

Studies on the proteolytic processing and binding of *Bt* toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 in the midgut of Western corn rootworm (*Diabrotica virgifera virgifera* LeConte)

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Abstract: The Western corn rootworm (WCR) is one of the most economically important corn pests worldwide. One possibility for controlling this pest is the cultivation of *Bt*-corn. However, widespread cultivation of *Bt*-corn may increase the probability of the development of pest populations resistant to the respective *Bt* toxins. To establish test systems for identifying resistance mechanisms in the case of resistance development, different parameters involved in the processing of *Bt* toxins in the midgut of third instars larvae of WCR (European strain) were studied. The proteolytic processing of *Bt* toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 by WCR midgut juice was examined, but no degradation of any of these toxins was observed. Ligand-blot binding analyses with Cry3Bb1 as well as Cry34Ab1 and Cry35Ab1 revealed specific receptors in the WCR midgut epithelium. The molecular weights for Cry3Bb1, Cry34Ab1, and Cry35Ab1 receptors were characterized as having molecular weights of approximately 30 kDa, 110 kDa, and 50 kDa, respectively.

Key words: Cry3Bb1, Cry34Ab1/Cry35Ab1, resistance mechanisms, midgut, gut juice, gut epithelium, proteolytic processing, binding analyses

Introduction

The Western corn rootworm (WCR) is one of the most economically important corn pests worldwide. In Europe, the WCR was observed for the first time at the beginning of the 90s, spreading since that time and emanating further from South East Europe. One possibility to control this pest is the cultivation of *Bt*-corn. For *Bt*-corn with activity against the WCR, different genes encoding *Bt* toxins were introduced into corn, such as Cry3A by Syngenta, the binary Cry34Ab1/Cry35Ab1 by DOW AgroSciences and Pioneer, and Cry3Bb1 by Monsanto.

However, widespread cultivation of the resulting *Bt*-corn may increase the probability of the development of pest populations resistant to the respective *Bt* toxins. The resistance can occur at any step of the toxic pathway, which includes as its main steps the enzymatic digestion of *Bt* toxins with proteases present in the midgut juice and the binding of *Bt* toxins to specific receptors of the midgut epithelium.

With this background, parameters of the processing of the *Bt* toxin Cry3Bb1 and the binary *Bt* toxin Cry34Ab1/Cry35Ab1 in the midgut of third instars larvae of the WCR (European strain) were examined. These examinations are of basic interest but also lead to test systems for the identification of resistance mechanisms in potentially resistant individuals. In addition, the binding sites of Cry3Bb1 as well as Cry34Ab1 and Cry35Ab1 were characterized.

Materials and methods

Midguts

In Germany, the WCR is under quarantine. Thus, the rearing of the required insects (third instars larvae of a WCR European strain) and the midgut preparation was done in the quarantine ward of the company BTL Bio-Test Labor GmbH in Sagerheide. From their laboratory, the midguts were sent to the Institute for Biological Control in Darmstadt. For studies on the proteolytic processing of *Bt* toxins, pure midgut juice was extracted by centrifugation (Eppendorf centrifuge 5417R) at 20,000 g for 30 minutes at 4°C. To prepare the midgut epithelia for binding studies, the midguts were cut lengthwise and the gut contents together with the peritrophic membrane were removed by rotating the epithelia three times in washing buffer (200 mM Tris, 20 mM CaCl₂, pH 5.75).

Bt toxins

The *Bt* toxin Cry3Bb1 was provided by Monsanto. The binary *Bt* toxin Cry34Ab1/Cry35Ab1 was produced according to the method described by Baum *et al.* (2004) using the *Bt* strain from which DOW AgroSciences transferred the toxin genes to the respective *Bt*-corn. To confirm the toxic effect of the 14 kDa (Cry34Ab1) and 44 kDa (Cry35Ab1) proteins, additional bioassays with a *Diabrotica*-related organism, the Green dock leaf beetle (*Gastrophysa viridula*), revealed a mortality of 80%.

