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Spodoptera littoralis detoxifies neurotoxic 3-nitropropanoic acid by conjugation with amino acids

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ABSTRACT

Spodoptera littoralis is a phytophagous generalist. Its host range includes more than 40 plant species, some of which produce 3-nitropropanoic acid (3-NPA), an irreversible inhibitor of mitochondrial succinate dehydrogenase. Growth in larvae fed an artificial diet with a sublethal admixture of 3-NPA (4.2 μmol per g) was slowed significantly, but larvae experienced no increase in mortality. In contrast, larvae injected with 25.2 μmol/g (bodyweight) 3-NPA experienced acute toxicity and death. To study the detoxification mechanism of 3-NPA in *S. littoralis*, the insect frass was analyzed by HPLC-MS. Comparative analysis of 3-NPA-treated and -untreated control samples using HR-MS^2 revealed a group of differential signals that were identified as amino acid amides of 3-NPA with glycine, alanine, serine, and threonine. When sublethal amounts of stable isotope-labeled 3-NPA were injected into a larva's hemolymph, 3-NPA amino acid conjugates were identified as putative detoxification products. Bioassays with synthetic standards confirmed that the toxicity of the amides was negligible in comparison to the toxicity of free 3-NPA, demonstrating that amino acid conjugation in *S. littoralis* represents an efficient way to detoxify 3-NPA. Furthermore, biosynthetic studies using crude fractions of the gut tissue indicated that conjugation of 3-NPA with amino acids occurs in epithelial cells of the insect's gut. Taken together, these results suggest that the detoxification of 3-NPA in *S. littoralis* proceeds via conjugation to specific amino acids within the epithelial cells followed by export of the nontoxic amino acid conjugates to the hemolymph via as yet uncharacterized mechanisms, most likely involving the Malpighian tubules.

1. Introduction

3-Nitropropanoic acid (3-NPA) is a naturally occurring neurotoxin known as bovinocidin that has a profound effect on human health and domestic livestock (Burdock et al., 2001; Francis et al., 2013; Mithöfer and Boland, 2012; Xingjie et al., 1992). 3-NPA is a neurotoxin that irreversibly inhibits succinate dehydrogenase, a key enzyme of the citric acid cycle (Hipkin et al., 2004; Olsen et al., 1999; Tarazona and Sanz, 1987). Livestock exposed to 3-NPA suffers from motor dysfunction and in some cases death (Akopian et al., 2012; Francis et al., 2013; Parry et al., 2011). 3-NPA mainly occurs in legumes; in particular several *Astragalus* species contain high levels of this compound (Burdock et al., 2001) and also certain endophytic fungi are able to produce 3-NPA (Chomcheon et al., 2005). The compound has been shown to be responsible for the toxicity of some weeds (Meldrum, 2000; Williams, 1994), and cases of 3-NPA poisoning have been widely documented in livestock, leading to significant economic losses (Williams, 1994).

In contrast, some grasshoppers such as *Melanoplus bivittatus* survive feeding on concentrations of 3-NPA that are lethal to mammals (Majak et al., 1998; Tarazona and Sanz, 1987). In the frass of *M. bivittatus* larvae 3-NPA conjugates of the amino acids glycine, serine, and glutamine were detected and assumed to be putative detoxification products. However, no information concerning the properties and toxicity of 3-NPA amino acid amides in insects has been reported (Majak et al., 1998). Many *N*-acyl amino acid conjugates have been described in Lepidoptera (Alborn et al., 1997; Stauber et al., 2012). Glutamine amides of fatty acids are known to be involved in plant–herbivore interaction (Alborn et al., 1997; Lait et al., 2003; Ping et al., 2007; Yoshinaga et al., 2005). The conjugates with glycine are products of glucosinolate metabolism in *Pieris rapae* (Stauber et al., 2012). However, despite this, the metabolic pathways of 3-NPA detoxification in the phytophagous generalist *Spodoptera littoralis* are poorly understood (Grossa et al., 2008). Larvae of *S. littoralis* feed on more than 40 plant families,

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including legumes that accumulate 3-NPA in concentration up to 100 μmol per g fresh weight (Hipkin et al., 2004). The aim of this study was to determine the metabolic pathways that facilitate the detoxification of 3-NPA in *S. littoralis*. We show that *S. littoralis* gains its resistance to 3-NPA by converting the free acid into several non-toxic amino acid amides.

