ORIGINAL ARTICLE

Potential of the bean α-amylase inhibitor αAI-1 to inhibit α-amylase activity in true bugs (Hemiptera)

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Abstract

True bugs (Hemiptera) are an important pest complex not controlled by Bt-transgenic crops. An alternative source of resistance includes inhibitors of digestive enzymes, such as protease or amylase inhibitors. αAI-1, an α-amylase inhibitor from the common bean, inhibits gut-associated α-amylases of bruchid pests of grain legumes. Here we quantify the *in vitro* activity of α-amylases of 12 hemipteran species from different taxonomic and functional groups and the *in vitro* inhibition of those α-amylases by αAI-1. α-Amylase activity was detected in all species tested. However, susceptibility to αAI-1 varied among the different groups. α-Amylases of species in the Lygaeidae, Miridae and Nabidae were highly susceptible, whereas those in the Auchenorrhyncha (Cicadellidae, Membracidae) had a moderate susceptibility, and those in the Pentatomidae seemed to be tolerant to αAI-1. The species with αAI-1 susceptible α-amylases represented families which include both important pest species but also predatory species. These findings suggest that αAI-1-expressing crops have potential to control true bugs *in vivo*.

Introduction

True bugs (Hemiptera) are characterized by their piercing–sucking mouthparts and extra-oral digestion. When feeding on crops, Hemiptera cause not only direct damage but also indirect damage by vectoring numerous plant pathogens, making them an important challenge for insect pest management (Chougule and Bonning 2012). Control of hemipteran pests has received growing attention, because the increasing adoption rate of insect-resistant genetically modified (GM) crops expressing lepidopteran- or coleopteran-active Cry toxins from *Bacillus thuringiensis* (Bt) and the associated decline in broadband synthetic insecticide applications in those crops may promote the rise of previously co-controlled secondary hemipteran pests (Naranjo 2011; Wilson et al. 2013). For example, the wide-scale adoption of Bt cotton in China

increased the likelihood of mirid (Hemiptera: Miridae) outbreaks in cotton and surrounding crops (Lu et al. 2010).

One approach to protect crops against hemipteran pests within integrated pest management strategies might be growing hemipteran-resistant transgenic crops. Despite the promise of hemipteran-active Bt toxins under development (Baum et al. 2012; Chougule et al. 2013), alternative gene sources should be considered as well. In their recent review, Chougule and Bonning (2012) discuss the use of lectins or protease inhibitors as likely control candidates. A group of plant-derived insecticidal compounds not considered in this review are α-amylase inhibitors. In insects, α-amylases are the only enzymes known to act preferentially on long α-1,4-glucan chains such as starch (Terra et al. 1996). These enzymes have been detected in various Hemiptera (e.g. Zeng and Cohen

2000a,b; Boyd et al. 2002), suggesting that α-amylase inhibitors might be an option for the control of hemipteran pests.

The α-amylase inhibitor αAI-1 from the common bean, *Phaseolus vulgaris*, has been studied extensively, and the gene coding for the inhibitor has been transferred into other legumes to confer resistance against bruchid beetles (Coleoptera: Chrysomelidae) (Shade et al. 1994; Ishimoto et al. 1996; Sarmah et al. 2004; Ignacimuthu and Prakash 2006; Solleti et al. 2008; Lüthi et al. 2013a) and into Arabica coffee, *Coffea arabica*, to confer resistance against the coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) (Barbosa et al. 2010).

In this study, we screened the *in vitro* activity of α-amylases from 12 Hemiptera species representing six families, and we measured the inhibition of the α-amylases by plant-derived αAI-1. Because α-amylase activity has also been documented in predatory Hemiptera (Zeng and Cohen 2000a,b), which are an important group of non-target insects that could be affected, we included both herbivorous and predatory species. We also included larvae of the bruchid *Callosobruchus chinensis* (Coleoptera: Chrysomelidae), which are highly susceptible to αAI-1 (Ishimoto and Kitamura 1989).

