CryAAC casi duplicó la toxicidad hacia larvas neonatas de *S. frugiperda* (LC_{50} 165 contra 288 ng/cm^2) y tuvo un efecto inhibidor más fuerte sobre el crecimiento de larvas de tercer estadio [inhibición del crecimiento (I.C.) 137% contra 112%). Las características de CryAAC en *S. frugiperda*, tales como, la activación *in vitro* de la protoxina con jugo gástrico y la unión de la toxina purificada por FPLC a proteínas de la membrana de borde en cepillo no se afectaron por la sustitución $R_{423}S$. Por el contrario, la activación de las protoxinas en presencia de vesículas de la membrana de borde en cepillo de *S. frugiperda* reveló diferencias en la concentración de la forma oligomérica generada por la toxina híbrida y la híbrida mutante y que podría ser la razón para el incremento de la toxicidad observada *in vivo* de CryAAC-$R_{423}S$.

Palabras clave: Bacillus thuringiensis, Cry, *Spodoptera frugiperda*, mutación $R_{423}S$, oligomerización

Introduction

Spray products based on the spore-forming bacterium *Bacillus thuringiensis* (Bt) have been used for decades as control agents of insect pests and disease vectors [1]. Since 1996, Bt insecticidal Cry toxins [2, 3] have been used commercially in transgenic crops, particularly cotton and maize, with 32 million ha of Bt crops being cultivated worldwide in 2006 [4]. Cry toxins have three structural domains of approximately 200 residues each. Domain I is formed by a bundle of seven anti-parallel a-helixes where a central amphipatic helix (α-5) is surrounded by the six others. The function of this domain has been associated with membrane lytic pore formation [5, 6]. Domain II consists of three anti-parallel β-sheets folded in a "Greek key" topology adopting a so-called β-prism conformation. Domain III is formed by two β-sheets in a β-sandwich structure with a "jelly roll" topology. Both domain II and III have been associated with the recognition and binding of a receptor in midgut cells [2, 7-10]. Domain III has been also associated with regulation of the pore activity [2].

Among Cry toxins, the Cry1-class contains several that are highly specific for lepidopteran pests. The primary site of Cry1 toxins action is the brush border membrane of midgut epithelia [11]. Once ingested, crystals are solubilized in the alkaline and reducing environment of the midgut lumen as protoxins that are truncated by midgut proteases to produce active toxins [12]. The toxin monomers then pass through the peritrophic matrix and bind to specific sites on microvilli, where oligomerise and insert into the apical membrane of brush border epithelial cells to form pores that disrupt functional membrane processes [13]. Toxin action induces a series of successive events that leads to the destruction of midgut cells, ultimately causing the insect death by inanition and septicemia [2, 3, 14].

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The general acceptance of Bt Cry toxins as substitute of chemical insecticides in spray formulations needs to overcome disadvantages as the narrow spectrum of activity and their low insecticidal potency to some economically important pests. Cry toxins form a large family of proteins, similar in overall structure, but differing in details that determine their activity for particular insect species [2, 15]. Within the small target ranges there are also dramatic differences in potency between species that are often closely related. Hybrids in domain III derived from toxins with different specificities have resulted in the construction of new chimerical toxins with a broader spectrum activity [7, 9, 10]. The toxicity of any existing toxin to an insect has also been improved by substituting its domain III [16-20]. This suggests that domain III, through an unknown mechanism, can play an important role in determining specific toxicity against insects. Moreover, such improved toxins could be used in resistance management strategies as alternatives for toxins to which insects have become resistant by losing or changing a receptor [21].

Genetic protocols for Cry toxin potency improvement may require not only major mutagenesis such us the construction of hybrid domain-swapped toxins, but also the additive effect of relatively minor incremental modifications at widely separated positions in the toxin structure. In a previous study, a substitution by serine of the arginine 423 (R_{423}) in the hybrid CryAAC (combining the Cry1Ac first and second domain with the Cry1Ca third domain) had a positive effect on the activity toward *Mamestra brassicae* [20]. Computer simulations suggested a greater fle-xibility in the CryAAC loop β7/β8 (G_{391}-P_{397}) caused by the R_{423}S substitution as the potential reason for the increase in *M. brassicae* toxicity. Since the R_{423} residue is located in domain II of CryAAC, an increased flexibility in this loop can be important for toxicity in terms of interaction with the receptor or oligomerisation of individual toxins to form a lytic pore complex. Since CryAAC has also resulted to be toxic against *Spodoptera frugiperda* [19], the aim of this work was to determine weather the R_{423}S substitution in CryAAC could enhance further its potency to this economically important pest. We also investigated the consequences of R_{423}S mutation in CryAAC through *in vitro* toxin binding and oligomerisation experiments.

