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In vitro and *in vivo* inhibition of α-amylases of stored-product mite *Acarus siro*

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Abstract. The stored-product mites are the most abundant and frequent group of pests living on the stored food products in Europe. They endanger public health since they produce allergens and transmit mycotoxin-producing fungi. Novel acaricidal compounds with inhibitory effects on the digestive enzymes of arthropods are a safe alternative to the traditional neurotoxic pesticides used for control of the stored-product pests. In this work, we explored the properties of acarbose, the low molecular weight inhibitor of α-amylases (AI), as a novel acaricide candidate for protection of the stored products from infestation by *Acarus siro* (Acari: Acaridae). *In vitro* analysis revealed that AI blocked efficiently the enzymatic activity of digestive amylases of *A. siro*, and decreased the physiological capacity of mite's gut in utilizing a starch component of grain flour. *In vivo* experiments showed that AI suppressed the population growth of *A. siro*. The mites were kept for three weeks on experimental diet enriched by AI in concentration range of 0.005 to 0.25%. Population growth of *A. siro* was negatively correlated with the content of AI in the treated diet with a half-population dose of 0.125%. The suppressive effect of AIs on stored-product mites is discussed in the context of their potential application in GMO crops

Introduction

Stored-product mites infest food during its storage (Sinha 1979; Iversen et al. 1990; Franz et al. 1997; Arlian 2002; Stejskal et al. 2003). The mite infestation decreases food safety because mites are allergen producers (van Hage-Hamsten and Johansson 1992) and transmit mycotoxin-producing fungi (Franzolin et al. 1999; Hubert et al. 2003). Traditional management of stored-product mites relies on chemical acaricides on good husbandry. However, in the past two decades the USA and EU legislative has increasingly restricted the use of methyl bromide and organophosphates, leaving the food industry without its only efficient class of registered acaricides, since most pyrethroids do not control mites satisfactorily (Wilkin and Hope 1973).

Juvenoids, plant oils and diatomaceous earth represent the only chemical alternatives to methyl bromide and organophosphate insecticides for control of the storage mites (e.g. Thind and Edwards 1990; Cook and Armitage 2000;

Eun-Hee et al. 2003). In addition, the boom genetic engineering techniques raises the possibility of using insecticidal and acaricidal biomolecules in GMO crops. The inhibitors of digestive enzymes (namely inhibitors of proteases and α-amylases) are among biomolecules that are currently intensively investigated to combat the insect and mite pests (Schuler 1999).

The α-amylase inhibitors were tested in natural and laboratory experiments with insects (e.g. Gatehouse et al. 1986; Pueyo et al. 1995; Chrispeels et al. 1998; Gatehouse and Gatehouse 1998; Hou and Fields 2003), however, little information is currently available about their effect on physiology and population dynamics of the stored-product mites.

In the present study, we characterized for the first time the interaction of an α-amylase inhibitor with the digestive apparatus of the stored-product mite. We present a high *in vitro* inhibitory potential of acarbose, the saccharidic α-amylase inhibitor of bacterial origin (Gilles et al. 1996), and demonstrate its suppressive effect in biotest with *Acarus siro*. The model stored-product mite species *A. siro* was selected as it is the most frequent and abundant pest of stored grain in the Czech Republic (Stejskal et al. 2003).

Material and methods

Experimental mites and rearing conditions

Acarus siro specimens originated from laboratory strains obtained in the Czech grain stores and were kept in RICP. The experiments were performed in rearing chambers (see Woodring and Cook 1962). The rearing diet consisted of wheat germs and oat flakes (1:1). The chambers were placed in the exicator (Secodar®) under standardized conditions (85% moisture, 25 ± 1 °C temperature) in darkness. From these chambers mites were removed and used for the experiments.

Enzymatically active extract from A. siro

Mites were removed mechanically using paint brush from the surface of rearing chambers, about 100 mg and of fresh weight (using Sartorius® microbalance) were placed into the plastic tubes. These samples were homogenized in a cooled Potter–Elvehjem glass homogenizer in 400 μl of 50 mM MES (2-Morpholinoethanesulfonic acid) buffer pH 6.0 containing 100 mM NaCl, 5 mM $CaCl_2$, 25% glycerol, 0.02% NaN_3 and protease inhibitors (3 μM E-64 and 0.3 μM bovine pancreatic trypsin inhibitor, Sigma®) then left for 30 min at 0 °C. The homogenate was centrifuged (10,000*g* for 10 min at 4 °C), and the supernatant was filtered trough a Micropure-0.22 Separator (Amicon®). The content of proteins in the extract was quantified using Bradford reagent (Sigma®). The mite extract containing the α-amylase activity was stored at −70 °C.