Materials and Methods

Insect samples

Twelve Hemiptera species from six families were included in this study: eight species are pests and four are considered generalist predators (table 1). In China, nymphs and adults of *Adelphocoris fasciaticollis*, *Adelphocoris lineolatus*, *Adelphocoris suturalis* and *Apolygus lucorum* (Hemiptera: Miridae) were collected in cotton fields at the experimental station of the Institute of Plant Protection, CAAS, near Langfang City, Hebei Province (39.5°N; 116.7°E). Nymphs and adults of *Cyrtorhinus lividipennis* (Hemiptera: Miridae) were collected in rice fields near Xiaogan City, Hubei Province (30.56°N; 113.54°E). In the USA, adults of *Empoasca solana* (Hemiptera: Cicadellidae), *Geocoris pallens*, *Geocoris punctipes* (Hemiptera: Geocoridae), *Nabis alternatus* (Hemiptera: Nabidae) and *Spissistilus festinus* (Hemiptera: Membracidae) were collected in cotton and alfalfa fields at the University of Arizona, Maricopa Agricultural Center farm (33.07°N; 111.97°W) in Maricopa, Arizona. Nymphs and adults of *Bagrada hilaris* (Hemiptera: Pentatomidae) were obtained from a greenhouse colony reared on broccoli at the USDA-ARS facility in Maricopa, Arizona. Upon transport from the field to the laboratory, living insects were frozen at −20°C, shipped to Switzerland on dry ice and stored at −20°C. *Euschistus heros* (Hemiptera: Pentatomidae) nymphs reared on soybean and adults reared on peanut of were provided by Syngenta AG (Stein am Rhein) from their colony. For comparison, third and fourth instar larvae of the αAI-1 susceptible bruchid beetle *C. chinensis*, taken from a colony reared on chickpea in our laboratory, were included in the study. The sex of the specimens and the instars of immature specimens were not identified. However, only later-instar nymphs (i.e. at least 3rd instar) were included.

Chemicals

The Megazyme Ceralpha assay kit and additional blocked *p*-nitrophenyl maltoheptasoide were purchased from Megazyme (Wicklow, Ireland). Diethylaminoethyl (DEAE) ion exchange and size fractionation column matrices were purchased from GE Healthcare (Upsala, Sweden). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Characterization of α-amylase activity

When nymphs and adults were available, the different stages were analysed separately. At least 15 specimens for each species were used to produce the whole-body extracts. Frozen samples of the same species and stage were pooled and homogenized in prechilled (4°C) 0.15 M NaCl. Homogenates were centrifuged at 10 000 *g* at 4°C for 5 min, and the supernatants were pooled and stored at −20°C. Total protein content was determined according to the Bradford (1976) method using bovine serum albumin (BSA) as the standard. Protein concentration in all samples was adjusted to 7 ± 2 µg/µl.

A pH range from 4.0 to 8.0 was generated using different buffers [0.1 M malate (pH 4.0–5.5), 0.1 M maleate (5.5–6.5) and 0.1 M Bis-Tris propane (6.5–8.0)]. All buffers contained 0.15 M NaCl and 5 mM $MgCl_2$. Assays were performed using a modified version of the Megazyme Ceralpha assay (McCleary et al. 2002) in 96-well microplates with a standard assay volume of 160 µl, which contained 2 µl of the insect protein extract, 108 µl of buffer and 10 µl of blocked *p*-nitrophenyl maltoheptasoide (BPNPG7) (27.25 µg/µl) as the specific substrate. Depending on their α-amylase activity, samples were incubated between 45 min and 24 h at 37°C to obtain *p*-nitrophenol concentrations within the quantifiable range (total amount of protein used and incubation time for each extract are

Table 1 Optimal pH and specific α-amylase activity (mg of *p*-nitrophenol released from BPNPG7/min/mg protein) of the various Hemiptera species and stages, and *in vitro* inhibition of the α-amylases by αAI-1 expressed as IC_{50} and IC_{90}. IC_{50} and IC_{90} values lower than in the reference species *Callosobruchus chinensis* are in bold