Materials and methods

Toxin preparation and purification

CryAAC and CryAAC-R_{423}S protoxins were obtained from recombinant Bt strains carrying plasmids pHY-AAC and pHY-AACR_{423}S, respectively [20], which were introduced by electroporation into the acrystalliferous Bt var. *israelensis* IPS-78/11 strain [22]. Crystals containing toxin were recovered using sucrose density gradients by the method of Thomas and Ellar [23]. Crystals from the parental toxins Cry1Ac and Cry1Ca were kindly provided by D.J. Ellar (Department of Biochemistry, University of Cambridge, UK). Crystals were solubilized in a solution containing 50 mM Na_2CO_3 and 10 mM dithiothreitol, pH 10.5, at 37 ºC for 60 min and the concentration subsequently adjusted to 1 mg/mL using a Bio-Rad protein assay (Bio-Rad, Richmond, Califor-nia, USA). Solubilized protoxins were then activated with trypsin at a protease:protoxin ratio of 1:10 (w/w) at 37 ºC for 60 min.

Further purification of activated CryAAC and CryAAC-R_{423}S toxins was accomplished by anion exchange fast protein liquid chromatography (FPLC) using a Hitrap Q HP (5 mL) column (Amersham-Pharmacia, Amersham, UK). Before loading toxins into the column (10 mg), toxin buffer was changed for column equilibration and loading buffer (0.02 M Tris-HCl, pH 8.01, 25 ºC) using a PD-10 column (Amersham-Pharmacia). For toxin elution from the anion exchanger, a lineal gradient of NaCl (ranged from 0 to 0.5 M) was used. Aliquots of 1 mL were automatically collected and 10 µL samples analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-12% PAGE) according to Laemmli [24]. Bands in gels were visualized with Coomassie brilliant blue staining. Aliquots containing the pure toxin were pooled, desalinated in a PD-10 column, and concentrated in an Amicon concentrator (model 8200; Amicon Division, W. R. Grace &. Co., Beverly, MA, USA) with an YM-30 membrane to a final concentration between 1000-1500 µg/mL. Protein purity was determined by scanning densitometry of Coomassie Blue stained toxin bands on a 12% SDS polyacrylamide gel. Toxins were quantified by the Lowry method [25] using bovine serum albumin as standard.

Insect and bioassays

S. frugiperda eggs were obtained from adults (moths) collected in maize plantations from the region of Quivicán, south of Havana, Cuba. Neonates were reared on artificial nutritive diet based on maize flour [26] at 30 ºC and 80% relative humidity under a 16:8 h light:dark cycle. The insect population had been maintained under laboratory conditions for ca. 50 generations prior to the experiments.

Bioassays were performed using the diet-surface contaminating procedure. Toxin preparations were serially diluted in six different toxin concentrations (2000, 1000, 500, 250, 125 and 63 ng/cm^2) and tested by using 24 neonate larvae per concentration. Toxin samples (20 µL) were uniformly applied into each well (Multiwell-24 plates) with the artificial nutritive preparation and then allowed to dry. Each larva was placed onto the nutritive surface and reared at 28 ºC with a photoperiod 16:8 h. Mortality was assessed after three days, where the effective dose (50% lethal dose of the toxins, LC_{50}) and 95% fiducial limits were calculated using PROBIT analysis of the results from three independent bioassay experiments [27].

A growth inhibition (GI) assay was performed by diet surface contamination with 2 µg/cm^2 of toxins. This value of toxin concentration was previously suggested by Herrero et al. [28] for GI experiments. Twenty pre-weighed early third-instar larvae per toxin were allowed to eat contaminated diet. After 48 h, larvae were weighed again and the GI, expressed as percentage, was calculated as described previously [29]. Values of GI higher than 100 mean a loss of weight of the larvae during the assay.