Proteolytic processing

Within the studies on the proteolytic processing, it was determined if the *Bt* toxins Cry3Bb1, Cry34Ab1, and Cry35Ab1 were digested by the WCR midgut juice. Proteolytic processing of the toxins was simulated *in vitro* by incubating the toxins with midgut juice. For two samples, 6 µl of the toxin solution (28 µg Cry3Bb1, 21 µg Cry34Ab1, or 29 µg Cry35Ab1) were diluted in 15.6 µl buffer (200 mM Tris, 20 mM CaCl₂, pH 5.75), and 2.4 µl midgut juice diluted 1:10, 1:25, or 1:100 was added. After incubation at room temperature, the reaction was stopped for 5 minutes at 95°C. For sample preparation, a Lämmli denaturation buffer (Roti-Load1 no. K929.1) was used in a ratio of 4:1 (12 µl of each sample and 4 µl buffer). Because *Bt* toxins are proteins, they and the products of their break down were described with the aid of SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) using 12% gels (BioRad). The sample input was 11 µl.

Binding analyses

To study the binding of *Bt* toxins to specific receptors in the midgut epithelium, ligand-blot binding analyses were carried out. First, intact brush border membrane vesicles (BBMV_s) were produced from midgut epithelia (Wolfersberger *et al.* 1987) and the *Bt* toxins were labelled with biotin (Dendolf *et al.* 1993). BBMV proteins - separated with SDS-PAGE and transferred to a PVDF membrane - were incubated with biotin-labelled toxins. Binding of biotin-labelled toxins was evidenced with a streptavidin horseradish peroxidase conjugate coupled for ECL detection.

Results and discussion

Proteolytic processing

The *Bt* toxin Cry3Bb1 showed a 77 kDa band. The toxin was not processed after a 60-minute incubation with 1:25 diluted WCR midgut juice (Fig. 1 a) or after a 60-minute incubation with 1:100 diluted WCR midgut juice (Fig. 1 b).

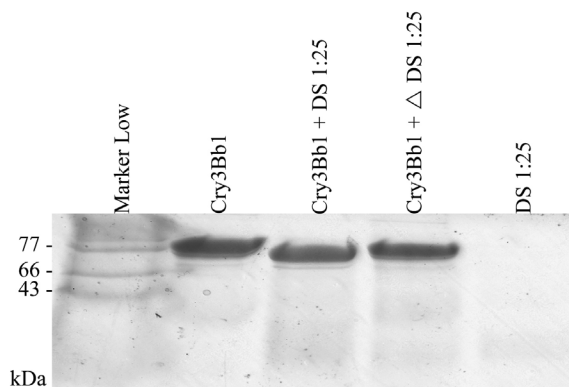


Figure 1a. Processing of *Bt* toxin Cry3Bb1 by 1:25 diluted midgut juice from *Diabrotica* (60 min incubation) [DS = midgut juice; Δ = heated]

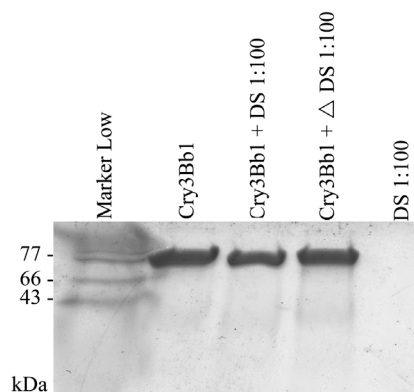


Figure 1b. Processing of *Bt* toxin Cry3Bb1 by 1:100 diluted midgut juice from *Diabrotica* (60 min incubation) [DS = midgut juice; Δ = heated]

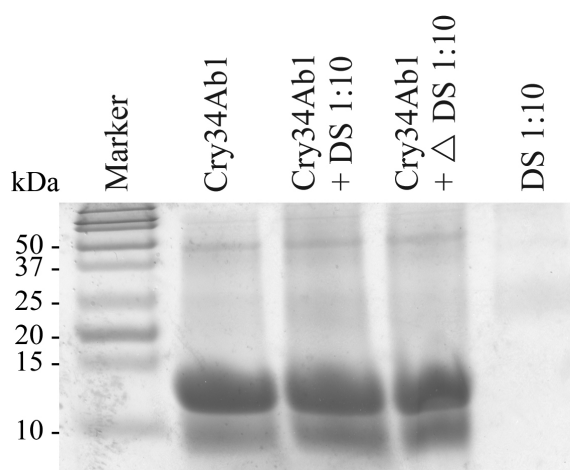


Figure 2a. Processing of *Bt* toxin Cry34Ab1 by 1:10 diluted midgut juice from *Diabrotica* (30 min incubation) [DS = midgut juice; Δ = heated]

