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Chlorophyll catabolites in the frass of the Small Tortoiseshell caterpillars (*Aglais urticae* L.)

B. Amarsanaa[1,2], and W. Boland[1]

Dedicated to Professor Badgaa Dagva on the occasion of his 80th birthday

[1] *Max Planck Institute for Chemical Ecology, Hans-Knull-Str. 8, Department Bioorganic Chemistry, Beutenberg Campus, Jena, Germany*

[2] *Institute of Chemistry and Chemical Technology, MAS, Peace ave., Ulaanbaatar 13330, Mongolia*

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Abstract: Herbivorous insects excrete most of the consumed chlorophyll as partly degraded derivatives lacking the phytol side chain and the central magnesium ion. To study common degradation patterns of chlorophyll in plant-feeding insects, the frass of the Lepidopteran caterpillar, *Aglais urticae* was analysed for chlorophyll catabolites. The major metabolites were determined as pheohorbide *a* and pyropheophorbide *a* by using LC-MS, LC-SPE-NMR and UV. These compounds are not present in fresh leaves of the food plants (*Urtica dioica*).

Keywords: Aglais urticae; Lepidoptera; frass; pheophorbide; pyropheophorbide

INTRODUCTION

Chlorophylls (Chl) are ubiquitous pigments present in nature. Chlorophyll is an extremely important biomolecule, critical in photosynthesis, which allows plants to absorb energy from light. Chls are also counted for dietary in the food of herbivores [1]. It is estimated that more than 10^9 tonnes of green Chls degrade annually on the land [2] to colorless linear tetrapyrroles in a highly conserved multistep pathway [3]. The pathway starts with the loss of the central Mg^{2+} ion by a chlorophyllase (CLH) and a metal-chelating substance (MCS), yielding pheophytins. After hydrolytic removal of the phytol side chain pheophorbides *a/b* (Phe) [4] and pyropheophorbides *a/b* (Pph) remain as stable products on the tetra-pyrrole level. Both, the biotic and the abiotic degradation of Chl generate a number of bioactive products. In particular, Phe *a*/*b* have a wide range of activities. Phe *a* has anti-oxidative properties and both, Phe *a*/*b,* are active against tumors [5]. In addition, some of their derivatives exhibit anti-microbial activities [6]. Moreover, Phe and Pph *a* are biotoxic [7] and act as photosensitizer disrupting the mitochondrial electron transport [8]. While the process of Chl degradation is well studied *in planta*, we faced a remarkable lack in knowledge on chemistry and biochemistry of the early and late events of Chl degradation in plant-feeding insects. Scattered information is available on Chl degradation in aquatic grazers, algae, bacteria and even aphids [9]. Recently, first evidence for an ecological role of Phe *a* was published, presenting Phe *a* as a powerful deterrent in the fecal shield of larvae of a tortoise beetle. For the first time, a Chl degradation product was assigned an interspecific, non-nutritive defense function, which might suggest a wider occurrence of Chl degradation products with still unknown functions in other ecological systems [10].

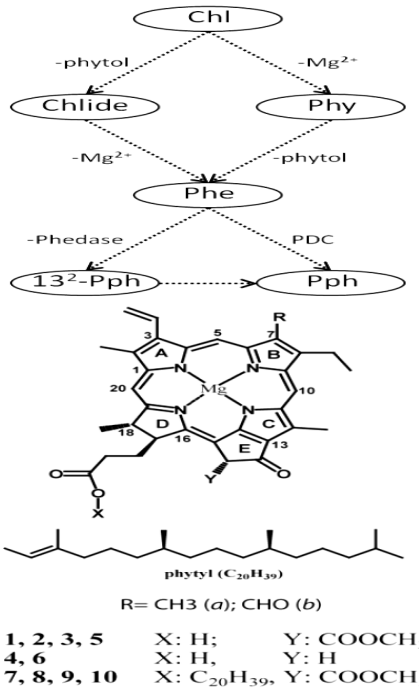

Fig. 1. The early degradation steps of chlorophyll in the insect alimentary tract and the basic structure of its derivatives. Chl is degraded by chlorophyllase – loss of phytol; and by a metal-chelating substance - loss of Mg^{2+}: producing Phe. Pheophorbidase (Phedase) and pheophorbide demethoxydecarbonylase (PDC) producing Pph.