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BBMV preparation

Brush border membrane vesicles (BBMV) to be used in binding assays were prepared according to the method described by Wolfersberger [30]. The same protocol was used for the preparation of the BBMV used in oligomerization assays, except that Ethylene glycol-bis(beta-aminoethyl ether)-N,N,N´,N´-tetraacetic acid (EGTA) was excluded from the buffers.

Ligand blotting

S. frugiperda BBMVs were dissolved in concentrated SDS-polyacrylamide gel electrophoresis sample buffer and heated to 100 ºC for 5 min before being loaded (6 mg per lane) on a 10% acrylamide gel. After electrophoretic separation, BBMV proteins were transferred to nitrocellulose by electroblotting. Duplicate strips were cut from the filter and washed in deionized water and subsequently in Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8, and 150 mM NaCl) containing 0.5% Tween 20. Next, the strips were incubated for 2 h in blocking solution (TBS containing 3% dried nonfat milk and 0.5% Tween 20). They were then incubated for 3 h with purified toxins at 1mg/mL in blocking solution, washed three times for 5 min each in TBS containing 0.5% Tween 20, and incubated for 1.5 h in rabbit anti-Cry1A serum [31] diluted 1:1 000 in blocking solution. After three more washes for 5 min each in TBS 0.5% Tween 20, bound toxin was detected by incubation for 45 min in mouse anti-rabbit-horseradish peroxidase conjugate diluted 1:3 000 in blocking buffer, followed by three washes for 5 min each in TBS-0.5% Tween 20 and visualization with the enhanced chemiluminescence kit (Amersham-Pharmacia).

Midgut juice isolation and *in vitro* processing of protoxins

Fifth-instar *S. frugiperda* larvae were chilled on ice for 10 min and midgut tissue was dissected. The midgut juice was separated from solid material by centrifugation (20 000 rpm, 20 min, 4 °C). The supernatant was removed and recentrifuged for 20 min at the same speed, and the resulting supernatant was removed and filtered through 0.22 µm filters. The midgut juice was stored at -70 °C until needed.

Five micrograms of solubilized protoxins in 50 mL of the solubilization buffer were incubated at 37 °C for 8 h in the presence of midgut juice at a concentration of 5% (vol/vol). Proteolysis was stopped by adding phenylmethanesulfonyl fluoride (1mM, final concentration), and the samples were separated into soluble and insoluble fractions by centrifugation (20 000 rpm, 20 min, 4 °C). In order to determine the extent of toxin proteolysis, 10 µL samples were subjected to a SDS-10% PAGE.

Oligomerisation assay

Oligomerisation assays were performed with small modifications of the method described by Rausell *et al*. [32]. Summarizing, 20 µl of solubilization buffer containing 10 pmol of protoxins was incubated with 10 µl of solubilization buffer containing 2 mg of *S. frugiperda* BBMV without EGTA for 15 min. The incubation was stopped by adding phenylmethanesulfonyl fluoride (1mM, final concentration), and the samples were separated into soluble and insoluble fractions by centrifugation (20 000 rpm, 20 min, 4 °C). The supernatant was mixed with SDS/PAGE loading buffer and heated at 70 °C for 10 min. Proteins in the sample were separated in an SDS-10% PAGE gel. Monomeric and oligomeric forms of toxins were detected using rabbit polyclonal antibodies raised against the Cry1Ac protoxin in a standard Western blot protocol [33] and visualized with the enhanced chemiluminescence kit (Amersham-Pharmacia).

Results

In order to determine the effect of R_{423}S substitution in the CryAAC hybrid on the toxicity against *S. frugiperda*, both CryAAC and CryAAC-R_{423}S proteins were expressed and purified from the acrystalliferous Bt strain IPS 78/11. Sucrose-gradient isolated crystals yielded protoxins of expected size (~130 kDa) on SDS-10% PAGE gels. *In vitro* trypsin-activated toxins were then used in bioassays against neonate larvae and in GI experiments against third-instar larvae.

