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A lepidopteran aminoacylase (L-ACY-1) in *Heliothis virescens* (Lepidoptera: Noctuidae) gut lumen hydrolyzes fatty acid–amino acid conjugates, elicitors of plant defense

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ABSTRACT

Fatty acid–amino acid conjugates (FACs) have been identified in Lepidopteran larvae as elicitors of plant defenses. Plant responses include the production of primary defense compounds and induction of secondary defense strategies including attraction of parasitoid wasps. These elicitors are present despite fitness costs, suggesting that they are important for the larvae's survival. In order to exploit FAC-mediated plant defense responses in agricultural settings, an understanding of FAC purpose and metabolism is crucial. To clarify their role, enzymes involved in this metabolism are being investigated. In this work a previously undiscovered FAC hydrolase was purified from *Heliothis virescens* frass by liquid chromatography and PAGE techniques and was identified as an aminoacylase-like protein (L-ACY-1) using MALDI-ToF/ToF and Edman sequencing. The full length gene was cloned and expressed in *Escherichia coli* and a polyclonal antibody against L-ACY-1 was made. L-ACY-1 was confirmed to be responsible for FAC hydrolysis activity through inhibition of *N*-linolenoyl-L-glutamine hydrolysis by titration with the polyclonal anti-L-ACY-1 antibody. L-ACY-1 activity is dependent on a divalent cation. This is the first time an aminoacylase has been described from an insect. L-ACY-1 appears to play a vastly different role in insects than ACYs do in mammals and may be involved in maintaining glutamine supplies for gut tissue metabolism. Identification of L-ACY-1, a FAC hydrolase, clarifies a previously uncharacterized portion of FAC metabolism.

1. Introduction

Several compounds that induce plant defenses, including fatty acid-amino acid conjugates (FACs) (Alborn et al., 1997), proteins (β-glucosidase) (Mattiacci et al., 1995), caeliferins (Alborn et al., 2007), and peptides (inceptin) (Schmelz et al., 2006) have been identified in the oral secretions or regurgitant of insect herbivores. The best known of these plant defense elicitors are FACs found in caterpillar oral secretions (Alborn et al., 1997, 2000; Kessler and Baldwin, 2001). FAC elicitors identified thus far are unsaturated 18-carbon fatty acids coupled to glutamine or glutamate and can be present in high concentration in the caterpillar gut (1.3–3 nmol/μL) (Alborn et al., 2000; Mori et al., 2001). The most common profile found in caterpillars consists of *N*-[17-hydroxylinolenoyl]-L-glutamine (volicitin), *N*-linolenoyl-L-glutamine (NLLG), and the linoleoyl analogues of both of these compounds. However, some caterpillars also produce compounds with a glutamate moiety instead of the more typical glutamine moiety (Kessler and Baldwin, 2001; Roda et al., 2004). Also, FACs containing other fatty acids, of differing chain length and saturation have been found in insect guts, but only in trace amounts compared to the 18-carbon FACs (Kessler and Baldwin, 2001). In addition to lepidopteran larvae, crickets and Drosophila larvae have been found to contain FACs, although there is no indication of the function of these compounds in the latter two insect groups (Collatz and Mommsen, 1974; Yoshinaga et al., 2007).

FACs must provide a significant advantage to the caterpillar since they are persistent in the gut tract and oral secretions despite the fitness cost of initiating plant defenses. Some early reports

Abbreviations: FAC, fatty acid–amino acid conjugates; ACY, aminoacylase; L-ACY-1, lepidopteran aminoacylase; NLLG, *N*-linolenoyl-L-glutamine; NAcLGln, *N*-acetyl-L-glutamine; NAcLGlu, *N*-acetyl-L-glutamate.

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suggest that FACs serve as biosurfactants in the gut to aid in lipid solubility (Collatz and Mommsen, 1974; Spiteller et al., 2000; Halitschke et al., 2001). However, recent labeling studies indicate that the role of FACs extends beyond emulsification of the gut environment, and may play a role in nitrogen assimilation (Yoshinaga et al., 2008). Without knowledge of what enzymes interact with FACs, experiments to elucidate the roles of FACs are severely limited.

Some efforts have been made to characterize FAC metabolizing enzymes, but none have identified the enzymes involved in the final steps of synthesis or hydrolysis. It has been shown that the FAC levels of freshly collected caterpillar regurgitant decrease over time if left unattended at room temperature, yet when the same regurgitant is boiled, the FAC levels do not change (Mori et al., 2001). Upon further investigation it became clear that an enzyme in the regurgitant is capable of hydrolyzing FACs into the constitutive fatty and amino acids. Interestingly the rate of hydrolysis appeared to be different in regurgitant collected from two closely related species, *Heliothis virescens* and *Helicoverpa zea*, although their FACs were similar in quality and quantity (Mori et al., 2001). Superficial investigation of the enzyme indicated that the optimum pH is 7 for *H. zea* and pH 8 for *H. virescens*. It is most active in the midgut of *H. virescens*, but significantly less active in *H. zea* gut content. There is some activity in the hindgut content of *H. virescens* which shows that the enzyme is still active in the excreted frass (feces) (Mori et al., 2001).

