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European corn borer (*Ostrinia nubilalis*): Studies on proteinase activity and proteolytical processing of the *B.t.*-toxin Cry1Ab in transgenic corn

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Abstract: One possibility to control the European corn borer (ECB) is the cultivation of *B.t.*-corn. However, this can result in the development of resistant pest populations. To analyse possible mechanisms of resistance, a reference system for the identification and quantification of physiological changes in the midgut was established. Studies on proteinase activities were conducted with a susceptible German ECB population. The digestive proteinases trypsin, chymotrypsin, elastase, and aminopeptidase were identified in the midgut sap of 5th instar larvae. In whole 1st and 2nd instars as well as in the midgut epithelium of 5th instar larvae, the proteinase aminopeptidase was provable. Besides, proteolytical processing of the *B.t.*-toxin (and protoxin) Cry1Ab as present in transgenic corn is described.

Key words: European corn borer, *Ostrinia nubilalis*, *B.t.*-corn, midgut proteinases, trypsin, chymotrypsin, elastase, aminopeptidase, *B.t.*-toxin Cry1Ab, protoxin, proteolytical processing

Introduction

In Europe, the economical most important pest in maize (*Zea mays* L.) is the European corn borer (ECB, *Ostrinia nubilalis*). Thus, transgenic corn (*B.t.*-corn) highly insecticidal to the larvae of ECB was developed based on a truncated Cry1Ab toxin of *Bacillus thuringiensis*. However, the cultivation of the respective cultivars may result in the development of resistant pest populations.

Depending on the mode of action of *B.t.*-toxins the potential of insect resistance to *B.t.*-toxins is generally located at any step of the toxic pathway: ingestion, solubilization, proteolytic processing, binding to specific receptors, membrane integration, pore formation, cell lysis, and insect death (Ferré & van Rie, 2002). Two main mechanisms of resistance to *B.t.*-toxins have been identified in other pest-*B.t.*-toxin-systems. One of them is proteinase-mediated and the other receptor-mediated (Oppert *et al.,* 1997, McGaughey & Oppert, 1998).

In order to establish preliminary reference systems for the characterization of potential available resistant individuals, first studies on proteinase activities in the midgut of Cry1Ab susceptible ECB larvae were carried out. Besides, the proteinases were tested for involvement in the digestion of the *B.t.*-toxin Cry1Ab and the respective protoxin.

Materials and methods

Isolation of midgut sap and BBMV

ECB larvae were reared on artificial diet up to the 5th instar. For the extraction of both, the pure midgut sap and the midgut epithelium, the larvae were calmed on ice and dissected. The total midguts were isolated and collected on ice. Due to the very small sizes of 1st and 2nd instar larvae it was not possible to separate their midguts. For the sap extraction the midguts as well as crushed whole 1st and 2nd instar larvae were centrifuged at 13.000 g for 15 min. The resuite was stored at -18°C until usage. For preparation of brush border membrane vesicles (BBMV) the isolated midguts were treated as described by Wolfersberger *et al.* (1987).

Photometrical tests

For the identification and quantification of proteinases, photometrical studies were conducted using typical proteinase-indicating chromogenic substrates and specific inhibitors according to the investigations of Wagner *et al.* (2002): Trypsin was tested with the substrate N-benzoyl-L-arg-p-nitroanilide (BApNA) and soybean-trypsin-inhibitor (SBTI). Chymotrypsin was tested with N-succinyl-ala-ala-phe-p-nitroanilide (SAAFpNA) and the inhibitor N-tosyl-L-phe chloromethylketone (TPCK). Elastase was tested with N-succinyl-ala-ala-pro-leu-p-nitroanilide (SAAPLpNA) and elastatinal. Aminopeptidase was tested with leu-p-nitroanilide (LpNA) and bestatin. Carboxypeptidase was tested with hippuryl-phe and hippuryl-arg.