Bioassays of the hybrid and mutant hybrid toxins on larvae of *S. frugiperda* revealed a biological effect of the mutation. As it is shown in Table 1, the R_{423}S mutation almost doubled the potency of CryAAC against neonate larvae (LC_{50} 165 versus 288 ng/cm^2). An increase in the activity of the mutant hybrid was also detected for larvae of third-instar stage, confirming that any effect in the mode of action of the hybrid toxin conferred by the mutation is maintained in the older larvae. Compared with the CryAAC toxin, GI expressed as percentage was higher for the mutant hybrid CryAAC-R_{423}S (Table 1).

Figure 1 shows the results of CryAAC and Cry AAC-R_{423}S purification by anionic exchange chromatography (AEC) in a FPLC system. The selection of an anionic exchanger was due to the basic isoelectric point of most Cry1 activated toxins (above pH 7) [34]. As it is shown in the chromatograms (Figure 1A and C), for both toxins a major elution peak occurred from C6 to C12 fractions corresponding with a NaCl at an approximate 0.3 M concentration. SDS-PAGE (12%) analysis of above mentioned fractions showed activated-form of toxins with an apparent molecular mass of 62 kDa (Figure 1 B and D). Though the same protein amount (10 mg) of CryAAC and CryAAC-R_{423}S was loaded into the column, the recovered amount of Cry AAC was higher according to the C6-C12 peak length. A fraction of the activated CryAAC-R_{423}S toxin eluted early as a complex peak from B8 to B12 fractions at around 0.1 M of NaCl (Figure 1C). This peak possibly corresponded to toxin aggregates formed after

Table 1. Toxicities of trypsin-activated toxins toward S. *frugiperda larvae*. LC_{50} is expressed as ng/cm2; confidence intervals (95%) are given in parenthesis. GI values are means ± standard error.

Toxin	LC_{50}	GI (%)
CryAAC	288 (202-354)	112 ± 0.7
CryAAC-R_{423}S	165 (117-198)	137 ± 0.4

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starting with NaCl-gradient elution. After the AEC, the purity of activated toxins ranged from 92-95%.

To identify CryAAC- and CryAAC-R_{423}S-binding components in *S. frugiperda* BBMV preparations, a "ligand" western blot experiment was performed. BBMV proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose, incubated with the pure toxin preparations, and subsequently detected with a rabbit anti-Cry1Ac antiserum. According to Figure 2, lane 1 and 2, this antiserum reacted efficiently in western blot experiments with the hybrid toxin but not with proteins from the *S. frugiperda* BBMV. The results of ligand blot in Figure 2, show CryAAC and CryAAC-R_{423}S's binding proteins in *S. frugiperda* BBMV. Both toxins bound to membrane proteins of approximately 210 and 195 kDa and to a smaller protein of ca. 40 kDa.

In the environment of Lepidoptera gut lumen, solubilised Cry1 protoxins face a highly complex mixture of trypsin -and chymotripsin- like proteases at a high proteases:protoxins ratio that could affect toxin potency in case of miss-processing or completely degradation of toxins [12]. *In vitro* processing experiments were carried out in order to characterize the activation pattern of CryAAC and CryAAC-R_{423}S toxins after long term incubation with *S. frugiperda* midgut juice (Figure 3). After 8 h of incubation with 5% *S. frugiperda* gut juice, CryAAC-R_{423}S generated a single soluble product of about 58 kDa in size that did not differ from that obtained with CryAAC. No detectable product was found in the insoluble fraction of both toxins.

According to the proposed mechanism of action of Cry1 toxins, oligomerisation of toxin monomers is an important step prior to the insertion into the apical membrane of epithelial gut cells to form lytic pores [14]. *In vitro*, formation of toxin oligomers from protoxins has been demonstrated when toxin activation occurs in the presence of BBMV from susceptible insect [14, 35]. Figure 4 shows a comparison of the activation of CryAAC and CryAAC-R_{423}S protoxins after incubation with *S. frugiperda* EGTA-free BBMV. An oligomeric form of the CryAAC-R_{423}S and to a lesser extent, of the CryAAC, with molecular weights slightly greater than 250 kDa were detected on immunoblot after incubation of the protoxins with *S. frugiperda* BBMV. This result showed a greater oligomerisation capacity for CryAAC-R_{423}S than for the hybrid toxin.