Source of extract				α-Amylase activity		Inhibitory concentration[3]	
Family	Species	Functional group	Stage[1]	Optimal pH	Specific activity[2]	IC_{50}	IC_{90}[4]
Cicadellidae	*Empoasca solana*	Herbivore	A	6.5	1.99±0.04	**0.67**	**(2.35)**
Lygaeidae	*Geocoris pallens*	Predator	A	5.5	8.54±0.09	**0.12**	**0.42**
	Geocoris punctipes	Predator	A	5.5	13.01±0.01	0.99	n.d.
Membracidae	*Spissistilus festinus*	Herbivore	A	6.5	0.50±0.01	2.78	(76.06)
Miridae	*Adelphocoris fasciaticollis*	Herbivore	N	5.5	5.46±0.25	**0.60**	**(2.18)**
			A	5.5	5.46±0.46	0.93	**(2.99)**
	Adelphocoris lineolatus	Herbivore	N	5.5	8.34±0.27	**0.23**	**1.87**
			A	5.0	4.25±0.03	**0.20**	**(2.08)**
	Adelphocoris suturalis	Herbivore	N	5.0	8.25±0.05	**0.17**	**(2.50)**
			A	5.0	10.58±0.22	**0.22**	**(3.96)**
	Apolygus lucorum	Herbivore	N	5.0	9.21±0.02	**0.34**	**(3.31)**
			A	5.5	7.78±0.05	**0.16**	**(4.03)**
	Cyrtorhinus lividipennis	Predator	N	6.0	12.43±0.11	**0.32**	**(3.13)**
			A	6.0	5.70±0.10	**0.35**	**(6.63)**
Nabidae	*Nabis alternatus*	Predator	A	6.0	9.95±0.58	**0.26**	**0.83**
Pentatomidae	*Bagrada hilaris*	Herbivore	N	5.5	9.71±0.18	n.i.	n.i.
			A	5.5	3.29±0.04	n.i.	n.i.
	Euschistus heros	Herbivore	N	6.5	7.44±0.10	n.i.	n.i.
			A	6.0	40.02±1.47	n.i.	n.i.
Chrysomelidae	*Callosobruchus chinensis*	Herbivore	L	6.5	30.29±0.89	0.82	7.2

[1]A, adult; N, nymph; L, larva.

[2]Values are means ± SE of triplicate measurements from a unique pooled extract. Where the optimal pH is at an intersection of two buffers, the higher of the measured values is given.

[3]μg αAI-1/ml, n.d., not determinable by ReaderFit software using the provided data; n.i., not inhibited.

[4]IC_{90} values in parentheses are beyond the highest αAI-1 concentration tested and were calculated using the equation of the best fit model determined by ReaderFit software.

provided in Table S1). The reaction was stopped by adding 50 μl of 5% Tris (unbuffered). Absorbance was read at 405 nm at room temperature with a Spectrafluor Plus plate reader (Tecan, Männedorf, Switzerland). α-Amylase activity was quantified as mg of *p*-nitrophenol released from BPNPG7/min/mg protein. All enzymatic assays were carried out in triplicate from a unique pool of extract, and appropriate blanks were used to account for spontaneous breakdown of substrates.

In vitro inhibitory activity of αAI-1

Transformation of cowpea expressing αAI-1 has been described elsewhere (Popelka et al. 2006; Higgins et al. 2013). αAI-1 was purified from seeds of GM cowpea of the variety Sasaque as described by Marshall and Lauda (1975) with slight modifications. Proteins were extracted in a 1% NaCl solution. The solution was centrifuged, and the supernatant was collected and incubated in a water bath at 70°C for 30 min. The solution was centrifuged and the supernatant was collected. Unwanted protein was precipitated by adding ammonium sulphate to make up 20% (w/v). After stirring and centrifugation, the supernatant was collected and made up to 55% (w/v) ammonium sulphate to precipitate αAI-1. The pellet was collected and re-suspended in deionized water. All subsequent purification steps were carried out at 4°C. The protein in the 20–50% ammonium sulphate cut was dialysed against water containing 0.2% sodium azide and centrifuged, and the supernatant was retained. This was dialysed further against 10 mm citrate/phosphate buffer for loading onto a DEAE-Sepharose CL-6B ion exchange column. Unbound protein was washed out with 10 mm citrate/phosphate buffer. Bound protein was eluted using a salt gradient of 100–500 mm NaCl in 10 mm citrate/phosphate buffer. Fractions of the outflow containing αAI-1 were identified using the Megazyme Ceralpha assay