2. Materials and methods

2.1. Insects and toxicity experiments

S. littoralis eggs were obtained from Syngenta Crop Protection Münchwilen AG (Switzerland) and cultivated (Tang et al., 2012). The eggs were placed in a box with artificial medium and hatched at 14 °C (Bergomaz and Boppre, 1986). The larvae were cultivated at room temperature (24 °C) (Tang et al., 2012). The insects were raised on an artificial medium prepared according to Spiteller et al. (2005), but without addition of the preservatives parabene and formaldehyde. Fresh food was provided to the *S. littoralis* larvae once a day. For the feeding experiments, 3-NPA or amino acid amides of 3-NPA were added to the artificial diet at different concentrations. The body weight and body length of each insect were determined every day during the experiment. The control group fed on the artificial medium (n = 20) and the experimental group fed on a medium containing 3-NPA (c = 4.2; 8.4; 16.8; 33.6 mmol/kg, n = 20). The experiments were repeated three times. For the injection experiments, a buffer was used (1.4 mM NaCl, 0.07 mM, Na_2HPO_4, 0.03 mM KH_2PO, 4 mM KCl, pH 7.4). The solutions were injected into 4th-instar larvae (n = 30). In the control group, only the buffer was injected into the insect. In the experimental group, a solution of 3-NPA (c = 8.4; 12.6; 16.8; 25.2; 33.6 μ mol/g b. w.) was dissolved in the buffer and injected into the larvae (1 μl/100 mg of larvae weight). After injection, the larvae were individually reared on an artificial diet in plastic boxes (25 °C ± 1 °C). Fifth-instar larvae were used for tissue sampling. The larvae were killed using CO_2 and dissected. Their guts were separated from the other tissue and hemolymph was sampled with a thin capillary after dissection. The regurgitate was sampled with a thin capillary after stimulation of the larvae by forceps as described by Turlings et al. (1993).

2.2. HPLC/MS analysis of the hemolymph and excrement samples

Samples of the hemolymph and the frass of treated and untreated control larvae were suspended in MeOH/H_2O (1:1, v/v). The samples were centrifuged and analyzed by LC-MS and LC-HRMS experiments. The samples were analyzed on a Thermo Finnigan LCQ with atmospheric pressure chemical ionization (APCI) in the negative mode (vaporizer temperature: 450 °C; capillary temperature: 275 °C; source current: 4 uA; capillary voltage: −38 V; tube lens offset: −33 V). The mixture was separated on an Agilent HP1100 HPLC system equipped with a Hibar column (250 × 4 mm, Purospher STAR, RP-18 endcapped, 5 μm) using a flow of 1 ml min^{-1} with 97% of solvent A (0.1% formic acid in water) and 3% of solvent B (0.1% formic acid in acetonitrile) for 5 min, followed by a gradient up to 60% B over 30 min, then to 100% B for 5 min. Natural compounds were analyzed by using high-resolution (HRMS) and tandem mass spectrometry (BRUKER MAXIS) connected to an Dionex UltiMate 3000-system equipped with an Hibar column (250 × 4 mm, Purospher STAR, RP-18e, 5 μm).

The samples of subcellular fractions were separated isocratically on a Thermo Finnigan LCQ with electro spray ionization (ESI) in the negative mode (capillary temperature: 275 °C; source current: 100 μA; capillary voltage: −44 V; the tube lens offset: −53 V). The separation was achieved on an Agilent HP1100 HPLC system equipped with a LiChroCART (250 × 2 mm, Purospher STAR RP-18 endcapped, 5 μm). The samples were eluted at a flow of 0.380 ml min^{-1} with 80% solvent A (0.1% formic acid in water) and 20% solvent B (0.1 formic acid in acetonitrile) for 10 min.

2.3. General synthesis of the isotopic labeled 3-NPA and 3-NPA amides of amino acids

The synthesis of 3-[1−^{13}C,3−^{15}N]-nitropropionic acid was performed according to Baxter et al. (1985) using $Na^{15}NO_2$ to introduce a second stable isotope label.

The synthesis of the described amides of 3-NPA was performed using dicyclohexyl carbodiimide (DCC), 3-NPA, and free amino acids in an aqueous reaction medium (Neises and Steglich, 1978; Becker et al., 2015). For details see supplementary material (appendix I).

2.4. Subcellular fraction preparation

Gut tissue was cut open and washed with tris buffer, 50 mM tris base, 2 mM $MgCl_2$, and 250 mM sucrose according to Tiedtke et al. (1988). Washed gut tissue was mixed with 1 ml of homogenization buffer, prepared by mixing the tris buffer with 10 μl protease inhibitor mix (Serva) dissolved in phosphate buffer system (PBS), and 1 μl of 1 M dithiothreitol (DTT). Iron bits were added to the sample, which was homogenized using a GenoGrinder (SPEX SamplerPrep 2010) for 1 min at 1210 rpm. After homogenization, the debris was sedimented by centrifugation for 10 min at 500 g. Mitochondria were isolated by differential centrifugation, and microsomes were isolated by ultracentrifugation at 100,000 g (Firstenberg and Silhacek, 1973). Twice-washed mitochondria and microsomes were used in assays for the *in vitro* biosynthesis of 3-NPA amides of amino acids.