* corresponding author: e-mail: boland@ice.mpg.de

The number of Chl catabolites corresponds to the number of the eluting peaks in the profile of Figure 2. *- presence of Mg^{2+} ion, **9/8**- chlorophyll (Chl) *a*/*b*, **2/1**- chlorophyllide (Chlide) *a*/*b*, **10/7**- pheophytin (Phy) *a*/*b*, **5/3**- pheophorbide (Phe) *a*/*b*, **6/4**- pyropheophorbide (Pph) *a*/*b*.

Since the Chl metabolites were present in the fecal shield of the larvae, a structure that is build from the digestive products of the insect, this prompted us to analyze the frass of other Lepidopteran species for Chl degradation products. In this study, we analyzed the early stages of Chl degradation in the frass of a Lepidopteran caterpillar, namely *Aglais urticae,* that feeds on young leaves of their host plant *Urtica dioica*. We demonstrate that the overall degradation process in the insect is identical with the degradative pathway in other Lepidopteran species, but differs on the quantitative level of the early degradation products Phe *a* and Pph *a*.

EXPERIMENTAL

***Sample preparation*:** Third instar caterpillars of the Small Tortoiseshell (*Aglais urticae* L.) were collected along with their natural host plant, the common nettle (*Urtica dioica* L.) close to the city of Jena, Germany. Larvae of *A. urticae* were reared on leaves of the common nettle in a plastic box (25 ±1°C; 14 : 10 h light : darkness) until compound analysis.

Collection and extraction of regurgitate and frass: Fecal excretions were collected 48 h after placing the caterpillars onto their food plants. Next, the collected frass (0.1 g) was covered with 2 ml acetone-water (8:2, v/v) and stirred for 1 h in the dark at 4°C. After centrifugation at 14,000 × g for 10 min at 4 °C, the supernatant was taken and removed under a stream of argon. The residue was redissolved in methanol (0.3 ml) and filtered through membrane filters (PVDF, Millipore, 0.22 μm) into a GC-vial. Samples were diluted with methanol (1:10) and directly analyzed (10 μl) by liquid chromatography-mass spectrometry (LC-MS).

LC-MS analysis of Chl metabolites: Chls and their degradation products profiled by LC-MS and parallel UV-monitoring(430 nm) [9]. For Chl *a/b* (Sigma Aldrich), Phe *a* and Pph *a* standards (Wako) were prepared in methanol. The sample was analyzed on a Thermo Finnigan LCQ with atmospheric pressure chemical ionization (APCI) in the full scan mode. Separation of compounds was achieved on an Agilent HP1100 HPLC system equipped with a Kinetex C18 column (Phenomenex, 5 μm column, 150 x 4.6 mm). Pigments were eluted by a gradient starting at a flow of 0.750 ml min^{-1} with 35% solvent A (0.01M ammonium acetate in water) and 65% solvent B (75% acetonitrile/25% methanol, MeOH/CH_3CN, *v/v*) up to 100% B and a flow rate of 1 ml min^{-1}. Elution was maintained for 10 min before returning to the initial conditions for equilibration. Calibration curves for Chl *a/b*, Phe *a* and Pph *a* were established with defined concentrations of the authentic references.

The standard solutions were prepared as described, and peak areas were sampled and averaged.

LC-SPE-NMR analysis of Chl metabolites: The HPLC was connected to a Prospect 2 SPE unit (Spark Holland) for trapping of compounds. The post-column eluent was diluted with H_2O by a make-up pump prior to trapping individual peaks on a poly(divinylbenzene) SPE cartridge (HySphere resin GP, Spark Holland). The SPE device was coupled to a Bruker AVANCE 500 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a cryogenically cooled TCI ^1H ^{13}C probe and a Cryofit^{TM} flow insert (30 μL active volume) [11].