as described above. The αAI-1-containing fractions were pooled, dialysed against water containing 0.2% sodium azide, freeze-dried, resuspended in 50 mM acetate buffer (pH = 5) with 5 mM $CaCl_2$ and fractionated on a Sephadex S-200 column. Collected outflow fractions containing αAI-1 were identified as above, pooled, dialysed against water containing 0.2% sodium azide, freeze-dried, re-suspended again in acetate buffer and fractionated on a Sephadex S-25 column. Collected outflow fractions containing αAI-1 were identified as above and run separately on a 15–25% PAGE gel. Fractions highly enriched in αAI-1 were pooled, dialysed against water containing 0.2% sodium azide, freeze-dried and stored at −20°C.

For calculation of IC_{50} and IC_{90} values (the concentrations of αAI-1 required to inhibit the maximal α-amylase activity by 50 and 90%, respectively), dose–response curves using six αAI-1 concentrations ranging from 0 to 2 μg/ml were constructed. For *G. punctipes*, *S. festinus*, *B. hilaris*, *E. heros* and *C. chinensis*, additional concentrations up to 8 μg/ml were tested. The α-amylase activity of the different extracts was assayed at their respective optimal pH values, and the extracts were incubated with αAI-1 for 15 min at 37°C before adding BPNPG7. The software ReaderFit (Advanced Desktop version, Hitachi Solutions America Ltd., San Francisco, CA) was used to construct dose–response curves and to calculate IC_{50} and IC_{90} values.

Results

Characterization of α-amylase activity

α-Amylase activity was detected in whole-body extracts of all species assayed (table 1). The qualitative pattern of the α-amylase activity as a function of pH was similar in all extracts. α-Amylase activity was nearly zero at pH 4.0, low at pH 4.5 but quickly increased and reached their maximum at pH 5.0–6.5 (fig. 1). Beyond the optimal pH, the activity continuously decreased to pH 8.0. The pH supporting maxi-
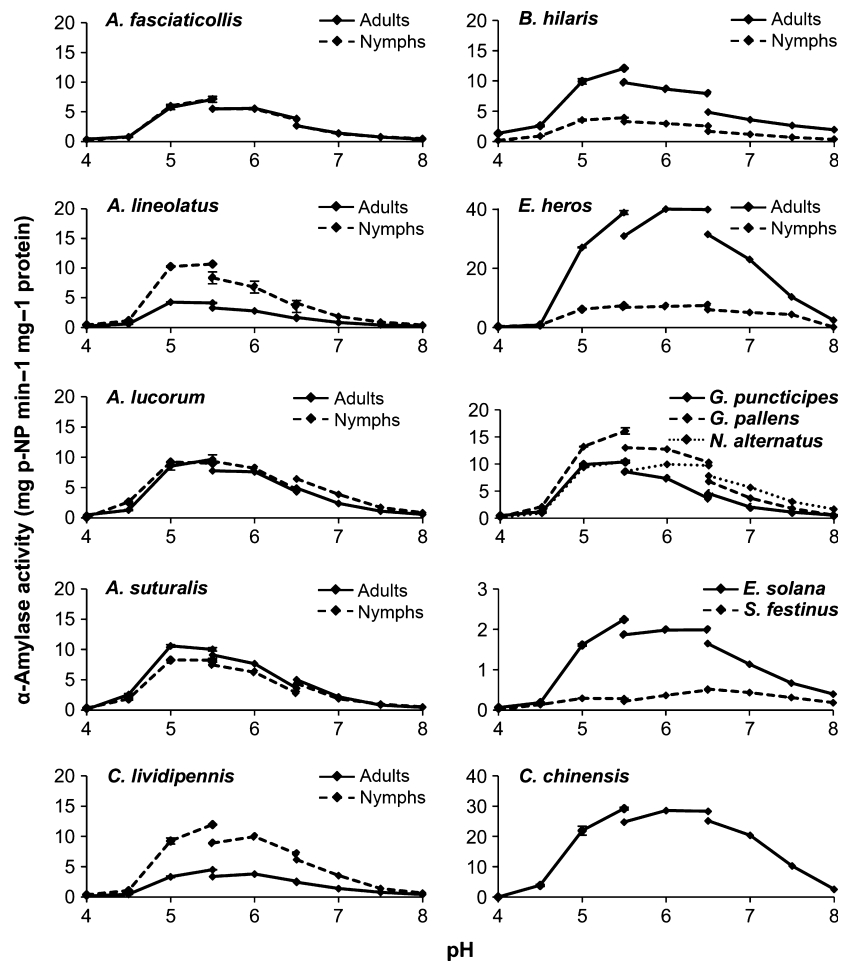