2.5. Assays for in vitro biosynthesis of 3-NPA amides of amino acids

Cytosolic proteins, and mitochondrial and microsomal fractions were added to 200 μl of homogenization buffer along with 1 mM coenzyme A (CoA), 25 mM adenosine triphosphate (ATP), 250 μg/ml glycine, and 250 μg/ml 3-NPA. The samples were incubated at room temperature for 20 h and lyophilized, and the residue was suspended in ethanol. The suspension was centrifuged, the supernatant was concentrated under reduced pressure, and the dry residue was taken up with MeOH and analyzed by LC-MS.

2.6. Assays for biosynthesis of 3-NPA amides in gut microorganisms

The gut content was separated from each insect's intestine, and diluted in 5 ml Todd-Hewitt-Bouillon (THB). 250 μg/ml 3-NPA and 250 μg/ml glycine was added, and the mixture was incubated overnight at 37 °C and shaken at 220 r.p.m. For cultivation under anaerobic conditions, the gut content in 2 ml THB was placed in a BBL GasPak anaerobic jar with Anaerocult A atmosphere generation kit (Merck)and incubated for 7 days at 27 °C in an anaerobic atmosphere composed of 18% CO_2 and traces of O_2.

3. Results

3.1. Toxicity of 3-nitropropanoic acid for S. littoralis by oral uptake

To study the susceptibility of *S. littoralis* larvae to the toxic host plant metabolite 3-NPA, feeding experiments were conducted.

3-NPA was added to the artificial diet of *S. littoralis* at 4.2 μmol 3-NPA per g (Chomcheon et al., 2005) to study the toxicological effects on the larvae. The body weight and length of *S. littoralis* larvae were determined over the time they fed on diet with 3-NPA

(experimental group) and without 3-NPA (control). Larvae of the experimental group grew significantly slower than those of the control group. After 16 days of feeding on the diet containing 3-NPA, a decrease of the average body length of 62% (Fig. 1B) and a decrease of the weight gain to 34% were observed (Fig. 1A). No effects on moulting or increase in mortality was detected in insects that fed on 4.2 μmol 3-NPA per g of the diet compared to insects in the control group.

To determine the overall resistance of *S. littoralis* to 3-NPA, increasing amounts of the toxin were added to the artificial diet of the larvae. After the application of 4.2, 8.4 16.8 and 33.6 μmol 3-NPA/g of artificial diet, the mortality of larvae in the experimental group was equal to that of larvae in the control group (data not shown). These data indicate that *S. littoralis* was highly resistant to 3-NPA upon feeding.

3.2. Toxicity of 3-nitropropanoic acid for S. littoralis after injection

The effect of directly injecting 3-NPA into the larval hemolymph of *S. littoralis* was studied. The following concentrations of buffered solutions of the toxin were injected: 8.4, 12.6, 16.8, 25.2, 33.6 μmol 3-NPA per g body weight. Mortality in insects fed with 3-NPA was lower than that in insects injected with ascending concentrations of 3-NPA (Fig. 2).

After injections of concentrations of 33.6 μmol 3-NPA per g body weight, all of the larvae died within 1 day, at concentrations of 12.6 μmol 3-NPA per g body weight 43% of the larvae survived (Fig. 2). These results show that *S. littoralis* larvae are more susceptible to injection than to oral administration of 3-NPA and suggest that *S. littoralis* is capable of detoxifying orally ingested 3-NPA.

3.3. Identification of 3-NPA derived metabolites

To study the detoxification of 3-NPA in the gut, the frass of larvae treated with 3-NPA was analyzed by HPLC-MS. Differential analysis

