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The effects of the broad-specificity lipase inhibitor, tetrahydrolipstatin, on the growth, development and survival of the larvae of *Epiphyas postvittana* (Walker) (Tortricidae, Lepidoptera)

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ABSTRACT

The effects of the lipase inhibitor, tetrahydrolipstatin (THL), on neonate *Epiphyas postvittana* (Walker) (Lepidoptera, Tortricidae) larvae were investigated by feeding on control artificial diets (with and without 2% ethanol) and diets containing 2% ethanol and one of three concentrations of THL (0.011%, 0.037% and 0.11%). Small but significant reductions in growth rate, percent pupation and time to pupation were observed for larvae feeding on 2% ethanol control diet compared with standard control diet, but larger reductions in all parameters occurred with increasing THL concentration. Third instar larvae fed 0.011% THL in the diet had 40% of the midgut lipase activity in the relevant control larvae and showed up-regulation of gene expression of the gastric lipase-like family but not the pancreatic lipase-like family of midgut lipases.

1. Introduction

Lepidopteran larvae have high nutritional requirements to match their need for an extremely high growth rate, with relative growth rates exceeding 1.0/day (Slansky and Scriber, 1985). There is thus a clear need to extract as much energy as possible from any diet. Lepidopteran larvae have well studied systems for the utilisation of proteins and carbohydrates, with an emphasis on characterisation of midgut proteinases and amylases (Markwick et al., 1998). However, much less is known about the digestion of lipids in these organisms, despite lipids being present at high concentrations in many lepidopteran larval diets and the energy-rich nature of lipids making it very unlikely that these compounds are not utilised nutritionally.

There are numerous reports of lipase-inhibitory secondary metabolite compounds in plants (Han et al., 2001; Hatano et al., 1997; Jang et al., 2008; Karu et al., 2007; Kim et al., 2009; Morikawa et al., 2009; Ninomiya et al., 2004; Raghavendra and Prakash, 2002; Sheng et al., 2006; Shin et al., 2004, 2003; Won et al., 2007; Xu et al., 2005; Yamada et al., 2010; Yamamoto et al., 2000; Yoshizumi et al., 2006; Zhang et al., 2008), based on inhibition of vertebrate pancreatic lipases. The mechanism of action of these chemicals has not been well studied. There are also rare reports of lipase-inhibitory proteins of vertebrate pancreatic lipases. These proteins, soybean lipoxygenase (Satouchi et al., 1998) and soybean β-amylase (Satouchi et al., 2002) are inhibitory by indirect means, perhaps by modifying the water-membrane interface where lipases are active (Chapman, 1987; Delorme et al., 2011). The inhibitory activity of these proteins may be fortuitous rather than evolved by physiological requirements, especially since they both have well-defined catalytic activities. To our knowledge, no specific lipase inhibitors of protein origin have been identified, a major difference compared to proteinase and amylase inhibitors.

The long-chain unsaturated fatty acids, linoleic and linolenic acids are essential for growth and development of lepidopteran larvae (Dadd, 1985). Larvae feeding on diets lacking these compounds exhibit failed pupal and adult ecdysis and wing deformities in any emerged adults (Canavoso et al., 2001). Most lepidopteran diets contain complex lipids from which the fatty acids are obtained by hydrolysis using lipases secreted into the midgut lumen.

A range of lipase and phospholipase activities have been reported from lepidopteran midgut extracts (Ponnuvel et al., 2003; Stanley et al., 1998) and high galactolipase activity has recently

Abbreviations: MLGR, mean larval growth rate; tetrahydrolipstatin, THL ((S)-2-Formylamino-4-methyl-pentanoic acid (S)-1-[(2S, 3S)-3-hexyl-4-oxo-oxetan-2-ylmethyl]v-dodecylester).

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been measured in phytophagous lepidopteran larvae (Christeller et al., 2011). Other studies have used midgut EST libraries to identify members of lipase families. Translated protein sequences with sequence similarity to those of classical vertebrate pancreatic/neutral lipases and gastric/acid lipases have been found (Christeller et al., 2010; Horne et al., 2009; Tan-Kristanto, 2008). The midgut-expressed members of the pancreatic lipase family constitute a new family within the pancreatic lipase superfamily (Christeller et al., 2010), with structural similarity to the N-terminal catalytic domain of pancreatic lipase-related protein 2 (PLRP2) family in that they share the feature of an extremely reduced catalytic cavity lid structure (Carrière et al., 1998; Eydoux et al., 2008; Withers-Martinez et al., 1996). The C-terminal domain of pancreatic lipase is, however, absent from these proteins. The members of lepidopteran larval midgut-expressed gastric lipase family appear structurally similar to classical vertebrate gastric lipases (Christeller et al., 2010; Horne et al., 2009; Tan-Kristanto, 2008). However, classical vertebrate phospholipase A2 appears to be absent from lepidopteran midgut, since no proteins have been identified by similarity in EST libraries (Simpson et al., 2007). An enzyme from the pancreatic lipase family with phospholipase A1 activity, Dolm1, has been identified in the venom of the hornet (*Dolichovespula maculata*) and like the lepidopteran putative lipolytic enzyme, this enzyme only contains a single domain (Soldatova et al., 1993). Galactolipase (Amara et al., 2010; Andersson et al., 1996; Sias et al., 2004) and phospholipase (Withers-Martinez et al., 1996) activity are consistent with the identification of these PLRP2-like lipases.