Fig. 1 α-Amylase activity of insect extracts as affected by pH. A pH range from 4.0 to 8.0 was generated using different buffers (malate: pH = 4.0–5.5, maleate: pH = 5.5–6.5, Bis-Tris propane: pH = 6.5–8.0). N = nymph, A = adult. Where no stage is indicated, adults were assayed (except that larvae were assayed for *Callosobruchus chinensis*). Note the differences in the y-axes scaling.

mal α-amylase activity never differed by more than 0.5 units for nymphs and adults of the same species and never by more than 1.0 unit within the same family.

α-Amylase activity ranged from 0.5 to 40.0 mg of *p*-nitrophenol released from BPNPG7/min/mg protein (table 1). α-Amylase activity was lower in the hempiteran extracts than in the extract from the reference species, *C. chinensis*. The only exception was the α-amylase extract from *E. heros* adults that had a similar activity to *C. chinensis*. α-Amylase activity of the extracts was similar within the families Lygaeidae and Miridae and within the groups of Auchenorrhyncha and the predatory species. α-Amylase activity of the extracts differed greatly between species and also between nymphs and adults of the two pentatomids. However, the difference in the α-amylase activity between life stages of a species seemed to be species specific.

In vitro inhibitory activity of purified αAI-1

α-Amylase activity was inhibited by αAI-1 in all extracts with the exception of those of the two pentatomids, *B. hilaris* and *E. heros* (table 1). Concentrations of αAI-1 as high as 8 µg/ml did not inhibit α-amylase activity in extracts of adults or nymphs of the pentatomids. Inhibition of α-amylase activity was also low in extracts of *S. festinus* and *G. punctipes*. Extracts of *A. fasciaticollis* nymphs and adults and *E. solana* had relatively high IC_{50} values but low IC_{90} values. In all other extracts, inhibitory activity of αAI-1 was greater than for the *C. chinensis* extract, which served as the highly susceptible control. Inhibitory activity was highest in the extracts of *G. pallens* and *N. alternatus* (fig. 2).

Discussion

α-Amylase activity of Hemiptera has been mainly studied to understand hemipteran diversity and flexibility in feeding habits (Cohen 1996, 1998; Zeng and Cohen 2000a,b,c; Boyd et al. 2002), but this important group of digestive enzymes might also be a potential target for pest management. Our results demonstrate that α-amylase activity is present in the whole-body extracts of all species in our study regardless of their taxonomic or functional group. The pH profile of activity was similar in all extracts, and the optimal pH ranged from 5.0 to 6.5, which is consistent with reports of other Hemiptera species (Zeng and Cohen 2000c; Kluh et al. 2005; Ramzi and Hosseininaveh 2010) and is in the typical pH range of gut

contents of Hemiptera (Terra and Ferreira 1994). Given that the optimal pH for the formation of the enzyme–inhibitor complex (α-amylase-αAI-1) is 5.0–6.0 (Powers and Whitaker 1978), αAI-1 seems to be very well suited for the potential use against hempiteran pests in GM crops.