Proteolytic assays and SDS-PAGE

The experiments were done with purified Cry1Ab toxin and protoxin which was prepared by Dr. J.A. Jehle (State Education and Research Center for Agriculture, Viticulture and Horticulture; SLFA Neustadt; Germany). Model proteinases were obtained from Sigma. Digestions were performed at 25°C with a final toxin concentration of 1 mg/ml and either a midgut sap dilution of 1:10 or a model proteinase concentration of 0.25 mg/ml, respectively. Proteolyses were stopped by heating the samples for 2 min at 95°C.

SDS-PAGE was done according to Laemmli (1970) using the Roti-Load1 no. K929.1 denaturation buffer from Roth and 15% polyacrylamide gels. Fluka standards no. 69810 (indicated as "low") and 69811 (indicated as "high") were used as reference standards.

Results and discussion

Proteinase activity in the midgut sap of 5th instar larvae

In the midgut of a Canadian ECB population, Houseman & Chin (1995) identified the digestive proteinases trypsin, chymotrypsin, elastase, and aminopeptidase. To compare their results with German ECB, a reference system, which is also intended to be used to characterize potential available resistant ECB´s, was established to identify and to quantify changes in proteinase-activities in the midgut of the pest insect. Thus, midgut sap of German susceptible 5th instar larvae was extracted and photometrical studies were carried out. Similar to the above described results, trypsin, chymotrypsin, elastase, and aminopeptidase were identified (Kaiser-Alexnat *et al.*, 2003). In additional tests the presence of other potential activities, e.g. carboxypeptidase, could not be highlighted (data not shown).

Beside the examined serine proteinases and metalloproteinases, other classes of proteolytic activities are unlikely to be present in the midgut sap of ECB due to physiological reasons. As reviewed by Terra *et al.* (1996), cysteine proteinases are generally common in the midgut of hemipteran Heteroptera or in slightly acid media and aspartic proteinases are only active at very acid pH values. Kaiser-Alexnat *et al.* (2003) demonstrated that the pH of pure

larval midgut sap of ECB 5th instar larvae is lightly basic, ranging between 7.2 and 7.5, depending on the rearing method before sample preparation.

Proteinase activity in whole 1st and 2nd instar larvae

Generally, early larval stages are known to be more sensitive to *B.t.*-toxin than late instars. Unfortunately, it was not possible to separate the midguts of 1st and 2nd instar larvae due to their very small sizes. Thus, with sap of whole larvae it was examined whether the activity of the above described proteinases is provable, too. As a control, no proteinase activity could be demonstrated in the haemolymphe (data not shown). In the sap of whole 1st (Fig. 1) and 2nd (Fig. 2) instar larvae, aminopeptidase activity was identified and quantified in photometrical tests. The diagrams show the means and the standard deviation of each experiment which was done three times. Columns indicated as "0" quantify the prevailing proteolytic activity; columns indicated with an increasing concentration of inhibitor show the specific inhibition of the proteolyses which is a tool to identify the type of proteinase.

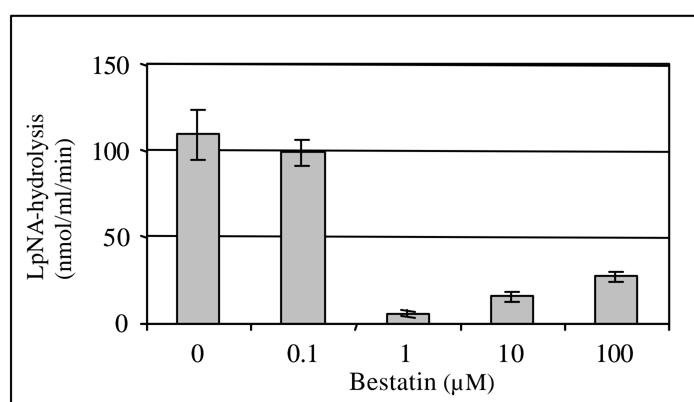

Figure 1. Aminopeptidase-activity (± sd) in the sap of whole 1st instar larvae.