RESULTS AND DISCUSSION

The degradation of Chl in the digestive tract of plant feeding *A. urticae* was addressed by analysis of fecal deposits. HPLC-UV-APCI-MS coupling as well as LC-SPE-NMR were used to separate and to identify the Chl metabolites. Authentic samples were used to identify the Chl catabolites in the feces (Fig. 2). The metabolite profile, extracted from the frass of *A.urticae,* showed pheophorbide (Phe) *a* (**5**) along with pyropheophorbide (Pph) *a/b* (**6**/**4**) as the major products. Interestingly, the ingested Chl *a/b* (**9**/**8**), Chlide *a/b* (**2**/**1**) and pheophytin (Phy) *a/b* (**10**/**7**) were only present as minor products demonstrating that the majority of ingested Chls is rapidly degraded in the alimentary canal. In senescent plants, the Chls *a/b* are degraded first to Chlide *a/b* or to Phy *a/b* followed by loss of the central magnesium ion or removal of the phytol side chain to give Phe *a/b*. Both compounds can exist as two stereoisomers that can be separated on C18 columns. The formation of the two isomers results from isomerization of the β-ketoester in the E-ring. All degradation products were identified by their characteristic UV absorption and molecular ions, for example ($[M+H]^+$) at 593.2745 Da characteristic for Phe *a*, consistent with the calculated mass for $C_{35}H_{36}N_4O_5$ (593.2759). Moreover, a strong UV absorption at 408 nm, along with smaller long wave absorption at 666 nm (data not shown) is typical for this compound. In plants and algae two different pathways lead to the Pph *a/b* [12]. In plants the pheophorbidase generates C-13^2-carboxyl-pheophorbide *a* by saponification releasing methanol. The resulting acid is unstable and suffers a spontaneous decarboxy-lation. Alternatively, a Phe demethoxycarbonylase [12] directly removes the ester moiety from the E-ring of Phe without obvious intermediates (Fig. 1). In addition to Phe *a*, we identified in the frass of *A. urticae* eight additional signals, which could be attributed to Chl *b* (**8**) at 36.59 min and Chl *a* (**9**) at 41.58 min (*m/z* = 907 and *m/z* = 893, respectively). long-wave UV absorption at 438 nm (Fig. 3), and a maximum fluorescence emission around 680 nm, which is due to the extended chromophore with the aldehyde group in ring B (Fig. 1). Since the metal-free Phe is still able to generate cell-toxic singlet oxygen by photo-activation

further degradation is achieved by the Phe-oxygenase which generates *in planta* open chain tetrapyrroles by cleavage of the bond between the A- and B-rings of the tetrapyrrole skeleton [13]. Pph *a* ($C_{33}H_{34}N_4O_3$) is characterized by a molecular ion at *m/z* =535.3, a UV absorption maximum at 409 nm (Fig. 4a), and a fluorescence emission maximum at 671 nm. The HR-ESI- MS yielded a quasimolecular ion at *m/z* = 535.2702 matching the calculated *m/z* = 535.2714 [14]. The denoted (*; Fig. 2) signals in the frass were not assigned. The natural concentration of Phe *a* and Pph *a* in the frass of *A. urticae* was determined to be 65.42 µg mg^{-1} and 75.51 µg mg^{-1}. The concentrations of these Chl catabolites in *A. urticae* were comparable to other Lepidopteran species [9].