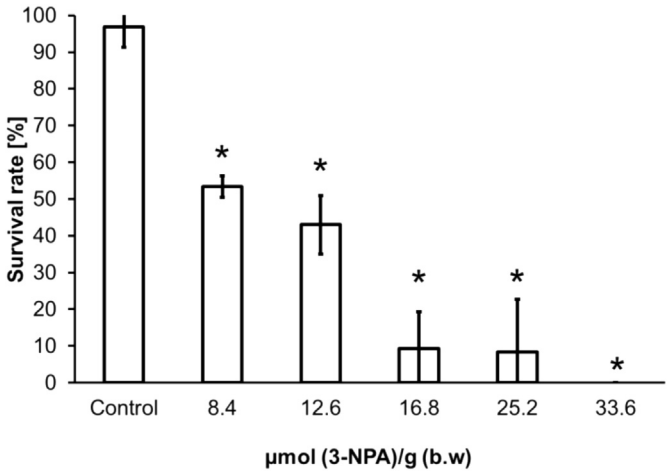

Fig. 2. Comparison of the survival rates of *S. littoralis* larvae determined 2 days after injection of increasing amounts of 3-NPA (one-way Anova, Dunnett's post test, $^{*}p < 0.05$; ±1 SD, n = 3).

of the frass of treated larvae versus the frass of untreated control revealed a 3-NPA-dependent signal that might represent a metabolite derived from 3-NPA (Fig. 3A). Its mass spectrum showed a pseudo molecular ion at *m/z* 175 [M−H] along with signals for the formic acid adduct and the dimer. HR-MS/MS analysis revealed the formula $C_5H_7N_2O_5^-$, which suggested an amide-bound glycine conjugate of 3-NPA (**1**) (Fig. S1). To unambiguously establish that the detected compound originates from 3-NPA, the stable isotope labeled [1−^{13}C,3−^{15}N]-3-nitropropanoic acid was synthesized and injected into the hemolymph of *S. littoralis* larvae. HPLC-MS analysis of the putative 3-NPA metabolite in the insect frass revealed a mass shift of Δ*m/z* +2 for the molecular ion along with Δ*m/z* +1 for the fragment ion originating from neutral loss of $^{15}HNO_2$ and confirmed the structure assignment of (3-nitropropanoyl)glycine **1**

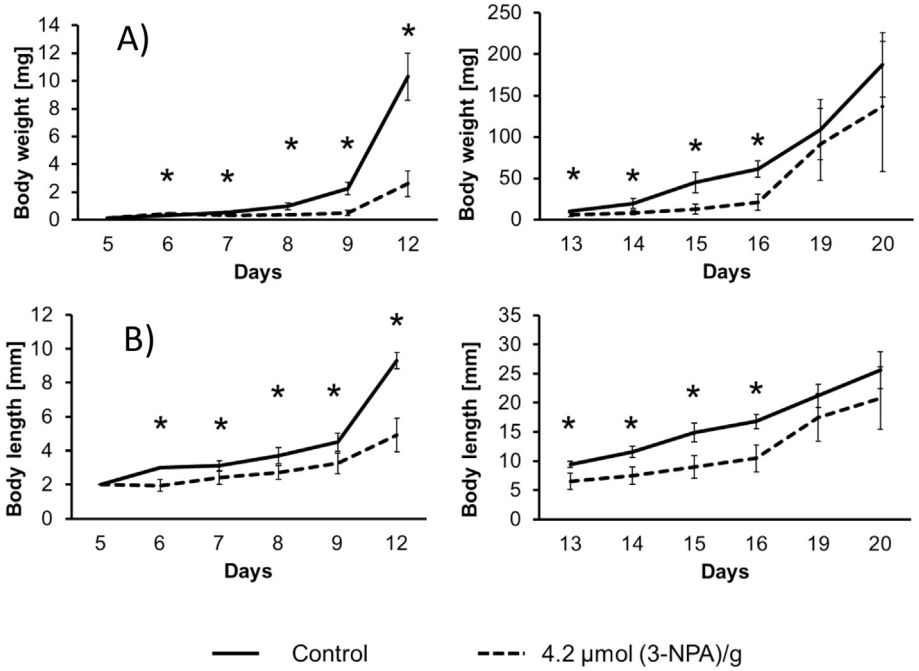

Fig. 1. Effect of the plant secondary metabolite 3-nitropropionic acid (3-NPA) on the body weight (A) and length (B) of *S. littoralis* larvae over time compared to the control (one-way Anova, Dunnett's post test, $^{*}p < 0.05$; ±1 SD, n = 20).

