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Digestive proteolysis in the Colorado potato beetle, *Leptinotarsa decemlineata*: Activity-based profiling and imaging of a multipeptidase network

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A B S T R A C T

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is a major pest of potato plants, and its digestive system is a promising target for development of pest control strategies. This work focuses on functional proteomic analysis of the digestive proteolytic enzymes expressed in the CPB gut. We identified a set of peptidases using imaging with specific activity-based probes and activity profiling with selective substrates and inhibitors. The secreted luminal peptidases were classified as: (i) endopeptidases of cathepsin D, cathepsin L, and trypsin types and (ii) exopeptidases with aminopeptidase (cathepsin H), carboxypeptidase (serine carboxypeptidase, prolyl carboxypeptidase), and carboxydipeptidase (cathepsin B) activities. The proteolytic arsenal also includes non-luminal peptidases with prolyl oligopeptidase and metalloaminopeptidase activities. Our results indicate that the CPB gut employs a multienzyme network of peptidases with complementary specificities to efficiently degrade ingested proteins. This proteolytic system functions in both CPB larvae and adults and is controlled mainly by cysteine and aspartic peptidases and supported by serine and metallopeptidases. The component enzymes identified here are potential targets for inhibitors with tailored specificities that could be engineered into potato plants to confer resistance to CPB.

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1. Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata*, is the most devastating defoliator of potato in the northern hemisphere. Both larvae and adults feed on potato plants, causing large losses in crop yield. Several factors have contributed to the great success of CPB as a pest species, including adaptation to its potato host, high fecundity, a flexible life cycle with adult diapause, and the capability to evolve resistance to a wide variety of conventional insecticides. Therefore, there are pressing reasons to develop alternative strategies for CPB management. One promising approach for pest control is to disrupt protein digestion with dietary peptidase inhibitors, causing reduction in survival, growth, and fecundity of the insects. This has been illustrated in feeding trials with various species, including CPB (e.g., Bolter and LatoszekGreen, 1997; Wolfson and Murdock, 1987).

Plant peptidase inhibitors have been proposed as protein insecticides that could be engineered into crops to confer resistance to insect pests. However, the effectiveness of such transgenic plants is limited due to the adaptation response of insects, which counteract the plant defense proteins. CPB has exhibited several adaptation mechanisms to peptidase inhibitors, including compensation through upregulation of digestive peptidase genes, expression of inhibitor-insensitive peptidases, and proteolytic inactivation of peptidase inhibitors (Bolter and Jongsma, 1995; Cloutier et al., 2000; Gruden et al., 2003, 2004). To prevent CPB adaptation, current research concentrates on peptidase inhibitors of non-plant

Abbreviations: ABP, activity-based probe; Abz, 2-aminobenzoic acid; Ald, aldehyde; Amc, 7-amino-4-methylcoumarin; Bodipy, Bodipy TMR-X; BoFC, Bodipy-AAF-Cmk; BoRC, Bodipy-FPR-Cmk; BPTI, bovine pancreatic trypsin inhibitor; BSA, bovine serum albumin; Bz, benzoyl; Cbz, benzyloxycarbonyl; CPB, Colorado potato beetle; Cmk, chloromethyl ketone; dF, D-phenylalanine; DFP, diisopropyl fluorophosphate; Dmk, dimethylketone; DTT, dithiothreitol; E64, trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid; F*, 4-nitrophenylalanine; Fmk, fluoromethyl ketone; FRET, fluorescence resonance energy transfer; nL, norleucine; PCDI, potato cathepsin D inhibitor; PCPI, potato carboxypeptidase inhibitor; PMSF, phenylmethanesulfonyl fluoride; PSPI, potato serine protease inhibitor; Suc, 3-carboxy-propionyl; STI, soybean trypsin inhibitor.

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Abbreviations

ABP	activity-based probe
Abz	2-aminobenzoic acid
Ald	aldehyde
Amc	7-amino-4-methylcoumarin
Bodipy	Bodipy TMR-X
BoFC	Bodipy-AAF-Cmk
BoRC	Bodipy-FPR-Cmk
BPTI	bovine pancreatic trypsin inhibitor
BSA	bovine serum albumin
Bz	benzoyl
Cbz	benzyloxycarbonyl
CPB	Colorado potato beetle
Cmk	chloromethyl ketone
dF	D-phenylalanine
DFP	diisopropyl fluorophosphate
Dmk	dimethylketone
DTT	dithiothreitol
E64	trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane
EDTA	ethylenediaminetetraacetic acid
F*	4-nitrophenylalanine
Fmk	fluoromethyl ketone
FRET	fluorescence resonance energy transfer
nL	norleucine
PCDI	potato cathepsin D inhibitor
PCPI	potato carboxypeptidase inhibitor
PMSF	phenylmethanesulfonyl fluoride
PSPI	potato serine protease inhibitor
Suc	3-carboxy-propionyl
STI	soybean trypsin inhibitor

origin and engineered inhibitors with improved potency, specificity, and stability (Brunelle et al., 2005; Goulet et al., 2008; Gruden et al., 1998). For selection and development of these inhibitors, more information is needed about the target digestive proteolytic system of CPB, its component enzymes, and their functional relationships.