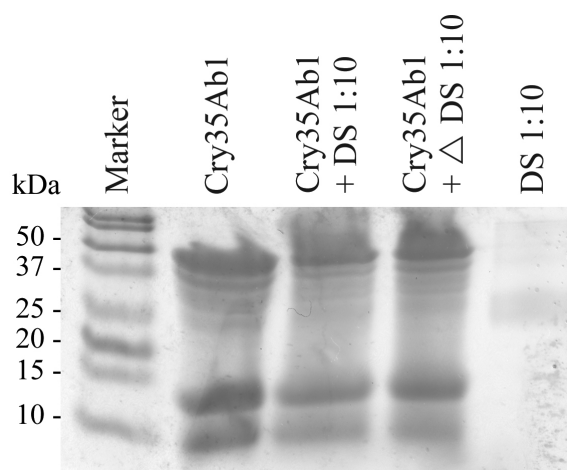


Figure 2b. Processing of *Bt* toxin Cry35Ab1 by 1:10 diluted midgut juice from *Diabrotica* (30 min incubation) [DS = midgut juice; Δ = heated]

The toxin Cry34Ab1 showed an approximate 14 kDa band and Cry35Ab1 an approximate 44 kDa band (Ellis *et al.* 2002). After a 30-minute incubation with 1:10 diluted midgut juice, neither toxin was processed and they remained unchanged (Fig. 2 a, Fig. 2 b).

Thus, in the studies on the proteolytic processing of Monsanto's transgenic corn toxin Cry3Bb1 and DOW AgroSciences binary toxin Cry34Ab1/Cry35Ab1 by midgut juice from the Western corn rootworm, no degradation of the toxins was observed. However, it is conceivable that in the case of genetically conditioned protease changes, the digestion of the toxins into ineffective breakdown products could occur.

Binding analyses

Because *Bt* toxins bind to specific receptors of the midgut epithelium, ligand-blot binding analyses with the toxins Cry3Bb1 and Cry34Ab1/Cry35Ab1 were carried out. The toxin Cry34Ab1 showed binding to an approximate 110 kDa WCR BBMV protein (Fig. 3 a) while the toxin Cry35Ab1 bound to an approximate 50 kDa protein (Fig. 3 b). After the addition of

increasing amounts of biotin-labelled Cry3Bb1, the bands for the biotin-labelled Cry34Ab1 and Cry35Ab1 receptors became weaker, whereas the approximate 30 kDa bands corresponding to the Cry3Bb1 receptor became stronger (Fig. 3 a, Fig. 3 b). Binding competition experiments have to be performed to establish whether both binary toxins Cry34Ab1 and Cry35Ab1 compete for Cry3Bb1 toxin binding sites.

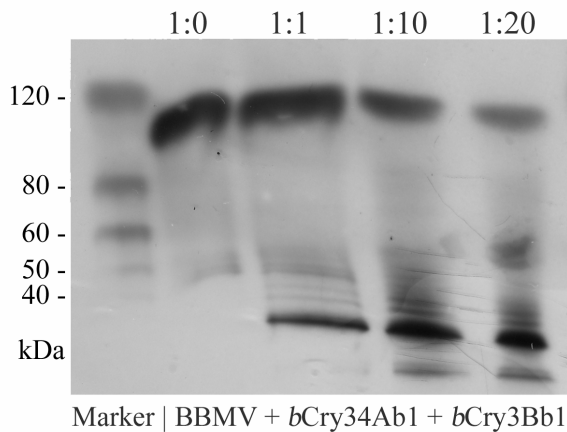


Figure 3a. Binding analysis with biotin-labelled toxins Cry34Ab1 and Cry3Bb1

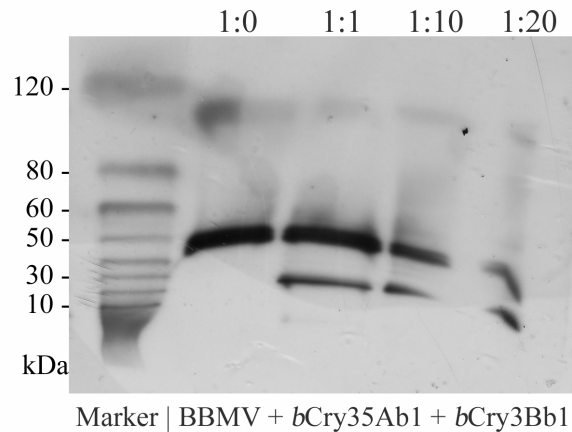


Figure 3b. Binding analysis with biotin-labelled toxins Cry35Ab1 and Cry3Bb1

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