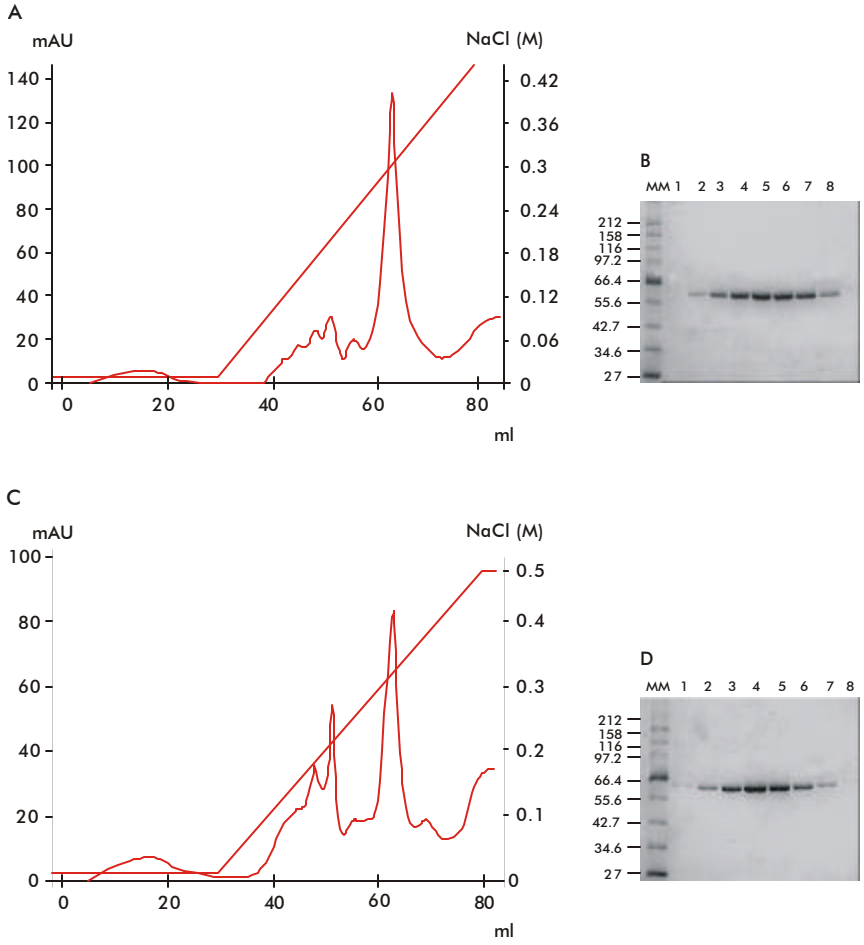

Figure 1. Purification profiles of the anionic exchange chromatography of CryAAC and CryAAC-R_{423}S toxins and SDS-12% PAGE analysis of the toxin peak fractions. A and B: CryAAC; C and D: CryAAC-R_{423}S. Gel lanes: 1 to 8 correspond to fractions C5 to C12, respectively. Lane MM: Broad range molecular weight marker. Sizes are expressed in kDa.

Discussion

Here we report the effect of R_{423}S mutation of the hybrid CryAAC (1Ac/1Ac/1Ca) on the insecticidal potency and toxin binding to BBMV proteins of *S. frugiperda*, as well as the oligomerisation capacity. *S. frugiperda* is the most economically important pest of maize in the Caribbean, Central and great part of South America. Previously, the hybrid toxin CryAAC showed the highest toxicity (lowest LC_{50}) among seven Bt Cry1 toxins (five wild type and two domain-swapped) against a strain of *S. frugiperda* from a Cuban maize field [19]. The mutation R_{423}S increased toxicity of CryAAC against neonate and third-instar larvae of *S. frugiperda* as was evidenced in bioassays and GI experiments. Previously, CryAAC-R_{423}S was also found to be more toxic than CryAAC against *Mamestra brassicae*, one of the most important lepidopteran pests of cabbage, thought differences in toxicity were not so high [20]. Differences in toxin potency against different Lepidoptera species could be attributed to other factors that are more insect-specific (*i.e.*, gut lumen environment, type of receptor molecules). The mutation at CryAAC arginine 423 (R_{423}) residue that changed it by serine increases predicted flexibility of loop β7/β8 (G_{391}-P_{397}) and had a positive effect in the toxicity of the hybrid. Since the substitution R_{423}S was not observed to produce any noticeable increase in inter-domain motion or major exchange in flexibility anywhere else in the molecule, any effect on CryAAC-R_{423}S activity could be related to the increased flexibility of loop β7/β8 that positively affected any of the steps of the proposed mechanism of action for Cry1 toxins in Lepidoptera. Loop β7/β8 is located in domain II of CryAAC, a domain mainly associated with the receptor binding on the apical membrane of insect midgut columnar cells [2]. Also, this loop could be involved in oligomerisation of individual toxins to form a lytic pore complex. A crucial role in toxicity for exposed loops of Cry2A [36], Cry3A [37], and Cry4A and Cry4B [38] has been previously proposed.

