Isomers of green leaf volatiles in Nicotiana attenuata and their role in plant-insect interactions
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Citation for published version (APA):

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Purification and properties of a (3Z):(2E)-enal isomerase from *Manduca sexta*’s oral secretion

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ABSTRACT

Plants release volatiles into the air upon herbivore attack and such herbivore-induced volatile blends can contain specific information about the attacker. We recently discovered that the oral secretions (OS) of the lepidopteran larva *Manduca sexta* contain a heat-labile compound that, when it comes in contact with leaf wounds, converts the leaf aldehyde (Z)-3-hexenal to its (E)-isomer, (E)-2-hexenal. This herbivore-specific conversion of the volatile profile tripled the foraging efficiency of the generalist predator *Geocoris* spp. for *M. sexta* eggs and reduced the oviposition rate of *Manduca* moths on these plants. Here we report our first results on the characterization and purification of (3Z):(2E)-enal isomerase activity from *M. sexta*’s OS. We show that the isomerizing compound is of proteinaceous nature, is larger than 50kDa and active over a wide pH range. With conventional biochemical fractionation techniques we were able to partially purify the activity. LC-MS/MS analysis of the most active fraction yielded the identification of several candidate peptide masses. These are the first steps that will allow us to identify the isomerase gene from *M. sexta*.

**Image description front page**

*Upper left panel*: Oral secretions are collected from 3rd to 5th instar caterpillars of *Manduca sexta*.

*Upper right panel*: FPLC equipment to fractionate the oral secretions of *Manduca sexta*.

*Lower left panel*: 10% Polyacrylamide SDS-Gel with OS samples of *Nicotiana attenuata* fed (lane 2) and artificial diet fed (lane 3) *M. sexta* caterpillars, and artificial diet fed *Spodoptera exigua* caterpillars (lane 4).

*Lower right panel*: MS/MS spectrum of tryptic peptide 1314.6 MH+ (active fraction)
INTRODUCTION

Plants are under continuous pressure to recognize pests and to tune defenses accordingly while these pests, in turn, are under selection to stay undetected and resist defenses. In the traditional view the plant is in control of direct and indirect defenses (Howe and Jander, 2008; Wu and Baldwin, 2010) since it synthesizes toxins to directly ward off attackers and volatiles to call for external help as the emission of herbivore-induced plant volatiles (HIPV), i.e. especially of terpenoids, aromatic compounds and C-6 based ‘green leaf volatiles’ (GLVs; Maffei, 2010), leads to the attraction of foraging natural enemies of herbivores and therefore is referred to as indirect plant defense (Mumm and Dicke, 2010). The induction of herbivore-induced direct and indirect defenses depends on the extent to which herbivores give away their presence and to which extent plants are able to recognize their presence. Elicitor compounds present in the oral secretions of the herbivore can play an important role in this process as many induced defense responses are initiated by the perception of such elicitors (Hilker and Meiners, 2010; Wu and Baldwin, 2010). Although scores of herbivore species exist, only few herbivore-derived elicitors have been identified so far and they can roughly be classified into two major groups: The first group consists of relatively small non-proteinaceous substances, like fatty acid-amino acid conjugates (FACs; Bonaventure et al., 2011) and sulfated fatty acids (caeliferin; Alborn et al., 2007), whereas the second group consists of proteinaceous substances, including proteolytic peptides, like inceptins but also digestive enzymes like β-glucosidase (Howe and Jander, 2008; Hilker and Meiners, 2010; Wu and Baldwin, 2010).

FACs are probably the best studied class of herbivore-induced elicitors. They consist of a plant derived fatty acid conjugated to an insect derived amino acid (Parè et al., 1998). FACs have been shown to play a role in the activation of phytohormone-mediated direct defense responses and the production and emission of HIPVs mediating indirect defense responses (Alborn et al., 1997; Halitschke et al., 2003; Bonaventure et al., 2011). By now FACs have been found in the oral secretions (OS) of many lepidopteran species and recently also in the OS of two cricket species (Teleogryllus taiwanemma and T. emma) and fruit flies (Drosophila melanogaster, Yoshinaga et al., 2007; Yoshinaga et al., 2010). Caeliferins, are a group of sulfated fatty acids which have been isolated from the OS of the grasshopper Schistocerca americana and they induce, like volicitin, the release of herbivore-specific terpenes from maize seedlings (Alborn et al., 2007; Hilker and Meiners, 2010; Wu and Baldwin, 2010). Both, volicitin and caeliferins were isolated by reversed-phase high performance liquid chromatography (Alborn et al., 1997; Alborn et al., 2007).
Inceptins are proteolytic peptides which are generated in the insect midgut by degradation of a plant’s chloroplastic ATP synthase γ-subunit (Schmelz et al., 2006). Even very small amounts are able to effectively amplify the accumulation of the phytohormones jasmonic acid, ethylene and salicylic acid and to induce the emission of the homoterpene (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) (Schmelz et al., 2006; Schmelz et al., 2007). Inceptins have been identified from the OS of S. exigua feeding on cowpea (Schmelz et al., 2006). Not only proteolytic peptides but also digestive enzymes can play a role in mediating plant defense responses. The ubiquitous enzyme (Cairns and Esen, 2010) β-glucosidase which hydrolyzes glycosidic bonds has been found in the OS of P. brassicae larvae, and application of their OS or pure β–glucosidase provoked a similar release of herbivore-induced volatiles in cabbage plants (Mattiacci et al., 1995). Recently Schaefer et al. (2011) discovered that lipase activity present in the OS of the grasshopper Schistocerca gregaria mediated the accumulation of oxylipins in Arabidopsis leaves.