Whereas the pH profile of α-amylase activity was similar in all extracts, its levels varied considerably. Extracts of nymphs and adults of all mirid species had similar levels of activity. Extracts of the adults of the three predator species in the Lygaeidae and Nabidae investigated had activity levels in the same range as the mirids. In contrast, the α-amylases in the extracts of the two Auchenorrhyncha, *E. solana* and *S. festinus*, had the lowest activity. These are only two species, however, in a large taxonomic group that contains many pests; determining whether Auchenorrhyncha in general possess a low level of α-amylase activity will require the assay of additional species. The variation among species was greatest between the two pentatomids. Adults of the pentatomid *E. heros* had the highest α-amylase activity of all extracts tested, even higher than for the bruchid *C. chinensis*. Bruchid beetles are well known to require these enzymes to obtain energy and metabolic water from carbohydrates (Southgate 1979; Murdock et al. 2012). Furthermore, the α-amylase activity was more than five times higher in the extract of adult *E. heros* than in that of the nymphs. Although the nymphs and adults were reared on different hosts (soybeans and peanuts, respectively), it is unlikely that this difference in activity is due to an adaptation to the different food source because both sources have similar carbohydrate levels and are rich in protein and lipids. Remarkably, *in vitro* α-amylase activity also differed greatly in adult vs. nymph of the second pentatomid, *B. hilaris*, but in the other direction: activity was higher in the extract of nymphs than in that of adults. Here, both life stages were taken from the same greenhouse host. In the case of the mirids, *in vitro* activity was similar for nymphs and adults of the herbivorous species but differed substantially for nymphs vs. adults of the predatory species *C. lividipennis*. These findings suggest that differences in the digestive systems of nymphs and adults might be species specific.

Agusti and Cohen (2000) suggested that the high α-amylase activity in the saliva of two herbivorous mirids, *Lygus lineolaris* and *Lygus hesperus* (Hemiptera: Miridae), indicates an adaptation to exploit starch from plant material via extra-oral digestion. Zeng and Cohen (2000a) reported that the α-amylase activity was dramatically lower in the salivary gland of the predator *G. punctipes* than in those of the herbivorous
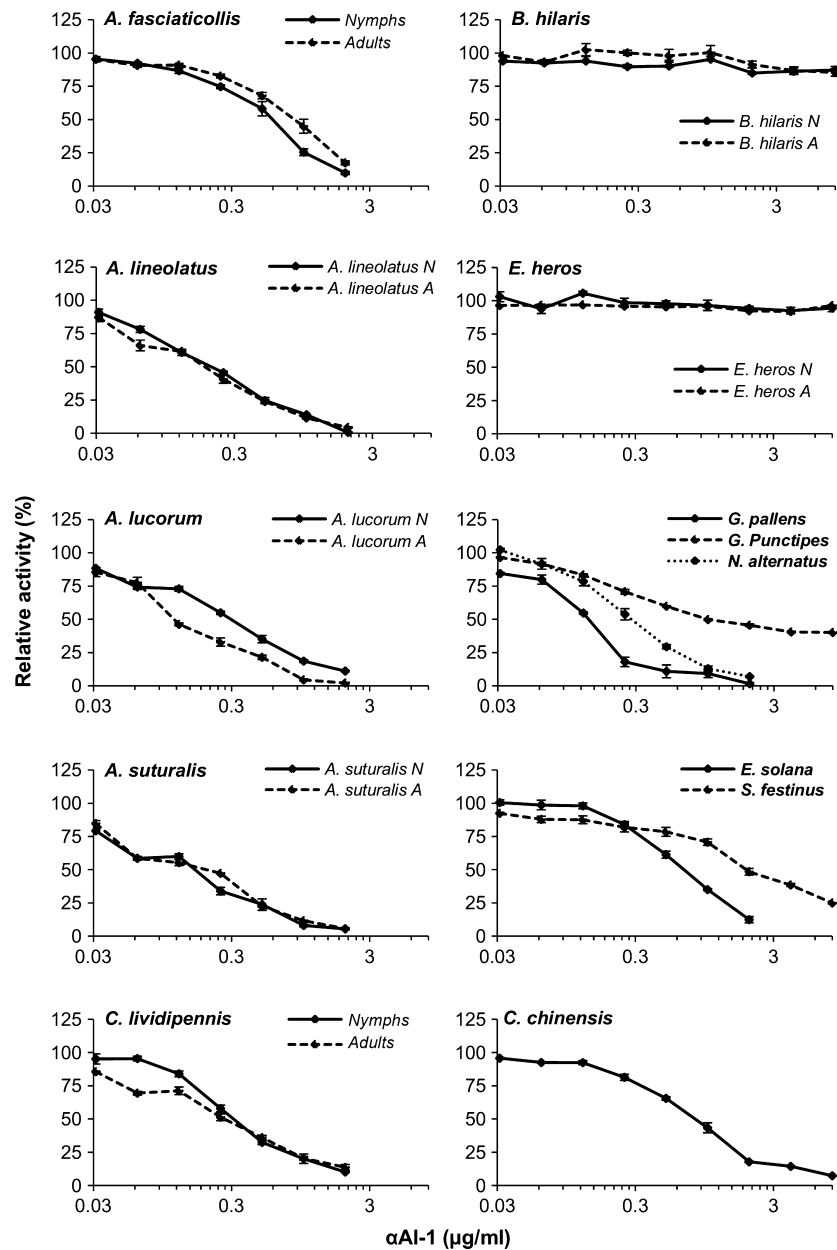
Fig. 2 Effect of αAI-1 on the relative *in vitro* activity of α-amylases in extracts from herbivorous Miridae, a predatory Miridae, Pentatomidae, predatory Lygaeidae and Nabidae, Auchenorrhyncha, and the bruchid *Callosobruchus chinensis*. Activity is expressed relative to that in the absence of the inhibitor. Bars represent means ± SE of three measurements from a unique pooled extract. N = nymph, A = adult, L = larva.

