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Protease activities in the midgut of Western corn rootworm (*Diabrotica virgifera virgifera* LeConte)

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ABSTRACT

The Western corn rootworm is one of the most economically important pests in corn. One possibility for controlling this pest is the cultivation of transgenic corn expressing *Bacillus thuringiensis* (Bt) toxins, such as Cry3A, Cry34Ab1/Cry35Ab1, and Cry3Bb1. However, widespread cultivation of the resulting Bt corn may result in the development of resistant pest populations. The Bt toxins are processed by proteases in the midgut of susceptible insects. Thus, protease activity studies were conducted using the midgut juice (pH 5.75) from third instars larvae of the susceptible Western corn rootworm. As a result, the activities of the serine endopeptidases trypsin, chymotrypsin, elastase, cathepsin G, plasmin, and thrombin; the cysteine endopeptidases cathepsin L, papain, cathepsin B, and cathepsin H; the aspartic endopeptidase pepsin; the metallo endopeptidase saccharolysin; the exopeptidase aminopeptidase, and the omegapeptidase acylaminoacylpeptidase were detected. These results are of basic interest but also lead to reference systems for the identification of protease-mediated resistance mechanisms in potentially resistant individuals.

1. Introduction

The Western corn rootworm (*Diabrotica virgifera virgifera* Le Conte) is one of the most economically important pests in corn (*Zea mays* L.). One possibility for controlling the pest is the cultivation of transgenic corn expressing *Bacillus thuringiensis* (Bt) toxins, such as Cry3A, Cry34Ab1/Cry35Ab1, and Cry3Bb1. The resulting Bt corn produces its own protective pesticide that is insecticidal to larvae. However, widespread cultivation of Bt corn may increase the probability of the development of resistant pest populations.

Depending on the mode of action, the potential for insect resistance to Bt toxins can occur at any step of the toxic pathway: ingestion, pH dependent solubilization, proteolytic processing, specific receptor binding, membrane integration, pore formation, cell lysis, and insect death (Ferré and van Rie, 2002). However, in other pest-Bt toxin systems, the resistance mechanisms are mainly protease- or receptor-mediated (Oppert et al., 1997; McGaughey and Oppert, 1998). Protease-mediated resistance includes qualitative and quantitative changes in protease activity in the midgut juice. Receptor-mediated resistance includes alternate toxin binding to specific receptors in the midgut epithelium.

The Bt toxins are proteins; thus, they are processed by proteases in the midgut of susceptible insects. In general, there is sufficient data in the literature to suggest that changes in protease activity within an insect gut can affect susceptibility to Bt toxins. In the present paper, protease activities are studied in the midgut juice from third instars larvae of the susceptible Western corn rootworm. These examinations are of basic interest but also lead to reference systems for the identification of protease-mediated resistance mechanisms in potentially resistant individuals.

2. Materials and methods

2.1. Insect rearing

In Germany, the Western corn rootworm is under quarantine. Insects were reared in the quarantine ward of BTL Bio-Test Labor GmbH, a company in the north of Germany. A European strain of Western corn rootworm was reared to the third instars using the standard method of the USDA-ARS North Central Agricultural Research Laboratory in Brookings, SD.

2.2. Midgut juice

For midgut preparation, the larvae were calmed on ice, dissected, and the total midguts collected in microcentrifuge tubes placed on ice. From BTL Bio-Test Labor GmbH, the midguts were transferred to the Institute for Biological Control in Darmstadt. Pure midgut juice was extracted by centrifugation (Eppendorf centrifuge 5417R) at 20,000*g* for 30 min at 4 °C. In the centrifugation residue of each sample, each of which consisted of approximately

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50 midguts, pH measurements were made with a microelectrode (MI-414-2-E, Microelectrodes, Bedford, USA). The single residues were pooled and centrifuged again. The subsequent residue represented the midgut juice and was stored at −28 °C until used. To extract 1 µl midgut juice, approximately five midguts were required.

2.3. Photometric tests

For the identification and quantification of protease activities in the midgut juice, photometric tests with specific chromogenic substrates, mainly peptidyl-*p*-nitroanilide (*p*NA), and specific inhibitors were conducted according to Wagner et al. (2002). If the protease of interest was in the midgut juice, the specific *p*NA substrate was hydrolyzed into oligopeptides and chromogenic *p*-nitroaniline. This reaction produced a yellow color that was measured photometrically. The level of substrate digestion, as determined by the intensity of the yellow color, is an indicator of the activity of the protease in question. In addition, using the respective specific protease inhibitor, substrate processing decreased with increasing concentrations of inhibitor, decreasing the intensity of the yellow color, if the protease of interest was present.

The test solution (500 µl) was prepared at room temperature in one-way polystyrol cuvettes. The samples with pure substrate were prepared using 10 µl midgut juice diluted 1:10 with tricine buffer, 10 µl tricine buffer (20 mM tricine; pH 5.75), and 480 µl substrate solution (0.2 mM of the respective *p*NA substrate in tricine buffer). For samples with inhibitor, 10 µl of inhibitor solution with increasing concentrations of the inhibitor were added instead of the 10 µl of tricine buffer. Measurements were made with a spectrometer (UVIKON Spectrometer 922) at 405 nm with 10 min between the first und last measurements. To convert extinction values to concentrations, the Lambert-Beer formula was used.

Photometric tests with synthetic substrates and inhibitors are a diagnostic guide for analyzing protease activities, but the results cannot be used absolutely; enzymes and inhibitors have particular requirements for activity (e.g., pH and temperature), and some enzymes can be very susceptible to degradation during the substantial processing necessary for obtaining gut extracts.