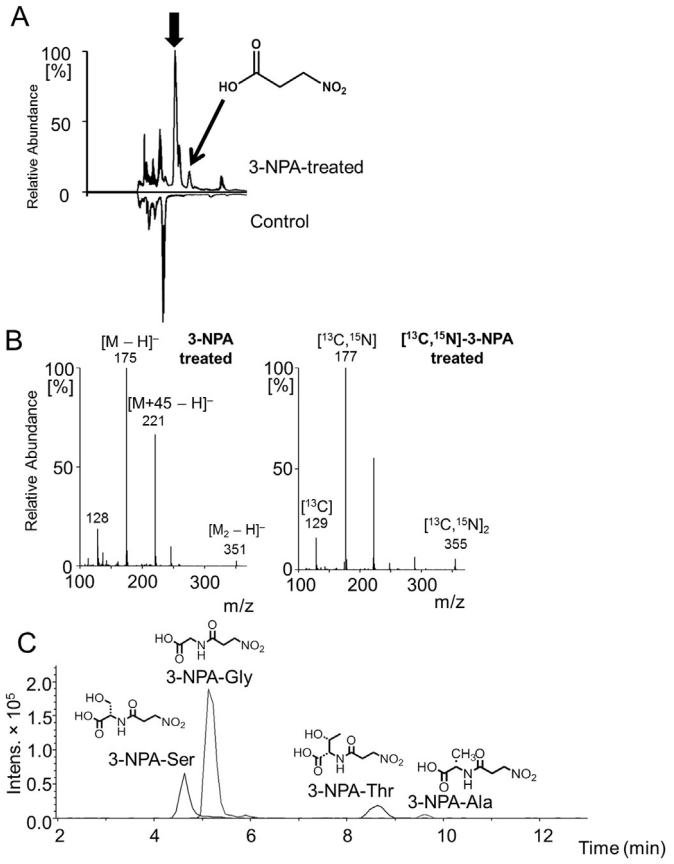

Fig. 3. (A): differential HPLC-MS analysis of the frass of 3-NPA treated and untreated control larvae; (B): Mass spectra of natural (3-nitropropanoyl)glycine **1** from *S. littoralis* after feeding on diet containing 3-NPA or [1−^{13}C,3−^{15}N]-3-NPA; (C): 3-NPA-amino acid conjugates **1**–**4** detected in the frass of larvae fed on diet containing 3-NPA.

(Fig. 3B). Screening for similar amino acid conjugates, we detected three additional differential signals in the chromatograms of the larval frass after the larvae had been injected with 3-NPA (Fig. 3C). Tandem- and HRMS analyses provided molecular formulas corresponding to amides of 3-NPA and alanine **2**, serine **3** as well as threonine **4**. The chromatograms of the frass of larvae treated with isotopically labeled 3-NPA showed a mass shift Δ*m/z* of +2. Consequently, amides of 3-NPA with glycine, alanine, and serine as well as threonine were synthesized and shown to be identical to the natural components (see supplemental material Fig. S2).

3.4. Toxicity experiments using amino acid amides *1*–*4*

To determine the toxicity of the amino acid amides **1**–**4** to *S. littoralis*, solutions of the synthetic standards were injected into the larval hemolymph. The application of compounds **1**–**4** at 25.2 μmol per g body weight showed a survival rate of ca. 95%, whereas after injection of 3-NPA at the same concentration only 8% of the larvae survived (Fig. 4). The control group showed a survival rate of 95%. These results clearly indicate that in contrast to free 3-NPA, the amino acid amides **1**–**4** are not toxic when injected into *S. littoralis* larvae. This result confirms our assumption that the conjugation of 3-NPA to an amino acid represents an efficient detoxification pathway in *S. littoralis*. Moreover, the non-toxic compounds **1**–**4** could also be detected in the hemolymph after insects fed on an artificial diet containing 12.6 μ mol/g 3-NPA. The conjugates were also found in the frass after insects were injected with 8.4 μ mol 3-NPA per g body weight (data are not shown).

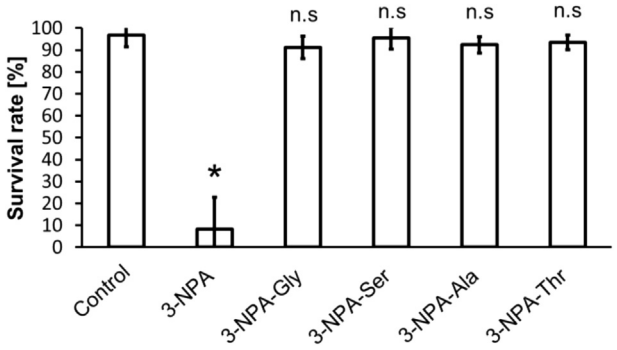

Fig. 4. Survival of *S. littoralis* larvae. Survival was determined 2 days after the injection of 3-NPA and 3-NPA amino acid conjugates at 25.2 μmol/g body weight. (one way Anova, Dunnett's post test, *p < 0.05; n.s. = no significant difference; ± 1 SD, n = 3).

