Insect Biochemistry and Molecular Biology 44 (2014) 44–53

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/ibmb

Sequestration, tissue distribution and developmental transmission of cyanogenic glucosides in a specialist insect herbivore

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ARTICLE INFO

Article history:
Received 4 October 2013
Received in revised form
13 November 2013
Accepted 14 November 2013

Keywords:
Zygaena filipendulae
Burnet moth
Cyanogenic glucosides
Sequestration
Plant–insect interactions

ABSTRACT

Considering the staggering diversity of bioactive natural products present in plants, insects are only able to sequester a small number of phytochemicals from their food plants. The mechanisms of how only some phytochemicals are sequestered and how the sequestration process takes place remains largely unknown. In this study the model system of *Zygaena filipendulae* (Lepidoptera) and their food plant *Lotus corniculatus* is used to advance the knowledge of insect sequestration. *Z. filipendulae* larvae are dependent on sequestration of the cyanogenic glucosides linamarin and lotaustralin from their food plant, and have a much lower fitness if reared on plants without these compounds. This study investigates the fate of the cyanogenic glucosides during ingestion, sequestration in the larvae, and in the course of insect ontogeny. To this purpose, double-labeled linamarin and lotaustralin were chemically synthesized carrying two stable isotopes, a ^{2}H labeled aglucone and a ^{13}C labeled glucose moiety. In addition, a small amount of ^{14}C was incorporated into the glucose residue. The isotope-labeled compounds were applied onto cyanogenic *L. corniculatus* leaves that were subsequently presented to the *Z. filipendulae* larvae. Following ingestion by the larvae, the destiny of the isotope labeled cyanogenic glucosides was monitored in different tissues of larvae and adults at selected time points, using radio-TLC and LC-MS analyses. It was shown that sequestered compounds are taken up intact, contrary to earlier hypotheses where it was suggested that the compounds would have to be hydrolyzed before transport across the gut. The uptake from the larval gut was highly stereo selective as the β-glucosides were retained while the α-glucosides were excreted and recovered in the frass. Sequestered compounds were rapidly distributed into all analyzed tissues of the larval body, partly retained throughout metamorphosis and transferred into the adult insect where they were distributed to all tissues. During subsequent mating, isotope labeled cyanogenic glucosides were transferred from the male to the female in the nuptial gift.

1. Introduction

Some insect species have the ability to take up, concentrate, and store potentially toxic bioactive compounds present in their food plants for exploitation in their own defense, and in some cases for intersexual communication and nuptial gifts (Rossini et al., 2001; Zagrobelny et al., 2013; Bowers, 1990; Conner et al., 2000; Dussourd et al., 1989; Cardoso and Gilbert, 2007; Zagrobelny et al., 2007b). This process is defined as sequestration (Duffey, 1980), and when it takes place in the insect larval stages, the sequestered compounds often have to be maintained through ontogenesis. Sequestered compounds may be distributed throughout the entire insect body, be mainly present in the haemolymph, or are sometimes restricted to specialized tissues in the insects, such as defensive glands (Nishida, 2002; Blum, 1983; Bowers, 1990).

Zygaena larvae (Zygaenoidea, Lepidoptera) sequester the cyanogenic defense compounds linamarin and lotaustralin from their food plant *Lotus corniculatus* (Fabaceae) (Nahrstedt and Davis, 1986). The insect also has the unique ability to biosynthesize the exact same cyanogenic glucosides *de novo* (Jensen et al., 2011;

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http://dx.doi.org/10.1016/j.ibmb.2013.11.003

Zagrobelny et al., 2007a; Davis and Nahrstedt, 1987), when feeding on *L. corniculatus* plants with limited amounts of the two cyanogenic glucosides. Larval sequestration of cyanogenic glucosides from their food plant plays the dominant role in the overall acquisition of their cyanogenic glucoside content, and *de novo* biosynthesis or metabolic turn-over does not allow the larvae to fully adjust to the optimal content and ratio of the two cyanogenic glucosides (Zagrobelny et al., 2007a). Larvae feeding on acyanogenic plants also show reduced fitness, probably due to the higher metabolic cost associated with *de novo* biosynthesis *vs.* sequestering (Zagrobelny et al., 2007a).

Zygaena larvae and moths are known to use cyanogenic glucosides as defense compounds distributed throughout their bodies. Cuticular cavities in the larval integument harbor defense droplets exuded upon attack and constitute the main storage site of the cyanogenic glucosides (Franzl and Naumann, 1985). In the course of the *Zygaena filipendulae* life cycle, content and ratio of the two cyanogenic glucosides are strongly regulated with an approximate 1:1 ratio of linamarin:lotaustralin present at the L4-L7 larval stages and an increased ratio of at least 2:1 in the adult stage (Zagrobelny et al., 2007a). Cyanogenic glucosides have gained additional important functions in the life cycle of *Zygaena filipendulae* as part of a nuptial gift from the male to the female during mating, in intersexual communication, and possibly in nitrogen metabolism during pupation (Zagrobelny et al., 2007b, 2013, 2007a; Zagrobelny and Møller, 2011). Cyanogenic glucosides are hydrolyzed by β-glucosidases, resulting in release of toxic hydrogen cyanide and ketones (Fig. 1). In plants, cyanogenic glucosides and their activating β-glucosidases are stored separately, constituting a two-component defense system that may be activated by tissue disruption (Morant et al., 2008; Neilson et al., 2013). In *Zygaena* the two components are present in the same tissues (mainly the haemolymph) (Franzl et al., 1989), and it is not understood why the two components do not immediately interact, since the neutral pH of the haemolymph (Franzl et al., 1989) is not inhibitory for β-glucosidase activity.