To control the photometric test systems, cross combinations of *p*NA substrates and inhibitors were tested. In general, the inhibitors reacted as expected. However, even though E-64 was supposed to be a specific inhibitor for cysteine endopeptidases, the digestion of most of the *p*NA substrates was most strongly inhibited by E-64. In addition, TLCK and Z-Phe-Tyr-CHO also strongly inhibited the digestion of many of the *p*NA substrates (data not shown).

For aspartic endopeptidases, no specific *p*NA substrate was available; thus, general protease substrates were used. The processing of the general substrates led to chromogenic breakdown products that remained in the test solution, whereas the intact proteins precipitated and were separated by centrifugation. The residue was measured photometrically. Details of the method were described previously (Beynon and Bond, 2001).

Many inhibitors of serine and cysteine endopeptidases are known (Turk and Bode, 1991) but only a few for aspartic endopeptidases (Ryan, 1990; Jouanin et al., 1998). The specific protease inhibitor pepstatin A has been described for the aspartic endopeptidase pepsin (Rymerson and Bodnaryk, 1995; Blanco-Labra et al., 1996; Oppert et al., 2003). However, specific protease inhibitors were available for all protease classes, and the tested substrate-inhibitor combinations are summarized in Table 1.

3. Results

Because protease activity correlates with the pH of the midgut juice, pH measurements were made in pure larval midgut juice after midgut centrifugation. The pH values of the samples were homogenous, as indicated by a very low standard variation (0.05). The average pH value was 5.75.

3.1. Serine endopeptidases

The trypsin substrate BA*p*NA (N-benzoyl-L-arginine *p*NA·HCl) was processed, but there was no inhibition with the trypsin-specific SBTI (soybean trypsin inhibitor, data not shown). Further experiments revealed that BA*p*NA was processed at 438 nmol/ml/min, and with a very low concentration (1 µM) of the trypsin-specific inhibitor TLCK (N-*p*-tosyl-L-lysine chloromethylketone·HCl), substrate digestion was increased. However, with increasing TLCK concentrations the digestion was finally reduced (Fig. 1, part 1). Thus, trypsin was detected in the midgut juice of the Western corn rootworm using the test system BA*p*NA and TLCK.

The chymotrypsin-specific substrate SAAF*p*NA (N-succinyl-Ala-Ala-Phe *p*NA) was processed with the midgut juice, but the highest concentrations of the chymotrypsin-specific inhibitor TPCK (N-tosyl-L-phenylalanine chloromethylketone) did not inhibit digestion (data not shown). In further experiments, the substrate SAAF*p*NA was processed at 85 nmol/ml/min, and increasing concentrations of the general serine endopeptidase PMSF (phenylmethansulfonyl fluoride) led to decreased digestion activity (Fig. 1, part 2). Thus, chymotrypsin was detected in the midgut juice of the Western corn rootworm using the test system SAAF*p*NA and PMSF.

Elastase was detected with the elastase-specific substrate SAAPL*p*NA (N-succinyl-Ala-Ala-Pro-Leu *p*NA) and the elastase-specific inhibitor elastatinal. The substrate was processed at 108 nmol/ml/min, and the activity decreased with increasing inhibitor concentrations (Fig. 1, part 3).

Five specific *p*NA substrates for other serine endopeptidases were also tested. The substrates for cathepsin G (N-methoxysuccinyl-Ala-Ala-Pro-Met *p*NA), plasmin (isovaleryl-Phe-Lys *p*NA·HCl), and thrombin (Sar-Pro-Arg *p*NA) were processed, but the substrates for granzyme B (Ac-Ile-Glu-Pro-Asp *p*NA), and subtilisin (Z-Gly-Gly-Leu *p*NA) were not processed.

The digestion rate of cathepsin G-specific substrate N-methoxysuccinyl-Ala-Ala-Pro-Met *p*NA was high (1237 nmol/ml/min), and it reduced with increasing concentrations of the serine endopeptidase inhibitor PMSF (Fig. 1, part 4).

The specific substrate for plasmin (isovaleryl-Phe-Lys *p*NA) was digested at a very high level (3694 nmol/ml/min), but no inhibition was seen with PMSF or elastatinal, which was recommended in the producer catalogue. Despite the high rate of substrate digestion, no complete test system was found for identifying plasmin.

The digestion of the thrombin substrate Sar-Pro-Arg *p*NA was also very high (3203 nmol/ml/min). PMSF was not useful as an inhibitor, but with increasing doses of TLCK, a clear reduction in substrate digestion was observed (Fig. 1, part 5). In the midgut juice of the Western corn rootworm, very high thrombin activity was detected using the test system Sar-Pro-Arg *p*NA and TLCK.

3.2. Cysteine endopeptidases

The substrate Z-Phe-Arg *p*NA HCl (N-benzyloxycarbonyl-Phe-Arg *p*NA) is specific for cathepsin L and papain. In the midgut juice of the Western corn rootworm, the substrate was digested within 2 min at a very high processing rate (3267 nmol/ml/min). Increasing concentrations of the general cysteine endopeptidase inhibitor E-64 (L-trans-epoxysuccinyl-L-leucylamide [4-guanidino] butane) continuously decreased the processing activity (Fig. 1, part 6). Thus, the cysteine endopeptidases cathepsin L and papain were detected in the midgut juice of the Western corn rootworm using the test system Z-Phe-Arg *p*NA HCl and E-64.