We are unaware of any reports of interactions between lipase inhibitors and insect lipases or any reports on the effects of lipase inhibitors on larvae. This contrasts with the extensive literature on proteinase inhibitor-insect digestive proteinase and amylase inhibitor-insect digestive amylase interactions, both *in vivo* and *in vitro*. There are many studies, however, of tetrahydrolipstatin (THL) with vertebrate and microbial digestive lipases (Asler et al., 2007; Crellin et al., 2010; Eydoux et al., 2007; Hadvary et al., 1987; Lobo and Wilton, 1997; Potthoff et al., 1998). THL appears to act by incorporation into the oil/water interface (Gargouri et al., 1991; Ko and Small, 1997; Ransac et al., 1991) and then by covalent bond formation to the active site serine (Asler et al., 2007; Hadvary et al., 1988, 1991; Lookene et al., 1994). Thus all types of lipases are susceptible to THL. Here we report the effects of THL on the larvae of the cosmopolitan generalist phytophage, *Epiphyas postvittana* (Tortricidae, Lepidoptera). The effects of THL on mortality, growth and development using an *in vivo* insect bioassay are determined and midgut lipase activity and steady state mRNA expression levels of lipase genes in these insects are measured.

2. Materials and methods

2.1. Insects and diets

E. postvittana larvae were obtained from a colony maintained at the New Zealand Institute for Plant & Food Research Ltd., (Auckland, New Zealand). This colony has been previously characterised (Simpson et al., 2007).

Leafroller assay diet (Suckling et al., 1996) was prepared by mixing the dry ingredients with a reduced volume of water (98 mL for every 100 mL specified in the recipe), microwaving on high and cooling to 60 °C before adding ascorbic acid, antibiotics and methyl-p-hydroxybenzoate. The diet was then cooled further to 45 °C before adding 2-mL aliquots of each treatment (see below) per 98 mL diet. The diets were then poured into square tissue culture dishes (100 × 100 mm) and cooled quickly by refrigeration before being held overnight in a sterile room (ambient conditions) to condition the diet by reducing the water content. The following day, about 1 mL diet was pressed into the bottom of 3.5-mL clear plastic autoanalyser (AA) cups.

2.2. Materials

The lipase inhibitor, tetrahydrolipstatin (THL), was extracted from a commercial source (Orlistat®) in absolute ethanol. Apart from polyvinylpyrollidone, a minor component, no other components listed are soluble in absolute ethanol. Complete extraction was confirmed by gravimetric analysis. The LC–MS system used to measure the concentration of THL of prepared solutions was a Dionex Ultimate® 3000 Rapid Separation LC system and a micrOTOF II mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray source operating in negative mode. The LC system contained a SRD-3400 solvent rack/degasser, HPR-3400RS binary pump, WPS-3000RS thermostated autosampler, TCC-3000RS thermostated column compartment. The analytical column used was a ZorbaxTM SB-C18 2.1 × 100, 1.8 μm (Agilent, Melbourne, Australia) maintained at 50 °C and operated in isocratic mode with 100% acetronitrile at a flow of 400 μL/min. The injection volume for samples and standards was 2 μL. The micrOTOF II source parameters were: temperature 200 °C; drying N_2 flow 8 L/min; nebulizer N_2 4 bars, endplate offset −500 V, capillary voltage + 3500 V; mass range 100–1500 Da, acquired at 2 scans/s. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each analysis. Exact ion chromatograms (EIC) composed of m/z 494.387 ± 0.01 $(M-H)^-$ and m/z 540.393 ± 0.01 $(M+HCOOH-H)^-$ were processed by QuantAnalysis (Bruker Daltonics) and showed only a single compound. An external calibration curve (1.0–20 μg/mL) was prepared from authentic THL (Cayman Chemical, Ann Arbor, Michigan). Sample solutions were diluted with methanol.

2.3. Experiment 1. Bioassay for growth and development

Treatments were sterile water, absolute ethanol, 5.5% THL in ethanol, 1.83% THL in ethanol, and 0.55% THL in ethanol, giving final concentrations of THL in the diets of 0.11%, 0.037% and 0.011%. *E. postvittana* neonate larvae (<24 h old) were weighed in batches of five and transferred into AA cups (one larva per cup), 20 larvae per treatment, and maintained at 21 ± 1 °C, and the whole experiment was replicated three times. Larval survival and instar were recorded weekly and larvae were weighed 14 and 21 days after being transferred onto the treatment diet to determine growth rates. Time to pupation, percentage pupation, weight and sex of pupae, and time to emergence as adults were also recorded weekly.

2.4. Experiment 2. Bioassay for lipase enzyme activity

Approximately 300 neonate *E. postvittana* larvae were transferred onto a diet containing 0.01% THL and 100 neonates were transferred onto each control treatment diet (water and ethanol) and maintained at 21 ± 1 °C. Larvae were dissected at the 3rd or 4th instar; for water controls, this was after feeding on the diet for ∼14 days, for ethanol controls at ∼21 days and for larvae on the 0.01% THL diet at ∼28 days. Following cold-anaesthetising, the midgut of each larva was removed under a dissecting stereomicroscope and carefully separated from potential contaminating tissues (silk glands, trachea, Malpighian tubules, fat body cells and haemocytes), and then immediately stored at −20 °C until required. Three samples of ∼15 mg midgut were then ground in 0.2 mL of 10 mM Tris, 20 mM NaCl, 2 mM sodium diethyldithiocarbamate, 2 mm sodium ascorbate and 2 mM cysteine, pH 7.5, cen-