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Cyanide detoxification in an insect herbivore: Molecular identification of β-cyanoalanine synthases from *Pieris rapae*

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ABSTRACT

Cyanogenic compounds occur widely in the plant kingdom. Therefore, many herbivores are adapted to the presence of these compounds in their diet by either avoiding cyanide release or by efficient cyanide detoxification mechanisms. The mechanisms of adaptation are not fully understood. Larvae of *Pieris rapae* (Lepidoptera: Pieridae) are specialist herbivores on glucosinolate-containing plants. They are exposed to cyanide during metabolism of phenylacetonitrile, a product of benzylglucosinolate breakdown catalyzed by plant myrosinases and larval nitrile-specifier protein (NSP) in the gut. Cyanide is metabolized to β-cyanoalanine and thiocyanate in the larvae. Here, we demonstrate that larvae of *P. rapae* possess β-cyanoalanine activity in their gut. We have identified three gut-expressed cDNAs designated *PrBSAS1-PrBSAS3* which encode proteins with similarity to β-substituted alanine synthases (BSAS). Characterization of recombinant PrBSAS1-PrBSAS3 shows that they possess β-cyanoalanine activity. In phylogenetic trees, PrBSAS1-PrBSAS3, the first characterized insect BSAS, group together with a characterized mite β-cyanoalanine synthase and bacterial enzymes indicating a similar evolutionary history.

1. Introduction

As an inhibitor of cellular respiration, cyanide is toxic for aerobic organisms. Herbivorous insects may be exposed to cyanide through the ingestion of cyanogenic compounds, such as cyanogenic glucosides, produced by their host plants as chemical defenses (Gleadow and Møller, 2014). Disruption of the plant tissue brings cyanogenic glucosides together with their hydrolytic enzymes, β-glycosidases, and free α-hydroxynitriles are formed. These are unstable and decompose spontaneously or enzymatically catalyzed into an aldehyde and toxic HCN (Fig. 1). Many generalist herbivores can tolerate low levels of cyanogenic compounds in their diet, but they avoid plants with high cyanogen content or, if forced to feed on such plants, suffer from serious symptoms of cyanide poisoning or die (Gleadow and Woodrow, 2002; Ballhorn et al., 2005). Some specialist herbivores have acquired adaptations which allow them to ingest high amounts of cyanogenic plant material without ill effects. Besides chemical modification of the intact glucoside to make it acyanogenic (Engler et al., 2000), they may circumvent cyanogenic glucoside hydrolysis by multiple mechanisms including feeding mode and plant glucosidase inhibition (Pentzold et al., 2014, 2015). If cyanide release cannot be prevented by such mechanisms, efficient means of cyanide detoxification are required (Duffey and Blum, 1977; Witthohn and Naumann, 1987; Meyers and Ahmad, 1991; Wybouw et al., 2014). Many organisms, including mammals and arthropods, detoxify cyanide by rhodanese (EC 2.8.1.1; Sörbo, 1955; Beesley et al., 1985; Cerletti, 1986) and/or β-cyanoalanine synthase (EC 4.4.1.9, Fig. 1; Floss et al., 1965; Miller and Conn, 1980; Meyers and Ahmad, 1991) activities.

β-Cyanoalanine synthases have primarily been investigated in plants (Hatzfeld et al., 2000; Yamaguchi et al., 2000) and rhodaneses in mammals (Cipollone et al., 2007). Only little information is available on the insect enzymes despite the widespread occurrence of β-cyanoalanine in insects (Duffey and Blum, 1977; Witthohn and Naumann, 1987). In the lepidopteran species *Spodoptera eridania* and *Trichoplusia ni* (Noctuidae), β-cyanoalanine synthase activity resides in the mitochondria (Meyers and Ahmad, 1991) while a soluble β-cyanoalanine synthase has been purified from the gut of the grasshopper *Zonocerus variegatus* (Orthoptera: Pyrgomorphidae) (Ogunlabi and Agboola, 2007). None of the insect enzymes has

Abbreviations: NSP, nitrile-specifier protein; BSAS, β-substituted alanine synthase; BSA, bovine serum albumin; DPD, *N,N*-dimethyl-*p*-phenylendiamindihydrochloride; ORF, open reading frame.