Since the original publication, only one other article was published reporting on the identity of a FAC hydrolase. In 2008 an enzyme was purified from a cultured gut symbiont, *Microbacterium* arborescens SE14, of *Spodoptera exigua* that was capable of hydrolyzing and synthesizing NLLG (Ping et al., 2007). The homododecamer, metalloenzyme was identified as a DPS-like protein (Ping et al., 2007), even though DPS family proteins are not known to form or break amide bonds. Although it is possible that bacteria in the insect gut may produce such an enzyme *in vivo*, it seems unlikely that it is solely responsible for the hydrolysis of FACs.

The aim of the research reported here was to identify the hydrolase responsible for degrading FACs in the gut lumen and thus open up new areas of FAC metabolism for future exploration. We have used liquid chromatography to purify the FAC hydrolase from *H. virescens* frass. The purified protein was analyzed by several biochemical methods to determine partial amino acid sequences, and based on these amino acid fragments, the full length gene was cloned and identified as an aminoacylase. By using a polyclonal antibody made to the expressed protein, we were able to inhibit the activity of the hydrolase *in vitro,* concluding that the aminoacylase is the enzyme responsible for hydrolysis of FACs.

2. Materials and methods

2.1. Insect rearing and frass collection

H. virescens eggs were purchased from Benzon Company (Carlisle, PA) and were maintained at room temperature until hatching. Neonates were transferred to 2oz cups containing about 1 mL of corn earworm artificial diet from Southland Products. The caterpillars were maintained at 25 °C for 14 h light followed by 10 h dark at 23 °C. After molting into the fifth instar, *H. virescens* individuals were transferred into new diet cups containing an artificial diet made from individual amino acids rather than protein as described by Vanderzant (1958). The caterpillars were allowed to feed for several days before frass was collected and stored at −20 °C for extraction.

2.2. Protein assays

Total protein was quantified using the Thermo Pierce BCA Protein Assay Kit and Thermo Pierce Micro BSA Protein Assay Kit with albumin standards according to manufacturer's protocol. To determine presence/absence and quantity of FAC hydrolase, each frass extract and chromatography fraction was assayed for its ability to hydrolyze NLLG. NLLG was synthesized from linolenic acid and glutamine by previously reported methods (Koch et al., 1999). A single aliquot of 90 μL of each extract or fraction containing separated proteins was added to 140 μL of 50 mM Tris, 100 mM NaCl, pH 8 containing 25 μg of synthetic NLLG and then divided into four aliquots. Aliquots were incubated at 20 °C for 0, 15, 60, and 180 min. The reaction was terminated by boiling for 5 min. After boiling, 20 μL of each sample was analyzed for disappearance of substrate using C18 reversed phase chromatography on a YMC-ODS 4.6 mm × 250 mm column (Waters), eluted with a 40–100% acetonitrile gradient over 7 min at 1 mL per minute, monitored at 210 nm (Fig. 1).

2.3. Protein purification

The FAC hydrolase was purified from *H. virescens* frass collected from fifth instar larvae. In a typical purification approximately 50 g of frass was homogenized in 100 mL of 50 mM Tris buffer pH 8.0. The slurry was mixed for approximately 30 min with a magnetic stir bar at 4 °C and then centrifuged to remove debris at 5000 rpm at 4 °C in 50 mL conical tubes. The supernatant was filtered through a 0.45 μm syringe filter (Millipore) and was diluted with Tris buffer to reduce the conductivity to below 6 mS/cm. The entire frass extract was loaded onto a 10 × 25 cm glass flash chromatography column packed with 10 cm bed height of Q-sepharose Fast Flow media (GE Healthcare). The column was rinsed with five column volumes of 50 mM Tris buffer, 30 mM NaCl pH 8, and then the FAC hydrolase was eluted with five column volumes of 50 mM Tris, 250 mM NaCl pH 8.0 buffer. Two milliliter fractions were collected and assayed for activity. The active eluted fractions were combined and concentrated using a Nanosep 30 kDa MWCO centrifugal device (Pall Laboratories) at 14,000 × g at 4 °C. The concentrated mix was divided into five equal portions and each was loaded onto a 1 × 45 cm column packed with Superdex 200 PG (GE Healthcare). The FAC hydrolase was eluted using 50 mM Tris buffer, 50 mM NaCl, pH 8.0 at 0.25 mL per minute. One half milliliter fractions were collected and assayed for activity. The active fractions from each of the five separations were pooled and injected onto a Mono-Q HR column (GE Healthcare) and eluted using a 0–500 mM NaCl gradient in 50 mM Tris buffer pH 8.0. The active fractions were pooled and concentrated to 200 μL using a 30 kDa MWCO centrifugal device (Pall Laboratories). The concentrate was injected onto a Superdex 200 HR column and eluted with 50 mM Tris, 50 mM NaCl pH 8.0 buffer at 0.25 mL per minute. The final active fraction was prepared as follows for either MALDI-ToF/ToF or Edman sequencing.

2.4. Edman sequencing

The amino acid sequence of the N-terminus of the purified enzyme was identified using Edman sequencing. Five hundred microliters of the active fractions from the final purification step were concentrated with a 30 kDa MWCO centrifugal device (Pall Life Sciences) and then separated by SDS-PAGE on a 4-20% Tris-Glycine gel (Jule Inc.) with Tris-Glycine running buffer system. The gel was then blotted to polyvinylidene fluoride (PVDF) at 50 V, 500 mA for 30 min in CAPS buffer pH 11 (10 mM *N*-cyclohexyl-3-aminopropanesulfonic acid, 10% methanol) in the Mini Trans-Blot