It is not known how *Zygaena* larvae sequester cyanogenic glucosides, but it has been hypothesized that the compounds are hydrolyzed before transport across the gut and then re-glucosylated afterwards (Nahrstedt and Davis, 1986). Furthermore, it is unknown how fast sequestration takes place, where in the insect tissues sequestered compounds are stored compared to biosynthesized compounds, and how quickly compounds are turned over during the insect life-cycle. In this study we demonstrate that linamarin and lotaustralin provided by the *L. corniculatus* food plant are ingested and sequestered by *Z. filipendulae* larvae as intact glucosides, and very quickly distributed to all tissues in the body of the larvae. Sequestered compounds are retained in adult tissues following ontogenesis, and finally transferred from the male to the female during mating as part of a nuptial gift. The cyanogenic glucosides directly sequestered in intact form from the food plant thus play numerous intimate roles throughout the entire life cycle of *Z. filipendulae.*

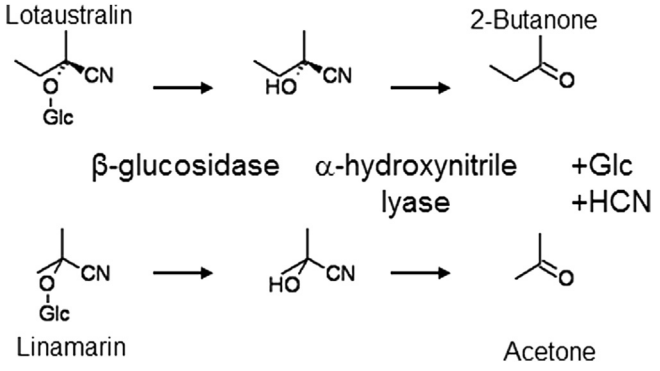

Fig. 1. Bio-activation of linamarin and lotaustralin. Liberation of toxic hydrogen cyanide, glucose and a ketone is catalyzed by a specific β-glucosidase and a α-hydroxynitrile lyase. The latter enzyme is only necessary if the pH is below 6, otherwise the reaction happens spontaneously.

2. Materials and methods

2.1. Biological material

Z. filipendulae larvae were collected from a natural population in the greater Copenhagen area in 2012 (N 55° 38.077′, E 12° 15.748′). Larvae were kept in boxes and fed cyanogenic *L. corniculatus* collected in the field ad libitum until used for experiments. 69 last instar larvae (L7) were moved into separate boxes each containing a single cyanogenic *L. corniculatus* leaf. Each leaf was painted with 3 μl each of ~1 mg/μl labeled linamarin and lotaustralin in 0.05% TritonX100 (0.24 μCi in total). This amount approximately corresponds to the entire amount of cyanogenic glucosides already present in the larva, and is therefore around a hundred times higher than what is naturally present in the leaf. Painted leaves were left to dry for a minimum of 2 h prior to being presented to larvae in feeding boxes. It was noted when larvae started to feed, and when they had ingested the entire leaf. A few larvae had to be left for several days before they had completed feeding, because they were close to pupation and therefore ingested slowly. 10 larvae were dissected less than 4 h after completion of feeding, 10 larvae were dissected 18–24 h after feeding, and 4 larvae were dissected 30–45 h after feeding. The last larval batch was allowed to feed on *L. corniculatus* devoid of labeled compounds after they had finished eating the leaf with labeled compounds, to avoid stress due to starvation. Larvae were dissected into the following tissues (See (Naumann et al., 1999) for illustrations): Defense droplets, haemolymph, frass, gonads (♀), Malpighian tubules, labial glands, gut (including ingested material at the early time-point, empty at later time points), integument, and fat body. Additionally, 33 larvae pupated after ingesting the leaf with labeled compounds, resulting in eight emerged adults. Four labeled virgin ♂ were mated to ♀ that had not eaten labeled compounds, and all were dissected at varying time points from 1 to 6.5 h after mating had ended. Two labeled virgin ♀ were also dissected. Adults were dissected into the following tissues (See (Naumann et al., 1999) for illustrations): wings (♂ + ♀), head + thorax (♂ + ♀), abdomen (♂ + ♀), eggs (♀), testes (♂), spermatophore (♀), internal genitalia (♂), ingluvie (♂ + ♀), corpus bursae (♀), rectal bladder (♂ + ♀), external genitalia (♂ + ♀), Petersens glands (♀), sebaceous glands (♀), and coremata (♂). ♂ internal genitalia includes ductus ejaculatorius, vas deferens and accessory glands. ♂ external genitalia includes aedeagus, bulbus ejaculatorius, lamina dorsalis, lamina ventralis, saccus, tegument, uncus, valva, vesica and vinculum. ♀ external genitalia includes papillae anales, abdominal tergite 8, “Schildchen”, lamella postvaginalis, lamella antevaginalis, ostium bursae.

2.2. Chemical synthesis of linamarin and lotaustralin specifically labeled with ^{2}H, ^{13}C and ^{14}C

The following procedure represents the chemical synthesis of D-glucose-$^{13}C_6$ (Sigma Aldrich 389374-3G)/2.5%D-glucose-$^{14}C_6$ (ARC 070315) labeled peracetylated glucosyl fluoride.

2.2.1. 1,2,3,4,6-Penta-O-acetyl-D-glucopyranose-$^{13}C_6$ (**2**, Fig. 2)

A suspension of anhydrous sodium acetate (3 g), acetic anhydride (30 mL) and D-glucose-$^{13}C_6$ (**1:** 3 g, 16 mmol) was heated under