Interestingly, a plant is not always in direct control of its own defenses: In a recent study Leroy et al. (2011) reported the first case in which an insect-associated bacterium, Staphylococcus sciuri, present in the honeydew of the pea aphid Acyrthosiphon pisum, produced volatile compounds that attracted the hoverfly, Episyrphus balteatus, a natural aphid enemy. Thus, in this special case neither the plant, nor the insect were responsible for the attraction of the third trophic level.

Trichome exudates of the wild tobacco Nicotiana attenuata contain O-acyl sugars which do not directly defend the plant against the specialist herbivore Manduca sexta. However, after ingestion by the feeding caterpillar the alkaline conditions in the caterpillar’s digestive tract probably caused hydrolysis of the O-acyl sugars and the resulting short chain fatty acids imparted the body and the frass of the caterpillars with a characteristic smell that was recognized by the omnivorous ant Pogonomyrmex rugosus. Thus trichome derived acyl sugars function as an ‘evil lollipop’ as they tag the feeding caterpillar with a distinct odor that is attractive to natural enemies of the herbivore (Weinhold and Baldwin, 2011).

We recently discovered that the increased emission of (E)-isomers of GLVs, induced in N. attenuata by M. sexta feeding, strongly increased the foraging efficiency of the generalist predators Geocoris spp. (Allmann and Baldwin, 2010 and Chapter 3) and decreased the oviposition rates of Manduca sexta conspecifics (Chapter 4) in nature. Surprisingly, the conversion of the plant’s (Z)-aldehydes to (E)-aldehydes was solely and directly due to isomerase activity in the herbivore’s oral secretions. Hence we were left with the puzzling
observation that *M. sexta* caterpillars are betraying themselves by making the plant’s volatile bouquet more attractive to their natural enemy.

Here we present our first results on the physico-chemical properties of the *M. sexta* derived (3Z):(2E)-enal isomerase and on the purification of this isomerase from oral secretions of *M. sexta* caterpillars.

**RESULTS**

*Enzyme characteristics*

We have developed an *in vitro* assay that allows to measure (3Z):(2E)-enal isomerase activity via the conversion of (Z)-3-hexenal to (E)-2-hexenal using headspace solid-phase microextraction (SPME) combined with gas-chromatography coupled to time-of-flight mass spectrometry (GC-ToF-MS; Fig. 5.1A). Details are described in the material and methods section. To determine the isomerase activity versus enzyme concentration we kept the amount of substrate constant (0.98 µg/reaction) and varied the amounts of *M. sexta* OS. Between 0 and 5 µg of total protein the substrate was not the limiting factor (Fig. 5.1B).

![Graph A) SPME-GC-ToF-MS chromatogram; ion 69](image)

![Graph B) Activity vs. enzyme concentration](image)

**Figure 5.1. Isomerase activity in *M. sexta* OS.** (A) GLV-related GC-ToF-MS extracted ion chromatogram (Ion 69) as output of the SPME-guided *in vitro* assay for measuring (Z)/(E)-isomeric activity. (Z)-3-hexenal was used as substrate; control (ctrl) = assay without OS; *M. sexta* OS = assay with OS. (B) Activity versus enzyme concentration curve of untreated *M. sexta* OS. Substrate concentration was kept constant.

In previous experiments we showed that the (3Z):(2E)-enal converting compound in the OS of *Manduca sexta* caterpillars is heat labile (Allmann and Baldwin, 2010 and Chapter 3). This already indicated that the compound is of proteinaceous nature and probably an enzyme. To test this we treated 1:20 (v/v) diluted *M. sexta* OS with Proteinase K (Prot. K), an
endolytic protease that cleaves peptide bonds at the carboxylic side of aliphatic, aromatic and hydrophobic amino acids and we compared the activity of Prot. K-treated against non-treated *M. sexta* OS using the SPME-guided *in vitro* assay (Fig. 5.1A). Indeed Prot. K-treated OS lost their ability to convert (Z)-3-hexenal to (E)-2-hexenal as conversion-rates did not differ from control treatments containing 0.02% Tween-20 instead of *M. sexta* OS (Fig. 5.2A).

In order to determine the influence of pH on the enzyme, its activity was studied in the range from 5.5 till 10.0 using different synthetic buffers. For the pH range 5.5 – 6.5 20 mM MES buffer was used, for the pH range 8.5 - 9.0 20 mM Tris-Cl buffer was used and for pH 10.0 we used 20 mM CAPS buffer. The enzyme is active over a broad range of pH values, but it is most active in the more alkaline conditions (Fig. 5.2B).