3.5. Determination of detoxification activity in the gut tissue

The formation of amino acid amides of 3-NPA has been shown to be a major detoxification reaction of this plant toxin in *S. littoralis* larvae. To determine where the detoxification occurs after insects feed on 3-NPA containing diet, different parts of the insect were isolated and incubated with 3-NPA and glycine.

At first, the gut content was isolated to investigate any conjugation activity of the gut microbiota. For this purpose, the gut liquid was separated from gut tissue and incubated for 24 h with a mixture of 3-NPA and glycine under aerobic or anaerobic conditions. However, none of the conjugation products could be detected, indicating that the gut microbes are not responsible for detoxifying 3-NPA in *S. littoralis*.

The enzymes that catalyze the amide formation may also be excreted by the insect's epithelial tissue into the gut lumen. To study whether the amide formation is carried out by secreted enzymes, the regurgitate of the larvae was collected and incubated with 3-NPA and glycine for 24 h; however, the conjugates were not detected (Fig. 5A). Since incubation experiments with the gut liquid failed, the tissue of the gut cells was separated from the gut content and adjacent tissues, homogenized, and the debris was filtered. 3-NPA, glycine, adenosine triphosphate (ATP), and coenzyme A (CoA) were added to the filtrate, and after 24 h of incubation, the 3-NPA-glycine conjugate **1** could be detected by LC-MS. This finding indicates that the conjugation activity is closely associated with the gut tissue. In addition, the fat body was separated from the other tissue and shown to catalyze the production of amino acid amides (Fig. 5B). To determine the conjugation activity in different subcellular organelles, the gut tissue was homogenized and fractionated. After centrifugation, the mitochondrial fraction was separated from the supernatant. Glycine and 3-NPA were added to both samples along with adenosine triphosphate (ATP), and coenzyme A (CoA). After incubation for 24 h, the 3-NPA-glycine conjugate **1** was detected in the supernatant but not in the mitochondrial fraction (Fig. 5C). To determine if the corresponding enzymes are present in the cytosol or in vesicles, cytosolic proteins were separated from the microsomes by ultracentrifugation. After the addition of 3-NPA, glycine, ATP, and CoA to both fractions and incubation for 24 h, compound **1** was detected in the cytosolic fraction (Fig. 5D). This finding shows that the enzymes involved in 3-NPA detoxification are present in the cytosol of epithelial cells in *S. littoralis*.

4. Discussion

The application of biologically relevant concentrations of toxic 3-NPA were shown to significantly reduce the growth rate of