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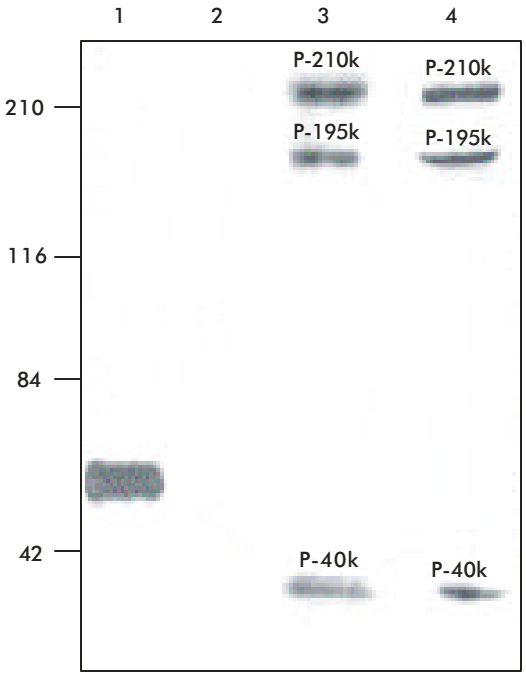

Figure 2. Detection of CryAAC and CryAAC-R_{423}S binding to *S. frugiperda* BBMV proteins by Ligand blot. Lanes: 1, CryAAC as the positive control for immunoblotting; 2, no toxin added; 3, CryAAC; 4, CryAAC-R_{423}S. The molecular weights indicated in kDa correspond to the Prestained molecular weight standard mixture (Sigma-Aldrich, St. Louis, Mo., USA).

Binding of Cry toxins is a complex process in which both, domain II and domain III seem to play a role, either by being involved in binding to receptors together or by having distinct functions in different binding steps. In ligand blot experiments, both CryAAC and CryAAC-R_{423}S recognized and bound to the same set of *S. frugiperda* BBMV proteins. Accordingly, the substitution R_{423}S did not affect the binding properties of the hybrid toxin. Hybrid CryAAC comprises domain I and II of Cry1Ac and domain III of Cry1Ca. Cry1Ac and Cry1Ab share same domain I and II and only differ in domain III. In

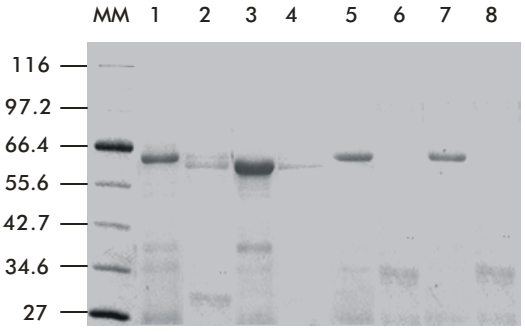

Figure 3. Proteolytic activation of δ-endotoxins with *S. frugiperda* gut juice. Cry1Ca (lanes 1 and 2), Cry1Ac (lanes 3 and 4), CryAAC (lanes 5 and 6) and CryAAC-R_{423}S (lanes 7 and 8). Lanes 1, 3, 5, and 7, soluble toxin fraction; lanes 2, 4, 6, and 8, insoluble toxin fraction. Lane MM: Broad range molecular weight marker (NEB, New England, USA). Sizes are in kilodaltons.