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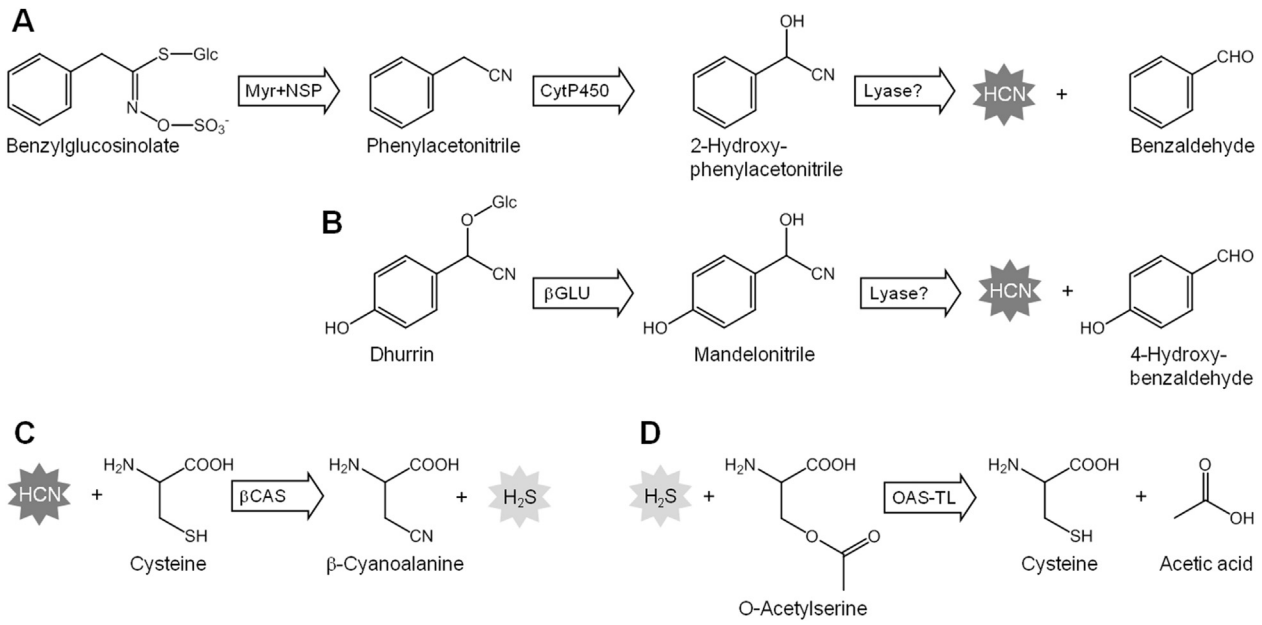

Fig. 1. Cyanogenesis and cyanide detoxification. **A.** Cyanide formation upon metabolism of benzylglucosinolate in larvae of *P. rapae*. In the presence of larval NSP, benzylglucosinolate hydrolysis by plant myrosinases (Myr) yields phenylacetonitrile which upon α-hydroxylation by larval gut cytochrome P450 enzymes (CytP450) decomposes into cyanide and an aldehyde. **B**. Cyanide formation from the cyanogenic glucoside dhurrin. Plant β-glucosidases (βGLU) convert dhurrin to its aglucone which decomposes to cyanide and an aldehyde. **C**. Reaction catalyzed by β-cyanoalanine synthase (βCAS). **D**. Reaction catalyzed by *O*-acetylserine (thiol) lyase (OAS-TL).

been identified at the molecular level. A β-cyanoalanine synthase from the two-spotted spider mite, *Tetranychus urticae* (Trombidiformes: Tetranychidae), has recently been cloned and characterized (Wybouw et al., 2014). Interestingly, the mites appear to have acquired this gene from bacterial symbionts by an ancient horizontal gene transfer event (Wybouw et al., 2014). Lepidopteran genomes contain sequences which group together with the β-cyanoalanine synthase sequence from *T. urticae* in phylogenetic analyses (Wybouw et al., 2014). It is presently unknown, if they are derived from the same or a separate gene transfer event (Wybouw et al., 2014).