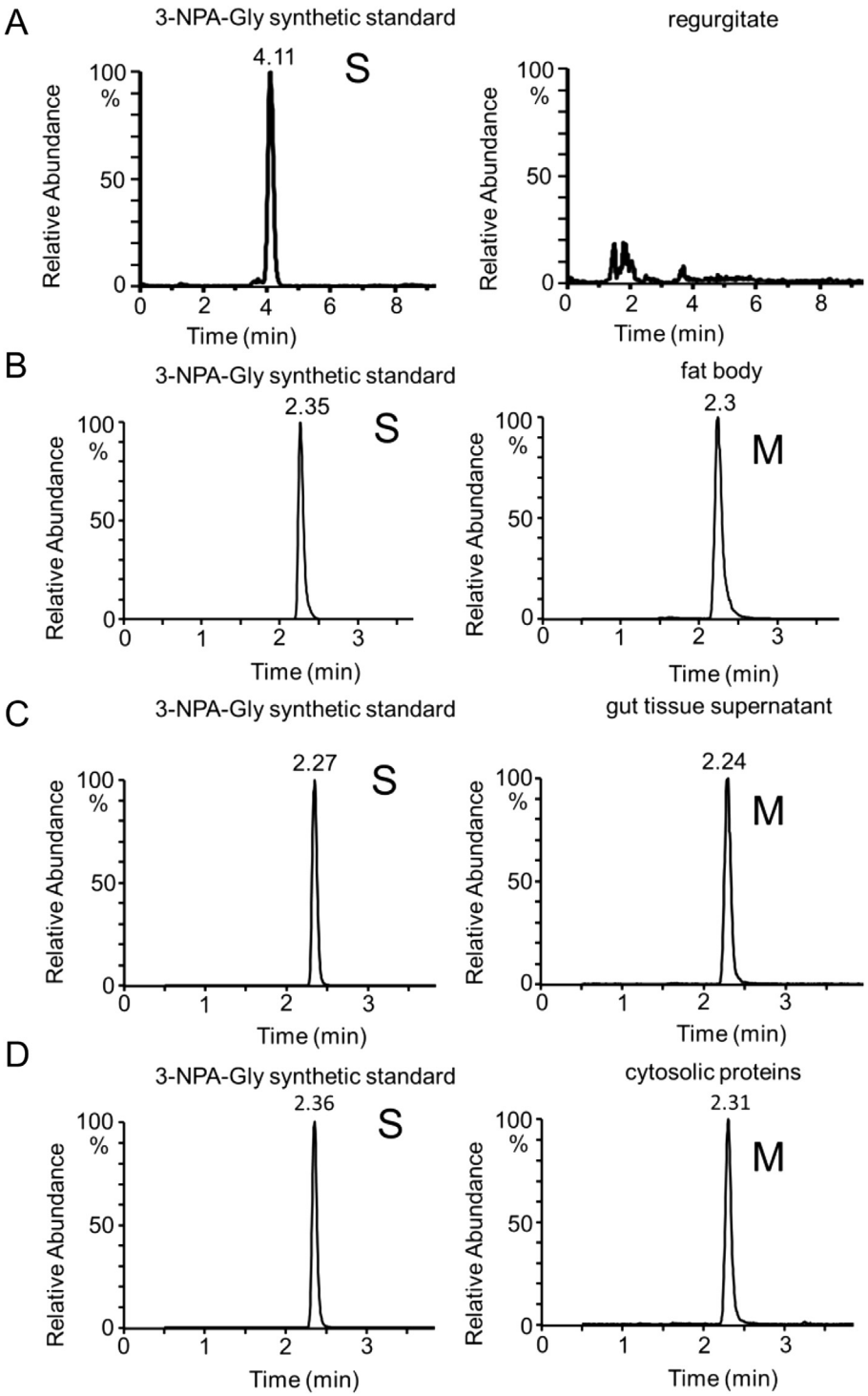

Fig. 5. The regurgitate (A), fat body tissue (B), and crude homogenized gut tissue (C), and isolated cytosolic proteins (D) were tested for conjugation activity. Samples were incubated with 3-NPA, glycine, ATP, and CoA for 24 h. The conjugate of 3-NPA with glycine was analyzed by LC-MS. (S- synthetic standard, M-metabolite).

S. littoralis larvae (Fig. 1A, B). Inhibition of larval growth by secondary metabolites is a frequent strategy how plants defend themselves against herbivores (Ibanez et al., 2012; Byers et al., 1977). Observed growth rate reduction by 3-NPA in *S. littoralis* suggests that 3-NPA, in fact, inhibits the growth of Lepidoptera (Cipollini et al., 2008; Wang et al., 2010).

Although larval growth is decreased after *S. littoralis* feeds on 3-NPA (Fig. 1A, B), its ingestion is not lethal; concentrations of 3-NPA up to 33.6 μ mol/g did not result in higher mortality compared to insects in the control group, demonstrating that *S. littoralis* is highly resistant to oral uptake of 3-NPA. In contrast, injecting the same amounts of 3-NPA into the insect's hemolymph resulted in mortality and death, suggesting that the total detoxification capacity of *S. littoralis* is based on conjugation of 3-NPA in the gut epithelium and the fat body.

Comparative analysis of the frass of *S. littoralis* larvae fed on a

diet with or without 3-NPA revealed the presence of new components shown to originate from 3-NPA by feeding stable isotope labeled precursors. Molecular structures of these 3-NPA conjugation products were deduced from HR-MS/MS data and confirmed by total synthesis (see supporting information). In the frass of 3-NPA fed *S. littoralis* larvae amino acid amide of 3-NPA was detected, but no glutathione derivative was detected. 3-Nitropropanoyl glycine (**1**) and 3-nitropropanoyl serine (**3**) have previously been described as metabolites in the grasshopper *M. bivittatus*. The grasshopper also produces a glutamine derivative which is absent in *S. littoralis* (Majak et al., 1998). Homologous 3-nitropropanoyl alanine (**2**) and (3-nitropropanoyl) threonine (**4**) were exclusively detected in *S. littoralis* and represent novel compounds. Although 3-NPA amino acid conjugates have previously been identified as putative detoxification products from *M. bivittatus*, no data concerning the toxicity of these compounds have been reported (Majak et al., 1998). We found that in *S. littoralis* the injection of the amides **1**–**4** into the hemolymph is not toxic. However, the injection of the same amount of free 3-NPA causes mortality in 92% of the larvae (Figs. 4 and 5) demonstrating that conjugation with amino acids represents an efficient way to cope with 3-NPA. Amino acid conjugation represents a common detoxification mechanism in insects and, as such, is similar to glycosylation or reaction with glutathione (Schramm et al., 2012) which force excretion.

Conjugation to amino acids is well known from Lepidoptera. Glycine conjugates have been described as products of glucosinolate metabolism (Stauber et al., 2012).