![Figure 5.2. The isomerase in *M. sexta* OS is of proteinaceous nature and is most active at pH > 8.5. (A) Percent conversion to (E)-2-hexenal (n=3). Proteinase (Prot K) K treated OS lost the (Z)/(E)- isomerase activity. Letters indicate significant differences between treatments (univariate analysis of variance (ANOVA), $F_{3,8} = 369.1$, $P < 0.001$, followed by a Scheffe post-hoc test). Ctrl = assay without OS in 0.02% Tween-20 solution; OS = 1:20 (v/v) diluted *M. sexta* OS; OS 37ºC = 1:20 (v/v) diluted *M. sexta* OS incubated at 37ºC for 75min; Prot.K OS 37ºC = Proteinase K treated 1:20 (v/v) diluted OS incubated at 37ºC for 75min. (B) Influence of pH on (3Z):(2E)-enal isomerase activity. *M. sexta*’s OS were diluted 1:20 in different 20 mM buffers and the % conversion to (E)-2-hexenal measured by SPME-GC-ToF-MS was compared to the % conversion of OS that had been diluted in distilled water. 100% activity = isomerase activity measured for OS sample diluted in H2O (red line). pH 5.5 and 6.5, 20 mM MES-buffer; 8.5 and 9.0, 20mM TRIS-buffer; 10.0, 20mM CAPS-buffer.

To estimate the size of the (3Z):(2E)-enal isomerase we applied *M. sexta* OS onto centrifugal filters with different nominal molecular weight limits. While all the activity was found in the retentate when using a 50 kDa cut-off filter, a 100 kDa cut-off filter retained most, but not all of the activity, assuming that the approximate size of the enzyme is around 100 kDa (Fig. 5.3A). Experiments with a Sephacryl S-200 column confirmed that the
isomerase has a rather high molecular weight as activity eluted shortly after the void volume (> 250 kDa; data not shown). We furthermore tested whether the isomerase is a membrane bound protein by ultracentrifugation at 100,000 g for 35 min of M. sexta’s OS. As the activity remained in the soluble phase and could not be recovered from the re-dissolved pellet it is very unlikely that the enzyme is bound to a membrane (Fig. 5.3B).

**Figure 5.3.** The isomerase has an approximate size of 100 kDa and is not membrane bound. (A) GLV-related GC-ToF-MS extracted ion chromatogram (Ion 69) of the isomerase activity in permeate and retentate of M. sexta OS samples centrifuged through 50 and 100 kDa centrifugal filters. Samples contain (Z)-3-hexenal as substrate. ctrl = assay without OS; M.s. OS = assay with OS. (B) Isomerase activity in M. sexta OS, centrifuged for 35 min at 100,000 g. Supernatant and re-dissolved pellet were tested with (Z)-3-hexenal as substrate. M.s. OSpellet-UC = re-dissolved pellet after centrifugation of M. sexta OS; M.s. OSsupernatant-UC = supernatant of M. sexta OS after centrifugation.

**Enzyme purification**

Each enzyme requires a different purification strategy. And even for the same enzyme different purification procedures might be necessary, depending on its origin and related to this, its physico-chemical properties (Schuurink et al., 1990). To determine the optimal purification strategy for the (3Z):(2E)-enal isomerase from M. sexta’s OS we first tested the suitability of several purification methods including precipitation methods and separation based on molecular size (see above), charge and hydrophobic interactions. We added different amounts of solid ammonium sulfate to test at what saturation the isomerase precipitates. Interestingly most of the enzyme stayed in solution even at a 100% saturation (Fig. 5.4). Because of the good solubility of the isomerase at saturated ammonium sulfate conditions we diluted 100 mL M. sexta OS after concentration with 30 kDa cut-off filters (Step 1, Fig. 5.5) in 2 M ammonium sulfate ((NH₄)₂SO₄) containing 100 mM Tris-Cl buffer (pH8.0; 1:1, (v/v);
Step 2, Fig. 5.5) to firstly, remove all proteins that precipitate already at a 1 M ammonium sulfate concentrations and secondly, to adjust the sample to the starting conditions for hydrophobic interaction chromatography (Step 3, Fig. 5.5). The sample was applied in sub-portions on a HiTrap Phenyl HP 5 mL column and the activity started to elute from the column at a salt concentration of approx 0.7 M (NH₄)₂SO₄ (Step 3, Fig. 5.5). The pooled active fractions were again concentrated and extensively washed with 20 mM Tris-Cl (pH 8.0), the starting buffer for anion exchange chromatography (AIC). The sample was applied on a HiTrapQ HP 5 mL column and the activity eluted from this column at a salt concentration of approx 0.4 M NaCl (Step 4; Fig. 5.5). We also tested the binding capacity and thus the purification efficiency of a cation exchange resin (CM-cellulose). However, we did not find any condition at which the isomerase either completely bound or did not bind at all to the resin and we therefore decided to not include cation exchange chromatography in our purification strategy.