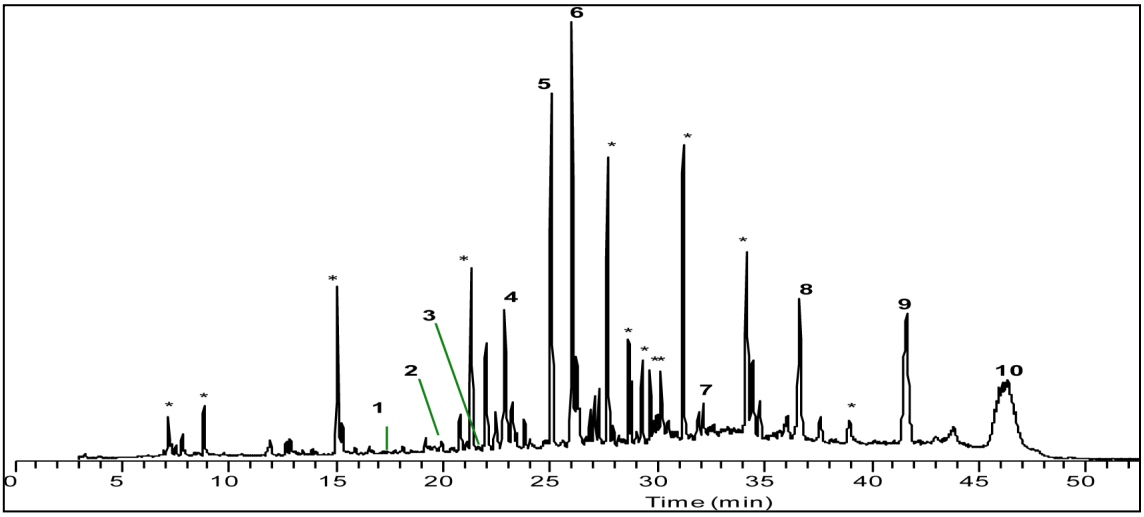

Fig. 2. Chromatographic separation and identification of Chl catabolites from the extracts of digestive products of *A. urticae* larvae. Total ion chromatogram (TIC) of LC-MS, APCI full scan (+) mode and peak assignment of Chl catabolites.

1- chlide *b*, **2-** chlide *a*, **3-** Phe *b*, **4-** Pph *b*, **5-** Phe *a*, **6-** Pph *a*, **7-** Phy *b*, **8-** Chl *b*, **9-** Chl *a* and **10-** Phy *a*, *-unidentified metabolites.

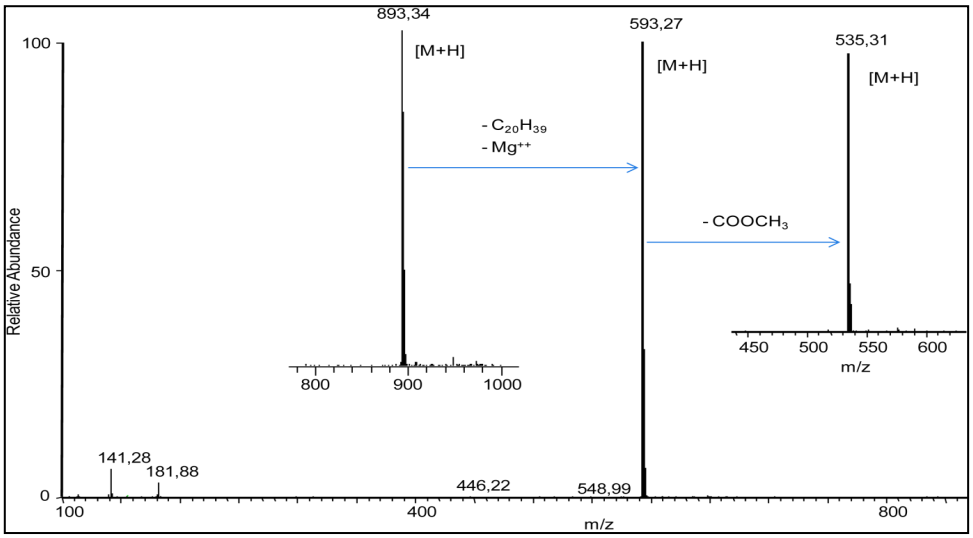

Fig. 3. Positive ion APCI mass spectrum of Chl and early metabolites from frass extracts of *A. urticae* monitored by LC-UV-DAD-MS. The difference between [M+H] peak of Chl *a* m/z 893.34 (small, left) to Phe *a* m/z 593.27 (big) as well as the decarboxylated peak of Pph *a* m/z 535.31(small, right) demonstrates the successive cleavage of Phe *a*.