Conjugation of long chain fatty acids to glutamine is mainly facilitated by gut tissue or membrane-associated enzymes (Alborn et al., 1997; Lait et al., 2003; Yoshinaga et al., 2005). However, occasionally the insect gut microbial community exhibited conjugation activity, as in the case of *N*-linolenoyl glutamine biosynthesis by *Microbacterium arborescens* in *Spodoptera exigua* (Ping et al., 2007). In *S. littoralis*, however, no 3-NPA conjugation activity of the gut microbes under aerobic or anaerobic conditions has been detected (Fig. 5). In contrast, cytosolic proteins, isolated from the *S. littoralis* gut tissue, were shown to catalyze the formation of 3-NPA amino acid amides in the presence of ATP and CoA (Figs. 4 and 5C). Dedicated enzymes catalyzing the conjugation of 3-NPA to amino acids from this or other organisms have not been described previously.

Taken together, these results indicate that amino acid conjugation via amide formation represents an important detoxification pathway in *S. littoralis*. Although the exact mechanisms of 3-NPA conjugation remains to be elucidated our results allow us to propose a functional model.

The epithelial cells protect the larval hemolymph from the free toxin taken up with the food (Fig. 6), as was previously shown for tannins (Appel and Michael, 1990; Engel and Moran, 2013; Giordana et al., 1989). Due to the high consumption rate of *S. littoralis* (consumed diet passes the gut within 2 h), larvae may accumulate a large amount of 3-NPA in the gut that can pass the membrane barriers via different membrane carriers (Lamp et al., 2011). Once taken up by the epithelium, as-yet unidentified cytosolic proteins detoxify 3-NPA by converting it to non-toxic amino acid conjugates. From the cytosol, the detoxification products **1**–**4** can be transported back into the gut lumen via apocrine secretion or passed onto the hemolymph (Cristofoletti et al., 2001; Ratzka et al., 2002; Terra and Ferreira, 1994; Wittstock et al., 2004) (Fig. 6). Compounds **1**–**4** were detected in the hemolymph after insects fed on a high concentration of 3-NPA (12.6 μ mol/g of artificial diet weight). In addition, compounds **1**–**4** were detected in the frass of the larvae after sublethal doses of 3-NPA (8.4 μ mol 3-NPA per g body weight) were injected into the hemolymph. When the toxin reaches the hemolymph after larvae have consumed high amounts of plant material, the fat body is also able to produce amino acid amides. Once amino acid amides are produced, they can be transported from the hemolymph to the hind gut via the malpighian tubules and finally excreted from the system.

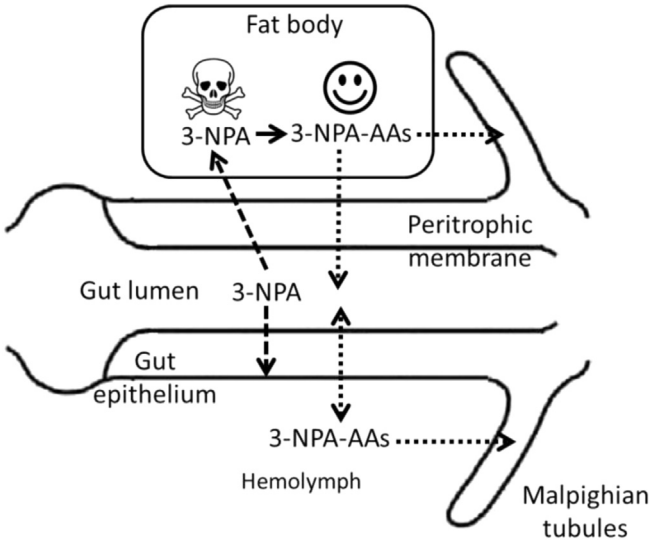

Fig. 6. Detoxification of 3-NPA and flux of metabolites in *S. littoralis*. 3-NPA can pass through membranes via different carriers. In the epithelial cells, the detoxification of 3-NPA proceeds via conjugation to amino acids by as-yet unidentified cytosolic proteins. 3-NPA conjugates are exported to the hemolymph, or retransported into the gut lumen by unknown mechanims. Also the fat body is able to produce amino acid amides. Conjugates reaching the hemolymph may be excreted via the malpighian tubules.

In conclusion, we demonstrated that the formation of 3-NPA amides of glycine, serine, threonine and alanine by cytosolic enzymes of the insect epithelium represents an important detoxification pathway in *S. littoralis*. Details of 3-NPA metabolism and the responsible enzymes in *S. littoralis* remain to be identified.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2015.05.013.

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