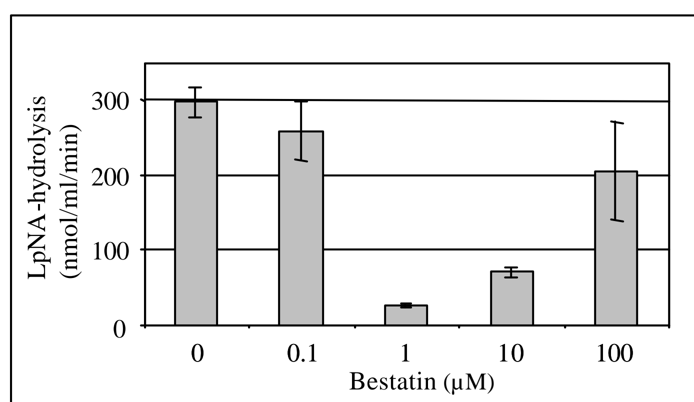

Figure 2. Aminopeptidase-activity (± sd) in the sap of whole 2nd instar larvae.

Proteinase activity of BBMV from 5th instar larvae

A membrane-bound aminopeptidase is one possible receptor for *B.t.*-toxins in the midgut epithelium (Oppert, 1999). Based on this fact and in context with the above described results,

aminopeptidase activity of BBMV isolated from 5th instar larvae was demonstrated using the established test system (Fig. 3). Binding analyses are presently carried out to show the interaction between Cry1Ab and the receptor.

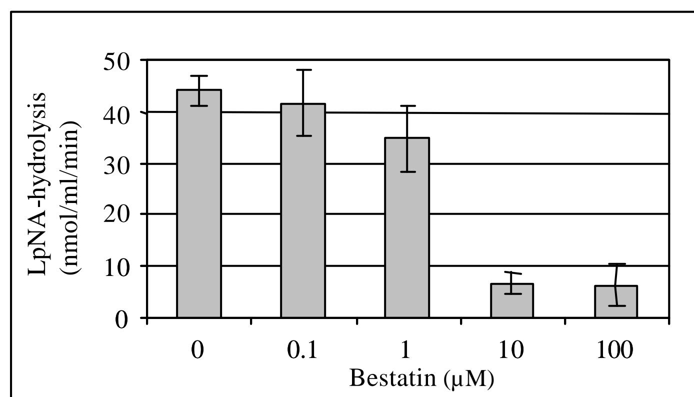

Figure 3. Aminopeptidase-activity (± sd) in the midgut epithelium (BBMV´s) of 5th instar larvae.

Proteolytical processing of B.t. toxin and protoxin Cry1Ab

After incubation with midgut sap from 5th instar larvae, the *B.t.*-toxin Cry1Ab was processed during the first minute. As a result, the 65 kDa toxin was digested for 2 kDa, resulting in a 63 kDa protein. This protein is stable for at least 60 minutes (Fig. 4). In order to show that this protein shortening is due to proteolytic activity, a control was performed using midgut sap that was heated at 95°C for 5 min. Due to this denaturation of the proteinases, no digestion of the *B.t.*-toxin took place (see also Fig. 4).

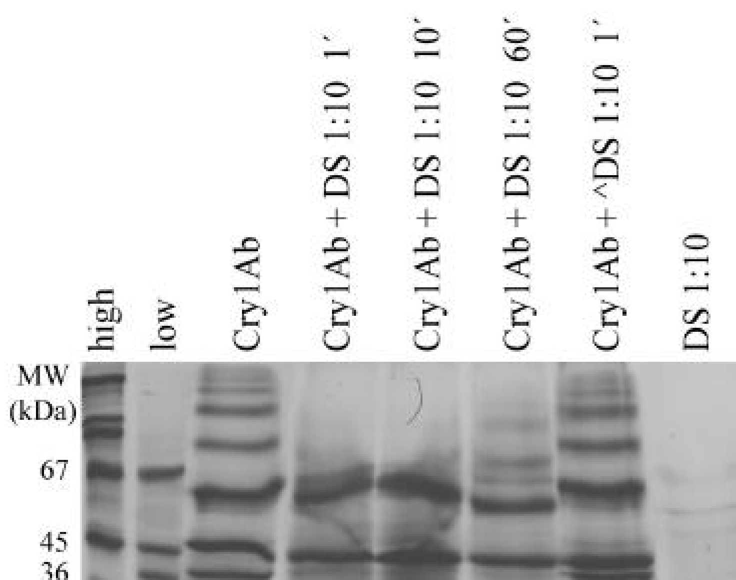

Figure 4. Proteolytical processing of *B.t.*-toxin Cry1Ab with midgut sap (DS) of 5th instar larvae.