L. lineolaris and *L. hesperus*; they concluded that these are adaptations to their respective feeding behaviours. Surprisingly, we found that the α-amylase activity in the extract of whole insects of *G. punctipes*, that is an extract that included both salivary glands and midguts, was higher than in any mirid species in our study. This indicates that it is important to distinguish between salivary and midgut α-amylase activity if α-amylase activity is to be used as an indicator of feeding habit.

Although α-amylase activity was detected in extracts of all species analysed, this activity was not always inhibited by αAI-1. The only extracts where α-amylase activity was not inhibited were those from nymphs and adults of the two pentatomid species. This is in line with a previous study reporting a lack of inhibitory activity of αAI-1 on the pentatomids *Eurydema oleracea* and *Graphosoma lineatum* (Hemiptera: Pentatomidae) (Kluh et al. 2005). However, the authors of this previous study concluded that Hemiptera, in general, might be tolerant to αAI-1. Our results show that while this may be true for Pentatomidae, it is not true for Hemiptera, in general. The extract of *S. festinus* not only had lowest α-amylase activity, also its inhibition

by αAI-1 was lowest of all species assayed. α-Amylase activity in the extract of the predator *G. punctipes* did not fall below 40% of original activity even at the highest concentrations of αAI-1 tested. Either the α-amylases of this species are only partially inhibited or it has multiple α-amylases, and not all are susceptible to αAI-1. This pattern was not observed in the second predator from this genus, *G. pallens*.

Unexpectedly, α-amylases of all other species were at least as susceptible to αAI-1 as those of the bruchid *C. chinensis*. This is interesting because these species include herbivorous as well as predatory species from four different families. It is encouraging that extracts of all species in the families Lygaeidae and Miridae were highly susceptible to αAI-1. These two families contain several important cotton pest species not controlled by Bt-transgenic varieties (Naranjo et al. 2008).

To further evaluate whether GM crops that express α-amylase inhibitors can be used to manage hemipteran pests, the following questions need to be answered with *in vivo* experiments: (i) to what extent are Hemiptera dependent on α-amylases? and (ii) what αAI-1 concentration in plant tissues is required to achieve a sufficient level of protection? Both questions could be answered by feeding insects artificial diets containing purified αAI-1 (Cohen 1985, 2000). Subsequently, greenhouse or field tests with αAI-1-transgenic plants are required to assess whether season-long suppression of hemipteran pest populations can be achieved. For the control of hemipteran species with α-amylases not or only partly inhibited by αAI-1, alternative bean α-amylase inhibitor isoforms, which have been shown to vary in specificity (Ishimoto et al. 1995), should be considered. Moreover, molecular evolution could be employed to generate αAI mutants with activity against α-amylases of hitherto insensitive insects (Silva et al. 2013).