The Small Cabbage White butterfly, *Pieris rapae* (Lepidoptera: Pieridae), is a specialist on plants containing glucosinolates, a group of chemical defenses found in the Brassicales (Halkier and Gershenzon, 2006). The defensive function of glucosinolates is mainly attributed to the toxic isothiocyanates (mustard oils) which are formed as a consequence of glucosinolate hydrolysis catalyzed by plant thioglucosidases (myrosinases, EC 3.2.1.147) upon tissue disruption ('mustard oil bomb'; Matile, 1980). Larvae of *P. rapae* circumvent this defense by expression of a gut nitrile-specifier protein (NSP). In the presence of larval NSP, myrosinase-catalyzed glucosinolate hydrolysis yields simple nitriles instead of the toxic isothiocyanates (Fig. 1; Wittstock et al., 2004). Aliphatic nitriles are excreted with the faeces while aromatic nitriles are further metabolized (Wittstock et al., 2004; Vergara et al., 2006; Agerbirk et al., 2010). Metabolism of the nitriles derived from benzyl- and 2-phenylethylglucosinolate (phenylacetonitrile and 3-phenylpropionitrile) exposes the larvae to cyanide as these nitriles are decomposed to cyanide and an aldehyde after α-hydroxylation by microsomal enzymes (Stauber et al., 2012, Fig. 1). When larvae feed on a plant with high content of benzylglucosinolate such as *Tropaeolum majus* (Tropaeolaceae), the amounts of cyanide formed per hour are estimated to be far above toxic levels for humans when related to body weight (Stauber et al., 2012). However, *T. majus* is a known host plant of *P. rapae* (Renwick and Huang, 1995), and feeding on a cyanogenic mutant of *Arabidopsis thaliana* (Brassicaceae) does not affect growth and survival of the larvae (Stauber et al., 2012; Pentzold et al., 2015). Thus, *P. rapae* larvae must be able to efficiently detoxify cyanide.

Feeding studies with aromatic glucosinolates or cyanogenic glucosides as cyanide precursors and gaseous [^{15}N]-labeled cyanide have shown that cyanide metabolites in *P. rapae* include β-cyanoalanine (Stauber et al., 2012). Here, we demonstrate β-cyanoalanine synthase activity in larval extracts of *P. rapae*. Furthermore, we report on the isolation from larval gut tissue of three cDNAs encoding proteins with β-cyanoalanine synthase activity. To our knowledge, this is the first report on molecular cloning and characterization of β-cyanoalanine synthases from insects.

2. Material and methods

2.1. General

β-Cyanoalanine was obtained from Sigma. Protein concentrations were determined with the Pierce BCA Protein Assay Kit (Thermo FisherScientific) using bovine serum albumin (BSA) as a standard according to the manufacturer's instructions. PCR was performed on thermocyclers PeqStar (PEQLAB Biotechnology) and TProfessional Gradient (Biometra). PCR primers were purchased from Invitrogen (Life Technologies). Unless otherwise stated, reactions were set up in a total volume of 50 μl DreamTaq buffer supplemented with 0.2 μM of each dNTP, 0.2 μM of each primer, 1 μl cDNA or appropriate amount of template DNA and 0.25 μl DreamTaq Polymerase (Thermo Scientific) and were subjected to the following temperature program: 95 °C for 5 min, 35 cycles of 95 °C for 45 s, appropriate annealing temperature for 1 min, and 72 °C for 1 min, and a final incubation at 72 °C for 10 min. Sequencing was done at Eurofins MWG Operon (Ebersberg, Germany).