To identify the activity among the midgut proteinases, which are responsible for the proteolytical reaction, available model proteinases were used to simulate the midgut conditions, i.e. bovine trypsin, bovine chymotrypsin, porcine elastase, and Aeromonas aminopeptidase. As a result, both the 65 kDa toxin as well as the 135 kDa protoxin were digested to 63 kDa by all types of proteinases proved in the midgut sap of ECB, except aminopeptidase. The proteolytical processing with trypsin and chymotrypsin is demonstrated in Fig. 5 and the one with elastase and aminopeptidase in Fig. 6.

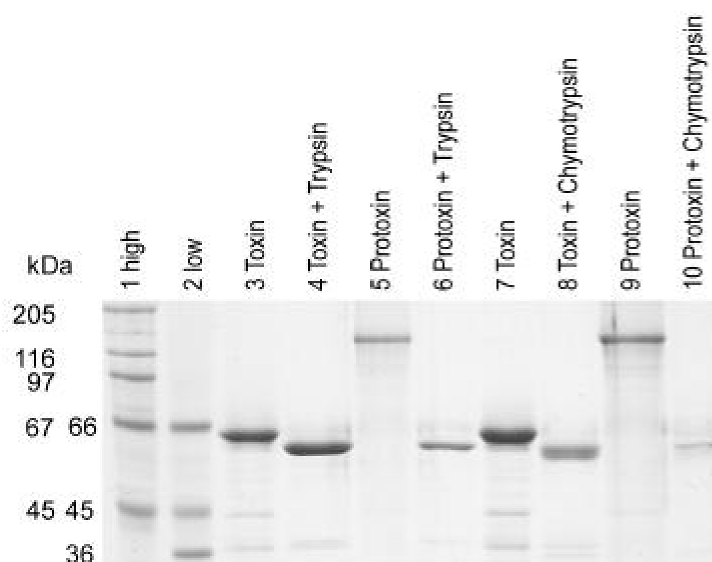

Figure 5. Proteolytical processing with trypsin and chymotrypsin.

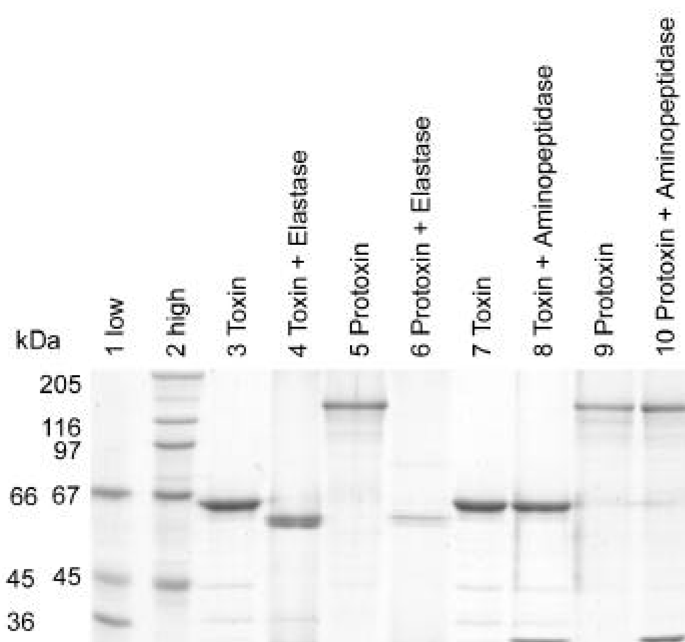

Figure 6. Proteolytical processing with elastase and aminopeptidase.

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