Our understanding of the proteolytic arsenal in the CPB gut is fragmented. Previous studies focused on individual enzymes have identified a group of cysteine peptidases from the papain family called intestains and analyzed their interactions with proteinaceous inhibitors (Gruden et al., 2003, 2004; Petek et al., 2012; Sainsbury et al., 2012; Vorster et al., 2015). Little information is available about aspartic peptidases and serine peptidases (Brunelle et al., 1999; Petek et al., 2012). In the 1990s, researchers applied a systematic enzymological approach to obtain initial information about the digestive peptidases in the CPB gut (Brunelle et al., 1999; Novillo et al., 1997).

The present work provides novel biochemical insight into the molecular proteolytic machinery for plant protein degradation in the CPB gut by using a functional proteomic platform. This has become feasible only recently, with the help of chemical tools such as activity-based probes (ABPs) for selectively imaging target peptidases (for review, see e.g. Sanman and Bogyo, 2014) and new selective inhibitors and substrates. Here, we uncover how plant proteins are proteolytically digested in the CPB gut. We characterize a multienzyme network of peptidases that orchestrates luminal proteolysis, and we define the contributions of individual peptidases to this process. Based on these findings, we discuss the molecular evolution of digestive proteolysis in CPB and identify critical digestive peptidases as novel targets for pest control strategies.

2. Materials and methods

2.1. Biological materials and protein extracts

A laboratory colony of CPB was obtained from the Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany. Larvae and adults were reared on young potted potato plants (*Solanum tuberosum* cv. Magda) at 22 ± 2 °C, 60–70% relative humidity, and a 16/8 h (light/dark) photoperiod in a greenhouse chamber. The 4th instar larvae or adult beetle were removed from food for 2 h, immobilized on ice, and guts were dissected and stored at −80 °C. Guts of starved 4th instar larvae were prepared after 2 days of starvation. Regurgitant was collected from 4th instar larvae and stored at −80 °C. Protein extracts (5–10 mg protein/ml) from pooled guts were prepared by homogenization in 50 mM sodium acetate (pH 4.5) or 50 mM MES (pH 6.5) containing 1% CHAPS, followed by 30 min incubation on ice and sonication (Horn et al., 2009). The extracts were cleared by centrifugation (16,000g, 10 min, 4 °C), filtered with Ultrafree-MC 0.22 μm (Millipore), and stored at −80 °C. The protein extract from potato leaves was prepared from pooled leaves damaged by larvae feeding as previously described (Horn et al., 2005) using the gut extraction buffer (pH 4.5).

2.2. Proteomic tools: substrates, inhibitors, and activity-based probes

Fluorescence resonance energy transfer (FRET) substrates containing 2-aminobenzoic acid (Abz) and 4-nitrophenylalanine (F*) were synthesized as previously described (Jilkova et al., 2011; Masa et al., 2006). The fluorogenic substrates with a 7-amino-4-methylcoumarin (Amc) leaving group; the substrates Cbz-FL (Cbz, benzyloxycarbonyl) and Bz-GF (Bz, benzoyl) with a free C-terminal carboxyl group; and the inhibitors Cbz-PP-Ald (Ald, aldehyde), dFPR-Cmk (dF, D-phenylalanine, Cmk, chloromethyl ketone), and AAF-Cmk were purchased from Bachem. The inhibitors BPTI, STI, DFP, chymostatin, Pefabloc, PMSF, bestatin, 1,10-phenanthroline, and PCPI were from Sigma. The Kunitz-family inhibitors PCDI and PSPI were purified as previously described (Mares et al., 1989; Valueva et al., 2000). The inhibitor Cbz-AP-Cmk was synthesized as described (Fajtova et al., 2015), and AzaN5s (Gotz et al., 2008) was kindly donated by J. C. Powers. The activity-based probes BoRC and BoFC were prepared from the inhibitors dFPR-Cmk and AAF-Cmk, respectively, by modification of the N-terminal amino group with the NHS ester-activated fluorescent dye Bodipy TMR-X (Molecular Probes). Inhibitor (0.2 mg in 10 μl dry dimethylformamide) was mixed with reactive dye (0.2 mg in 10 μl dry dimethylformamide) and 1 μl N-ethyldiisopropylamine and incubated for 24 h. The probes were purified by RP-HPLC using a trifluoroacetic acid/acetonitrile system and characterized by mass spectrometry. The probe FAP09 was synthesized as described (Nussbaumerova et al., 2010). The probes Green-DCG-04 (Greenbaum et al., 2002) and Fhex-PD-AOMK (Sexton et al., 2007) were kindly donated by M. Bogyo.

2.3. Activity profiling of CPB peptidases

Proteolytic activities were identified and characterized by hydrolysis of the substrates listed in the Supplementary materials and methods; Abz- and Amc-containing substrates were applied at

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