In Table 5.1 the results of the (3Z):(2E)-enal isomerase purification of M. sexta’s OS are summarized. After step 3 a 35 fold increase in the specific activity was achieved with a recovery of approx. 30%. Since the estimated protein concentration after step 4 was below the detection limits, we cannot estimate the overall purification rate. However, since the activity of the active fractions after step 4 was very high and clearly above the linear range of the SPME-guided in vitro assay (data not shown) while the absorbance at 280 nm for the active fractions was very low (Fig. 5.5), the last purification step must have been efficient.

Identification of candidate peptide sequences

The trypsin-digested pooled active fractions (dark grey bar; step 4 Fig. 5.5) were analyzed by LC-MS/MS and resulted in the identification of eight doubly charged (MH+) MS/MS peptide
masses. However, so far these could not be matched with significant scores to in situ digested peptide masses of the translated *M. sexta* midgut database or to peptides available from the NCBI nr protein database (nrdb90) and all other public accessible databases. Future analyses using a transcriptomic database of *M. sexta* salivary glands (provided by H. Vogel and David Heckel, MPI for chemical Ecology, Jena, GER) and a genomic database (Agricultural Pest Genomics Resource Database: www.agripestbase.org) will help to identify candidate peptide sequences of our *M. sexta* derived (3Z):(2E)-enal isomerase.

To shorten the list of candidate peptide masses we tested two samples that flanked the active pooled fractions (Fig. 5.4; (2)) on the left (1) and right (3) side (light grey bars). While sample 1 only yielded 3 MS/MS peptide masses (MH+) of which none could be assigned to in situ digested peptide sequences of the translated *Manduca* midgut transcriptome, sample 3, which contained minor amounts of isomerase activity resulted in the identification of 71 MS/MS peptide masses (MH+) of which two could be associated with a high score to a *Manduca* midgut sequence which in turn showed high similarity with trypsin-like serine proteases of several lepidopteran species. However, there was no overlap in the peptide masses between active and non-active fractions.

A second analysis of the active fraction with a Synapt G2 LC-MS/MS system (Waters) increased the number of selected peptides for MS/MS from 8 to about 120 due to the superior sensitivity of this newer generation LC-MS/MS. This data could not be matched with significant scores using the same databases as described above.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>total protein (mg)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>total activity (units)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>centrifuged OS</td>
<td>209</td>
<td>7136</td>
<td>34</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>Concentrated &gt; 30 kDa</td>
<td>202</td>
<td>2618</td>
<td>13</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>1M Ammonium sulfate</td>
<td>114</td>
<td>4650</td>
<td>41</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>Phenyl Sepharose column (pooled active fractions)</td>
<td>2</td>
<td>1975</td>
<td>1182</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>Sepharose-Q column (pooled active fractions)</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
<td>n.m.</td>
</tr>
</tbody>
</table>

<sup>a</sup> Protein concentration determined by Bradford assay using BSA as a standard protein.

<sup>b</sup> 1 unit causes 50% conversion to (E)-2-hexenal in 5min incubation time

<sup>c</sup> not measured as estimated protein concentration was too low

**DISCUSSION**

This study provides first insights into the properties of a (3Z):(2E)-enal isomerase present in the OS of *M. sexta* caterpillars. We have set up an SPME-based assay to measure (3Z):(2E)-enal isomerase activity and we have shown that the (Z)- to (E)-converting compound is of
proteinaceous nature, as activity vanishes after treatment with Prot. K (Fig. 5.2A). This is supported by our purification experiments as we were able to increase the specific activity by more than 35-fold using classical biochemical protein fractionation techniques (Table 5.1).

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**STEP**

0 100mL pure OS

1 concentration with 30kDa cut-off filter

2 precipitation with 1M (NH₄)₂SO₄

3 HIC (HiTrap Phenyl HP; Tris pH 8.0)

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4 AIC (HiTrapQ HP; Tris pH 8.0)