Because the *in vitro* susceptibility of α-amylases of non-target Hemiptera to αAI-1 was similar to that of target Hemiptera, the impact on non-target species, especially the important group of predatory Hemiptera, should be assessed using the same approach. Non-target Hemiptera might be exposed to αAI-1 in two ways: (i) predatory Hemiptera might feed on herbivores that have ingested αAI-1 and, thus, be indirectly exposed to the inhibitor. However, the fate of the inhibitor after ingestion by herbivores has never been investigated. Hence, whether the predators would be exposed to active forms of αAI-1 and how high this exposure would be is unknown. (ii) As many Hemiptera consume a mixed diet, some might occasionally feed on αAI-1-expressing GM crops without being considered as pests and thus be exposed to the inhibitor. However, they would have a lower exposure and are thus expected to be less affected. In this context, the impact of αAI-1 on non-target insects has been investigated for another important group of biological control agents. α-Amylase activity and the inhibition of those α-amylases by αAI-1 have been demonstrated for parasitoid wasps belonging to the order Hymenoptera in both *in vitro* and *in vivo* studies (Álvarez-Alfageme et al. 2012). However, tri-trophic studies using αAI-1-transgenic cowpea (*Vigna unguiculata*) seeds, *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae) as the host, and *Dinarmus basalis* (Hymenoptera: Pteromalidae) as the parasitoid did not reveal any adverse effects on the latter (Lüthi et al. 2013b). A likely reason for this lack of effect was the low level of exposure to the inhibitor.

The expression of αAI-1 in plant tissues of non-legume GM crops also requires additional research. Expression of αAI-1 in transgenic legumes is restricted to the cotyledons and the embryonic axis of the developing seeds because the bean phytohemagglutinin-L (dlec2) promoter is used (Shade et al. 1994) and the pro-protein requires post-translational modifications to become active (Pueyo et al. 1993). For αAI-1 to be expressed in other tissues, an alternative promoter must be used, and the problem of post-translational cleavage of the pro-protein into the two subunits must be overcome.

In conclusion, α-amylase activity was detected in extracts of all 12 Hemiptera species tested. The bean α-amylase inhibitor αAI-1 inhibited this activity in extracts of all species except those in the Pentatomidae. Moreover, inhibition of this activity by αAI-1 in the extracts of susceptible species was in a similar range as in larvae of the bruchid beetle *C. chinensis*, which is known to be highly susceptible to this specific inhibitor. The susceptible families contain many important pest species, suggesting that this approach for pest management is worth investigating.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

  Table S1 Total amount of protein used and incubation time at 37°C in the *in vitro* α-amylase activity and inhibition assays for all samples included in the study

Supporting Table 1. Total amount of protein used and incubation time at 37 °C in the *in vitro* α-amylase activity and inhibition assays for all samples included in the study.

Family	Species	Stage[a]	Soluble protein (μg)	Incubation time (min)
Cicadellidae	*Empoasca solana*	A	12.42	480
Lygaeidae	*Geocoris pallens*	A	9.20	120
	Geocoris punctipes	A	9.74	120
Membracidae	*Spissistilus festinus*	A	13.44	1440
Miridae	*Adelphocoris fasciaticollis*	N	12.08	120
		A	17.60	180
	Adelphocoris lineolatus	N	11.66	120
		A	12.64	180
	Adelphocoris suturalis	N	14.18	180
		A	12.82	180
	Apolygus lucorum	N	14.42	180
		A	12.42	120
	Cyrtorhinus lividipennis	N	12.16	120
		A	10.56	180
Nabidae	*Nabis alternatus*	A	11.54	120
Pentatomidae	*Bagrada hilaris*	N	10.08	120
		A	9.92	360
	Euschistus heros	N	11.54	180
		A	15.20	45
Chrysomelidae	*Callosobruchus chinensis*	L	13.18	45

[a]A = adult; N = nymph; L = larva.