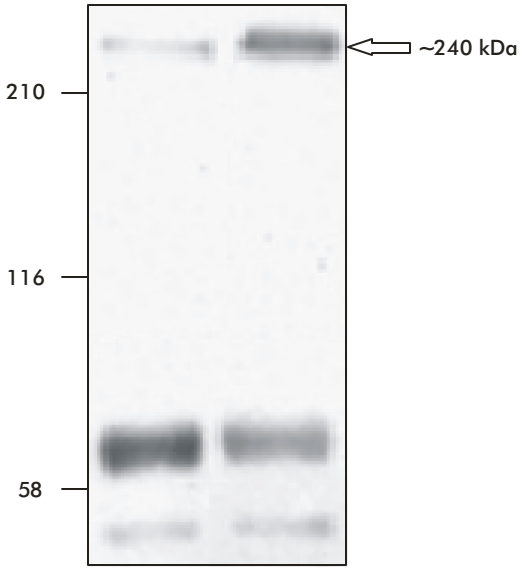

Figure 4. Oligomer formation of CryAAC and CryAAC-R_{423}S by activation with *S. frugiperda* BBMV. Lane 1: CryAAC; lane 2: CryAAC-R_{423}S. The molecular weights indicated in kDa correspond to the Prestained molecular weight standard mixture (Sigma-Aldrich, St. Louis, Mo., USA).

Manduca sexta, the isolated receptors for Cry1Ac [39] and Cry1Ab [40] (both isolated by using ligand blotting for detection) are aminopeptidase N (APN) and cadherin-like (Bt-R1) proteins with molecular masses of 120 and 210 kDa, respectively. Cry1Ac binding to APN has been shown to involve in part domain III that recognize a N-acetylgalactosamine residue in the receptor [41]. In our study, we found as the CryAAC and CryAAC-R_{423}S binding proteins on *S. frugiperda* BBMV, two polypeptides of approximately 210 and 195 kDa and one smaller protein of approximately 40 kDa. The lack of recognition of a 120 kDa APN in the hybrid and the mutant hybrid could be related to the substitution of the Cry1Ac domain III by the corresponding in Cry1Ca. In fact, Cry1Ca binding in BBMV from both *S. frugiperda* and *Spodoptera exigua* has been reported toward a single protein of ca. 40 kDa [42, 43]. Future studies will have to determine the relative importance of the observed types of binding and their relevance for *in vivo* binding and toxicity.

In the experiment of *in vitro* processing of protoxins, both hybrid and mutant hybrid were completely processed by *S. frugiperda* gut juice after 8 hours of incubation providing a soluble product of ~58 kDa corresponding to the activated toxins. In order to study if the R_{423}S mutation affected the formation of CryAAC oligomers, protoxin processing with *S. frugiperda* BBMV was also investigated. Toxin oligomerisation is considered an important step in the mode of action of Cry toxins and involves interactions with domain II loops [44, 45]. Previously, mutations in domain II loop 2 of Cry1Ca was shown to affect toxin oligomerisation and toxicity against *S. exigua* [28]. Even when CryAAC toxin formed oligomer

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structures, they were more prominent in the mutant hybrid. The possible explanation for this observation could be the predicted increased flexibility of loop β7/β8 in CryAAC-R_{423}S toxin [20] that could be indirectly beneficing oligomerisation step after a receptor(s) recognition and hence, the observed increased toxicity against *S. frugiperda*. Also, detected increased oligomerisation of CryAAC-R_{423}S toxin might be the reason for the yield lost during the AEC purification. Potential binding of monomers to the positively charged functional groups in the resin could simulate *in vivo* toxin binding to membrane receptors and trigger the oligomerisation process. As the mutant hybrid variant has an increased capacity of oligomerisation, it could form aggregates in the column more easily than CryAAC and eluted early during the purification.

Finally, these results represent the first evidence that a higher flexibility of loopβ7/β8 in Cry1Ac domain II increases oligomerisation of toxin monomers that produce the prepore intermediate necessarily to open lytic pores in target membranes. The higher activity against *S. frugiperda* showed by the chimeric toxin CryAAC-R_{423}S opens a possibility for its use as part of Integrated Pest Management programmes addressed to control this pest in open maize fields.

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