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5 concentration and analysis with LC-MS/MS
As the oral secretions of *M. sexta* caterpillars are a mix of gut-derived digestive secretions and labial and mandibular gland-derived saliva (Eichenseer et al., 2010) we cannot make any predictions on the origin of the isomerase. Additionally, as vertical transmission of gut bacteria from mothers to larvae via egg contamination have been postulated for *M. sexta* (Brinkmann et al., 2008), the isomerase might also originate from gut colonizing bacteria which have been found in the gut lumen of *Manduca sexta* and the hindgut epithelia (Toth-Prestia and Hirshfield, 1988; Dillon and Dillon, 2004; Brinkmann et al., 2008). We also performed dissection experiments to unravel the enzyme’s origin. However, for both dissected and homogenized salivary glands and midgut tissue, we were so far unable to detect any (Z)/(E)-converting activity (data not shown). Sometimes the pH-optimum of an enzyme can give a first hint on its biosynthetic origin: Tunaz and Stanley (2004) discovered two phospholipases A2 (PLA2), of which one had its origin in the salivary glands of *M. sexta* caterpillars, and the other in the midgut. While the midgut derived PLA2 preferred higher pH conditions of 9-10 and showed little activity at pH 8, the PLA2 synthesized in the salivary glands of the caterpillar showed highest activity at a pH of 8 (Tunaz and Stanley, 2004). Here we tested the pH optimum of the isomerase in a range between 5.5 and 10.0. However, so far we can only conclude that the optimal pH lies in the alkaline region. This is not surprising as luminal pH measurements of 10–11 pH units in the anterior and middle regions of *M. sexta*’s midgut are normal, and even pH values of 12 have been reported (Clark et al., 1998; Lait et al., 2003). The OS that we collected and used to characterize and purify isomerase activity had a pH between 9-10. Such alkaline conditions for *M. sexta*’s OS (pH 9.3) have already been reported in previous studies (von Dahl et al., 2006). However, this is probably not the pH-region at which the (3Z):(2E)-enal isomerase functions when it comes in contact with the leaf surface as the oral secretions of plant-feeding *M. sexta*’s caterpillars mingle with crushed leaf material. Higher plant cells contain two main compartments, the cytoplasm which is slightly

**Figure 5.5. Partial purification of (3Z):(2E)-enal isomerase from *M. sexta* OS.** 100mL of *M. sexta* OS was concentrated using 30 kDa cut-off filters (Step 1). Concentrated OS was diluted 1:1 (v/v) with 2M (NH₄)₂SO₄, 100mMTris pH 8.0 and centrifuged (Step 2). One quarter of the supernatant was applied on a HiTrap Phenyl HP 5mL column and proteins were eluted with a linear gradient of decreasing (NH₄)₂SO₄ concentration. This step was repeated with the remaining three quarters (Step 3). Combined active fractions were applied on a HiTrapQ HP 5mL column and proteins were eluted with an increasing, linear gradient of NaCl (0-1 M) (Step 4). Pooled active fractions (2) and neighboring inactive or only slightly active fractions (1, 3) were analyzed using advanced protein sequencing (liquid chromatography-tandem mass spectrometry [LC/MS/MS]) methods (Step 5). Protein elution in Step 3 and 4 was continuously monitored by UV absorption at 280nm and is given in milli absorbance units (mAU). The activity of each fraction was monitored using the SPME-guided in vitro assay and (3Z):(2E)-enal isomerase activity is given in % conversion to (E)-2-hexenal (dotted line).
alkaline (7.4-7.5) and the central vacuole which is acidic with a pH of 4.5-6 in many plant species (Gout et al., 1992). *Manduca* feeding causes cell disruption and thus a complete mixing of all cell contents, and for crushed leaf material rather acidic environments have been reported (pH 4-6; Schultz and Lechowicz, 1986). This is in line with our observation that the (3Z):(2E)-enal isomerase is still active at low pH levels (Fig. 5.2B).

The SPME-guided assay that we used to determine (3Z):(2E)-enal converting activity cannot be used for absolute quantifications, but only for relative comparisons, and this has mainly two reasons: Firstly, the assay is not performed in a closed system and volatiles could thus escape from the system e.g. during transfer from the small GC vials to the bigger SPME vials. Secondly, *M. sexta’s* OS does not only contain a compound that converts (Z)-3- into (E)-2-hexenal but also other unknown components which seem to have an aldehyde-buffering capacity. This caused a high variation in the detected absolute aldehyde levels between samples and made replicated quantifications almost impossible.

In 1965 Moore isolated a subcellular fraction from bovine brain and proteins within this fraction were called S100 since they were partially soluble in 100% ammonium sulfate at a neutral pH (Moore, 1965). This remarkable characteristic holds also true for the *M. sexta* derived isomerase, as activity remained in the soluble phase even at a 100% ammonium sulfate. This high solubility made it easy to perform hydrophobic interaction chromatography (HIC) as for this purification technique samples are often adjusted to a moderately high ammonium sulfate concentration as starting conditions, typically at 1-2 M. Different matrices and ligands are available to perform HIC and we tested the Butyl-S-ligand which was the least hydrophobic ligand available and the Phenyl-ligand, which has a mixed behavior in which aromatic and hydrophobic interactions, as well as lack of charge, play a role in the final chromatographic properties. Interestingly, the enzyme did not bind to Butyl-S Sepharose (data not shown), while it did when using Phenyl Sepharose as medium (Fig. 5.5). The enzyme additionally bound to an anion exchanger (Q Sepharose) in slightly alkaline environments (pH 8.0; Fig. 5.5) indicating that there were enough negative charges on the protein “surface” of the isomerase.