The structures of the two major degradation products of Chl *a*, namely Phe *a* and Pph *a,* were additionally confirmed by *LC-SPE-*^{1}*H-NMR*. Phe *a*: 500mHz, CD_3CN: 9.72, 9.49, 8.81 (3s, H10, 5, 20), 8.18, 6.40, 6.23 (3dd, J_{AX}=17.9, J_{BX}=11.7Hz, J_{AB}=1.0Hz, H_X, H_A, and H_B), 6.27 (s, 1H, $H13^2$), 4.56 (q, J=7.5, 1H, H18), 4.16 (d, 1H, J=7.6Hz, H17), 3.86 (s, 3H, $H13^{3COOCH3}$), 3.78 (q, 2H, $H8^1$, J=8.1Hz), 3.65 (s, 3H, $H12^1$), 3.43 (s, 3H, $H7^1$), 3.26 (s, 3H, $H2^1$), 2.42 (t, J=6.9Hz, 2H, $H17^1$), 1.71 (m, 2H, $H17^2$), 1.81 (m, 3H, $H8^2$), 1.27 (br.s, 3H, $H18^1$), 0.13 (br. s, NH, A-cycle), 0.06 (br. s, NH, C-cycle); Pph *a*: 500mHz, CD_3CN: 9.72, 9.49, 8.81 (3s, H10, 5, 20), 8.18, 6.40, 6.24 (3dd, J_{AX}=17.9, J_{BX}=11.7Hz, J_{AB}=1.0Hz, H_X, H_A, and H_B), 6.27 (s, 1H, $H13^2$), 4.57 (q, J=7.5, 1H,

H18), 4.16 (d, 1H, J=7.6Hz, H17), 3.87 (s, 3H, $H13^{3COOCH3}$), 3.78 (q, 2H, $H8^1$, J=8.1Hz), 3.65 (s, 3H, $H12^1$), 3.43 (s, 3H, $H7^1$), 3.26 (s, 3H, $H2^1$), 2.42 (t, J=6.9Hz, 2H, $H17^1$), 1.72 (m, 2H, $H17^2$), 1.81 (m, 3H, $H8^2$), 1.27 (br., 3H, $H18^1$), 0.13 (br. s, NH, A-cycle), 0.06 (br. s, NH, C-cycle). These data identified the compound as Pph *a* [15]. Besides the early degradation products of Chl (Table 1, Figure 2) no catabolites corresponding to an oxidative cleavage were found in the frass. While these degradation processes are rather well understood in plants, almost nothing is known on Chl degradation in feeding insects. Since insects prefer to feed on young and fresh leaves, the ingested leave tissue does not possess the typical Chl degrading enzymes of the senescence program. Therefore all catabolic activities can be linked to processes and conditions in the insect gut (Fig. 1). None of the Chl catabolites Phe *a/b* and Pph *a/b* were found in the leave tissue (data not shown), but all Lepidopteran larvae possessed substantial amounts of Phe *a* and Pph *a* in their regurgitate and frass [9]. In fact, most reactions, namely the hydrolytic removal of the phytol side chain, the removal of the central magnesium ion, and the hydrolysis of the ß-ketoester with subsequent spontaneous decarboxylation, can be achieved by the alkaline gut pH [16], especially in combination with non-specific hydrolases and esterases present in the gut. The different compo-sition of the early Chl degradation products in frass of the selected species supports that in the Lepidopteran insects, several enzymes may contribute to a certain extent to the degradation process. Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are assumed to have Mg-dechelation activities [17]. Thus, the presence of different GSTs along with hydrolytic enzymes present in the gut at different quantities and activities may account for the observed differences in the degradation profiles of the Lepidopteran cater-pillars [9]. Since the late Chl degradation products, linear tetrapyrrolles, require specific enzymes [13] and the presence of oxygen to cleave the stable aromatic system of the tetrapyrroles, the anaerobic digestive system of insects facilitates only the early steps of Chl degradation.

CONCLUSIONS

Due to the absence of oxygen in the gut of Lepidopteran larvae, documented by the presence of obligate anaerobic Clostridia [18], the oxidative cleavage of the tetrapyrrole core of Chl *a/b* is not observed and, hence, the early catabolites of Chl are qualitatively identical in all of the studied species. Only the quantitative composition may vary dependent on the enzymatic activities in the digestive tract [9]. Phe *a* and Pph *a/b* are the major chlorophyll catabolites in the frass of the investigated larvae and the isolated Chl catabolites indicate a breakdown pathway comparable to higher plants.

After defaecation, in the presence of oxygen, stable plant proteins surviving decompositon in the insect gut [19] could achieve such ring-cleavage reactions. Hence, we can expect linear chlorophyll catabolites only in the deposited frass resulting from oxidative cleavage of Chl *a/b*. Whether or not such linear Chl catabolite resemble those of the late steps of plant catabolism of Chl [20] are present in deposited feces remains to be established.

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