Insect saliva has been shown to play a role in antimicrobial defense (Musser et al., 2005), digestion (Lenz et al., 1991), detoxification (Ahmad and Hopkins, 1993; Mathews et al., 1997), and suppression of host plant defenses (Musser et al., 2002; Musser et al., 2005; Bede et al., 2006). This makes it an interesting insect derived fluid to study. However, only few proteins have been identified so far from the salivary glands of *Manduca sexta* caterpillars or their oral secretions and this only by indirectly measuring their enzyme activity.
Minor amounts of β-glucosidase (Allmann and Baldwin, 2010) as well as Glucose oxidase (Diezel et al., 2009; Allmann and Baldwin, 2010; Eichenseer et al., 2010) activity have been found in *M. sexta’s* OS and the latter also specifically in the salivary glands (Eichenseer et al., 1999). GOX is an enzyme with an approximate size of 82 kDa found in several lepidopteran and few hymenopteran species (Eichenseer et al., 2010). It catalyses the oxidation of glucose leading to the production of gluconic acid and hydrogen peroxide. Interestingly, GOX does not elicit defense responses in the host plant, but it rather suppresses induced JA-dependent nicotine accumulation of *Nicotiana tabacum* (Musser et al., 2005) and the expression of genes involved in volatile production (Bede et al., 2006) while it increases salicylic acid levels in *N. attenuata* (Diezel et al., 2009). Tunaz and Stanley (2004) identified Phospholipase A2 activity in the fat body, hemocytes, midgut and also the salivary glands of tobacco hornworms. The most obvious function of these PLAs is to facilitate fatty acid absorption by catalyzing their hydrolysis (Tunaz and Stanley, 2004). However, PLA2 from salivary glands and midgut tissue have not been purified yet.

While only little attention has been paid to the salivary proteome of *M. sexta* caterpillars much more is know about the haemolymph (Furusawa et al., 2008) and midgut proteome of tobacco hornworms (McNall and Adang, 2003; Pauchet et al., 2009). These studies were conducted using classical 1D- or 2D-polyacrylamide gel electrophoresis (PAGE) combined with peptide mass fingerprinting but a recent study by Pauchet et al (2010) used both classical 2D-PAGE and AIC combined with SDS-PAGE.

Two dimensional PAGE is a powerful technique as it separates proteins by their isoelectric points and their size, thus making use of two different properties of the protein. This technique allows separating hundreds of proteins in a single run and is thus ideal for full proteome analysis or for comparative proteomic experiments such as difference gel electrophoresis (DIGE Issaq and Veenstra, 2008). However, this technique has also its limitations as proteins which are too acidic or too basic, too big (>100 kDa) or too small, or have a too low abundance are only poorly resolved (McNall and Adang, 2003; Issaq and Veenstra, 2008; Pauchet et al., 2009). As we estimated an approximate size of 100 kDa for the isomerase and a low abundance in *M. sexta’s* OS, we decided not to use 2D-PAGE as purification strategy, but to rather focus on biochemical fractionation techniques to enrich for our low abundant protein. For the purification of our (3Z):(2E)-enal isomerase we first tested the quality of each purification step by 1D-PAGE and we focused on those bands that were enriched or only present in the active fraction and not in the surrounding inactive fractions. However the detectable and thus most abundant proteins in the isolated bands turned out to be
midgut derived digestive enzymes including a carboxypeptidase, chitin deacetylase, lipase and several proteases as we were able to match the resulting peptide masses to those of the translated in situ digested Manduca midgut database. Since we predicted that our enzyme of interest might not be abundant enough to be seen as a single band on a polyacrylamide gel we decided to use the entire pooled active fraction (Fig. 5.5; sample 2 (dark grey bar)) for protein identifications and to reduce the number of candidate peptides by comparing the identified peptides masses against those of two nearby fractions that were either inactive (Fig. 5.5; sample 1) or contained only little activity (Fig. 5.5; sample 3). The trypsin digested pooled active fraction resulted in the identification of more than 120 doubly charged (MH+) MS/MS peptide masses. Interestingly, none of these peptide masses could be matched to the databases used in this study, assuming that the M. sexta OS-derived isomerase belongs to a rather unknown group of enzymes which is likely not produced in Manduca’s midgut. Clearly, more work is needed to identify the (3Z):(2E)-enal isomerase from M. sexta’s oral secretion. New available databases or additional purification steps will clearly help to succeed in identifying the “treacherous” compound in the insect’s oral secretion and further work will reveal whether the isomerase activity is simply maladaptive for M. sexta caterpillars and hence under negative selection; if there is a life-stage dependent or ecological trade-off with respect to the effects of enzyme expression or whether it is the plant that has adapted to make isomerase- expression backfire by synthesizing (Z)-3-hexenal i.e. as a “green booby-trap”. Thus, taking the evolutionary time scale into account, it is still open to debate whether it is the herbivore betraying itself or the plant betraying the herbivore.

MATERIAL AND METHODS

Collection of Manduca sexta’s oral secretions

Tobacco hornworm (Manduca sexta) eggs, purchased from Carolina Biological Supply and bred in an in-house colony, were cultivated in climate chambers until hatching and reared on N. attenuata wild-type plants in the greenhouse until the third to fifth instar. Oral secretions (OS) were collected twice a day on ice as described in Roda et al. (2004) centrifuged at 13,000 g for 5min to remove particles and stored at -20°C until use.

Wild type N. attenuata plants for caterpillar feeding were from an inbred line in its 30th generation that originated from seeds collected in Utah in 1988. Seeds were germinated on Gamborg’s B5 medium (Duchefa) as described in Krügel et al. (2002). In short, seeds were sterilized and incubated in 1 mM GA3 (Roth) and 1:50 diluted liquid smoke (v/v; House of
Herbs) before germination on Gamborg’s B5 medium at a 26°C/16 h 155 μmol s⁻¹ m⁻² photosynthetic photon flux (PPF): 24°C/8 h dark cycle (Percival). Plants were grown in the glasshouse with a day/night cycle of 16 h (26°C-28°C)/8 h (22°C-24°C) under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600-W sodium lights (Philips).

**SPME-GC-ToF activity assay**

Enzyme activity was determined by SPME-GC-ToF-MS. 200 μL of the eliciting solutions (water, diluted OS or purified fractions) containing 0.02 % Tween-20, were transferred into a 1.5 mL GC vial equipped with a 200 μL insert and 1-2 μL of (Z)-3-hexenal (0.48 μg/μL) was added to the solution. A volume of 200 μL was chosen to minimize the headspace and thus also the chance of (Z)-3-hexenal to volatilize without reacting with the eliciting solutions. The GC vial was closed and gently shaken for 5 minutes. Subsequently the liquid solution was transferred into a 20 mL SPME vial which was immediately closed with a Teflon lined crimp cap and incubated under moderate shaking (250 rpm) for 5 min at 30°C. Volatiles were sampled with a Solid Phase Micro Extraction fiber (SPME; Carboxem/PDMS) for 10 min at 30°C. The fiber was desorbed for 1 min in an Optic injector port (ATAS GL Int. Zoeterwoude, NL) which was constantly kept at 250°C. Compounds were separated on a DB-5 column (10m x 180μm, 0.18 μm film thickness; Hewlett Packard) in an 6890 N gas chromatograph (Agilent, Amstelveen, NL) with a temperature program set to 40°C for 1.5 min, increasing to 250°C at 30°C per minute and 250°C for an additional 2.5 min. Helium was used as a carrier gas, the transfer column flow set to 3 ml per minute for 2 min, and to 1.5 ml per minute thereafter. Mass spectra were generated with the ion source set to -70 V at 200°C and collected with a Time-of-Flight MS (Leco, Pegasus III, St. Joseph, MI, USA), with an acquisition rate of 20 scans per second. GLVs were identified and quantified using standard solutions of (Z)-3-hexenal, (E)-2-hexenal (Sigma-Aldrich; http://www.sigmaaldrich.com/nederland.html).

To account for the high variability between samples we first calculated the summed amount of hexenals (sum of (Z)-3-hexenal and (E)-2-hexenal) which was set to 100 %. From this we calculated the proportion of (E)-2-hexenal and called this “percent conversion to (E)-2-hexenal”.

145
Enzyme characterization

To test whether the (3Z):(2E)-enal converting compound in the OS of *M. sexta* is of proteinaceous nature we treated *M. sexta*’s OS with Proteinase K (Fermentas, St. Leon-Rot, GER; http://www.fermentas.de/index.php?c=NL). For this purpose 10 µL OS was pipetted into a 1.5 mL Eppendorf tube and diluted 1:20 (v/v) with 0.02 % Tween-20 solution. After adding 10 µL of Proteinase K (Prot. K; end concentration of 0.95 µg/µL) the tube was incubated for 1h 15min at 37°C and gently shaken in between. To ensure that the loss of activity was due to the Prot. K treatment and not to the heat-treatment we additionally incubated 1:20 diluted OS at 37°C for the same time, but without adding Prot. K. Two hundred µL of each sample were used for the *in vitro* enzyme assay and each treatment was done in triplicates.

To estimate the size of the enzyme we applied *M. sexta* OS onto centrifugal filters (Millipore, Amsterdam, NL; http://www.millipore.com/offices/cp3/nl) with different nominal molecular weight limit (NMWL, 3, 10, 30, 50 and 100 kDa cut-off). 150 µL OS was applied onto the filter device which was placed in a filtrate collection tube and samples were centrifuged at maximum speed for 10-30 min, depending on the NMWL of the filter device. Concentrate was re-suspended in same amounts as the filtrate and both concentrate and filtrate were tested for their (3Z):(2E)-enal converting activity.

We also estimated the size of the enzyme by Gel-filtration. We therefore applied OS samples which had been dissolved in elution buffer (20 mM Tris-Cl pH 9.0, 50 mM NaSO₄) onto a Sephacryl S-200 HR (GE Healthcare, Hoevelaken, NL; http://www.gehealthcare.com/nlnl/) column and eluted samples with a fraction size of 3 mL using a constant flow of 0.5 mL/min. The void volume of the column was determined by applying Dextran Blue onto the column which has a MW of 2,000 kDa. The void volume of the column was approx. 30 mL. To test whether the enzyme is a membrane bound protein we centrifuged pure OS for 35 min at 100,000 g using an Airfuge (Beckman, Woerden, NL; https://www.beckmancoulter.com) and we tested the activity of the supernatant and the pellet which had been dissolved in 0.02 % Tween-20 using the SPME-guided *in vitro* assay.

To determine the isomerase activity versus enzyme concentration we used our SPME-guided enzyme assay. We added 2 µL of (Z)-3-hexenal (0.48 µg/µL) to each reaction to keep the amount of substrate constant (0.98 µg/reaction). We gradually increased the amount of total protein in pure *M. sexta* OS and monitored the percent conversion to (E)-2-hexenal (Fig. 5.1B).
Enzyme purification

100 mL of crude *M. sexta* OS was concentrated using Centrifugal devices (Macrosep 30K Omega centrifugal filter) with a NMWL of 30 kDa. The filtrate was tested, but did not contain any activity. The concentrate was diluted 1:1 (v/v) with 2 M (NH₄)₂SO₄ in 100 mM Tris pH 8.0 and subsequently centrifuged for 20 min at 4100 g. The precipitate (approx. 1 mL) was dissolved in 6 mL MiliQ H₂O and tested for activity. To remove small particles we pushed the supernatant through a 0.45 µm filter and applied the filtered solution to a HiTrap Phenyl HP 5 mL column (GE healthcare, Hoevelaken, NL; http://www.gehealthcare.com/nl/) equilibrated with starting buffer (1 M (NH₄)₂ SO₄, 50 mM Tris pH 8.0 buffer). The adsorbed proteins were eluted with a linear gradient from 1 to 0 M (NH₄)₂ SO₄ in 50 mM Tris pH 8.0 and a constant flow of 3 mL/min (1-0M (NH₄)₂SO₄ in 18 column volumes (CVs)). As parts of the activity did not bind to the column, probably due to overloading, these active, unbound fractions were split into three samples and each sample was re-loaded separately on a clean HiTrap Phenyl HP 5 mL column. Active fractions were pooled and concentrated to exchange buffers. The concentrate of the active fractions was dissolved in 2 mL of 20 mM Tris pH 8.0 and applied onto a HiTrapQ HP 5 mL column (GE healthcare, Hoevelaken, NL; http://www.gehealthcare.com/nl/) which had been equilibrated with starting buffer (20 mM Tris pH 8.0). The adsorbed proteins were eluted with a linear gradient of NaCl in the same buffer (0 – 1 M NaCl in 18 CVs) and a constant flow of 2 mL/min. Active fractions were pooled and concentrated with centrifugal filters (30 kDa cut-off) to exchange buffers. The final active concentrate was dissolved in 100 µL 0.1M Tris pH 7.4 buffer for further analysis (Fig. 5.5).

Both fractionation steps in which we used HiTrap columns were done on a FPLC (ÄKTA; GE healthcare, Hoevelaken, NL; http://www.gehealthcare.com/nl/) and protein elution was continuously monitored by UV absorption at 280 nm. After each step of purification, all fractions were tested for activity using the SPME-guided *in vitro* assay.

In the various steps of the purification procedures protein concentration were determined by Bradford assay using BSA for comparison.

**In solution digestion of active protein sample and LC-MS/MS measurements**

The concentrated active sample and two non-active samples, which eluted shortly before and after the active fractions, were digested with 2 µg trypsin (Gold, Promega) in 0.1 M Tris pH 7.4. Before starting the overnight digestion at 37°C the samples were reduced with 5 mM dithiothreitol for 30 minutes at 55°C and after cooling down alkylated with...
15 mM iodoacetamide. The digested peptides for each sample were collected and washed on a 2 µg capacity OMIX tip (Varian) and eluted in 10 µL 50% acetonitrile (CAN), 0.1%TFA. The tryptic peptide mixtures were analyzed after dilution in 0.1 % TFA with liquid chromatography (LC)-nano-electrospray ionization tandem MS (ESI-MS/MS). Details of the LC method, data acquisition and processing have been described by Sorgo et al (2010).

The generated data were used to search against different databases using MASCOT software (Matrixscience release 2.3.02). In MASCOT, search parameters were as follows: allowance of one missed cleavage, fixed modification of carbamidomethyl cysteine and variable modification of oxidation on methionine, an error tolerance of 0.3 Da for calculated peptides and their corresponding MS/MS spectra. The databases used were known contaminants, NCBI, and the *in situ* digested translated *Manduca* midgut transcriptome (Pauchet et al., 2010).

*Statistics*

All statistics were done using SPSS 17.0 (Chicago, IL, USA).

**ACKNOWLEDGEMENTS**

We thank Eva Rothe, Benjamin Naumann, Stefan Kruse, Anke Rauch and Nora Petersen for collecting enormous amounts of *M. sexta* oral secretions, Jos Arents for his invaluable help and constant advice on protein purification techniques, Heiko Vogel for providing the *M. sexta* midgut transcriptome database and Winfried Roseboom for his help with uploading the *Manduca* databases to MASCOT.

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