α-Amylase activity and inhibition assay

The measurement of α-amylase activity was performed with chromolytic substrate of Remazol Brilliant Blue dyed starch (RBB-Starch, Fluka®). As aliquot of mite extract (containing 1.8 μg of proteins) was incubated with 0.3% RBB-Starch in 1 ml of 0.1 M Britton–Robinson buffer adjusted to pH 4 to 10 for 20 min at 26 °C. The reaction was stopped by adding 200 μl of 1 M NaOH. The mixture was then centrifuged. The absorbance of the supernatant was measured at 620 nm against the corresponding control sample prepared without the mite extract. One unit of α-amylase activity liberates the chromolytic product to the final absorbance $A_{620} = 1$.

The assay on inhibition of α-amylase activity was performed as above, the only difference was the initial pre-incubation step: the mite extract was pre-incubated (10 min at 26 °C) with the α-amylase inhibitor acarbose (Bayer®) in the concentration range of 0–7.5 μM and, afterwards, the reaction was then started by adding the RBB-Starch. The inhibition was expressed as remaining α-amylase activity (%) compared with the uninhibited sample. The α-amylase activity and inhibition assay were performed in triplicates.

Biotest with A. siro

In vivo effect of the inhibitor was evaluated as inhibition of population growth of *A. siro* fed on experimental diets compared to those fed on the control diet. The experimental diets were prepared from the control diet (see above) by addition of the inhibitor in the concentration range of 0.005–0.25% (w/w). The inhibitor was homogenously incorporated as a suspension in distilled water into the diet, followed by lyophilization and moisturization.

The population increase of flour mite (*A. siro*) was observed. Each experimental chamber contained 0.05 g of the experimental or control diet and 50 adult mites in 10 replicates per both experimental and control diets (cf Hubert et al. 2004). The final population was recorded after 21 days. Mites and food were removed on the circles of filter paper placed in the Berlese-Tullgren apparatus. The extraction temperature was 25 °C for 12 h and 35 °C for the next 12 h. The mites migrated from the filter paper were trapped in picric acid solution and counted.

The effect of inhibitor on mite population was analyzed by linear regression using SPLUS® software (with number of mites as depended variable and the concentration of amylase inhibitor as independent variable).

Microanatomical observation of A. siro *digestive tract*

The microanatomical methods were applied to 20 specimens originated from the control diet and the experimental diet containing 0.005% inhibitor,

respectively. Mites were fixed in modified Bouin-Dubosque-Brazil fluid (Smrž 1989), embedded in paraplast, sectioned (thickness 5–7 μm) and stained in Masson's triple stain. The content of food boli and the features of the digestive tract were observed (Hubert and Šustr 2001). The presence of food boluses in mesenteron, colon and rectum was used as indicators of consumption (Smrž and Čatská 1989). The nomenclature of the gut is given by Hubert et al. (2002a).

Results

In vitro *analysis*

The activity of α-amylases in whole-body extracts from *A. siro* was detected with macromolecular starch substrate and quantified with the highest level of 235 units per mg of the extracted proteins. The α-amylases showed activity at pH values from 4.5 through 7.5 with the optimum at 6.0 (Figure 1). The potential of acarbose to interact with and to block the activity of α-amylases of *A. siro* was analysed. The inhibition curve clearly revealed a concentration-dependent and efficient inhibition with IC_{50} value of 4.2 μM acarbose (Figure 2).

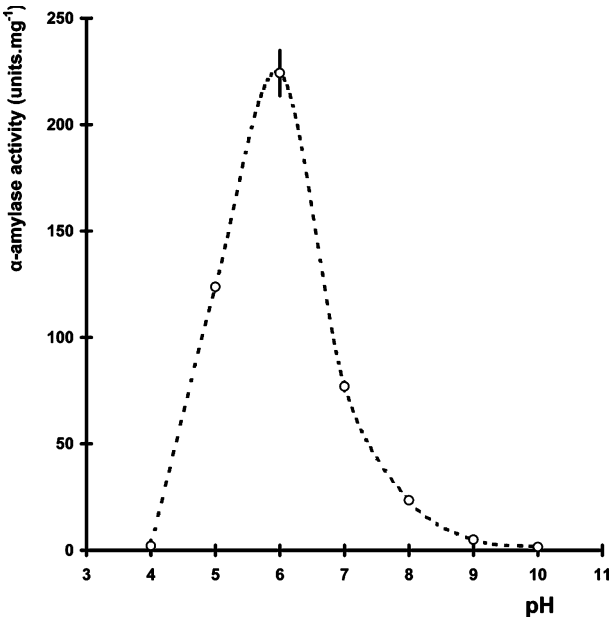


Figure 1. The pH profile of enzymatic activity of α-amylases from *Acarus siro*. The measurement of α-amylase activity of the mite whole-body extract (1.8 μg of protein per assay) was performed with chromolytic substrate of 0.3% RBB-Starch in 0.1 M Britton–Robinson buffers. Bars indicated standard deviations.

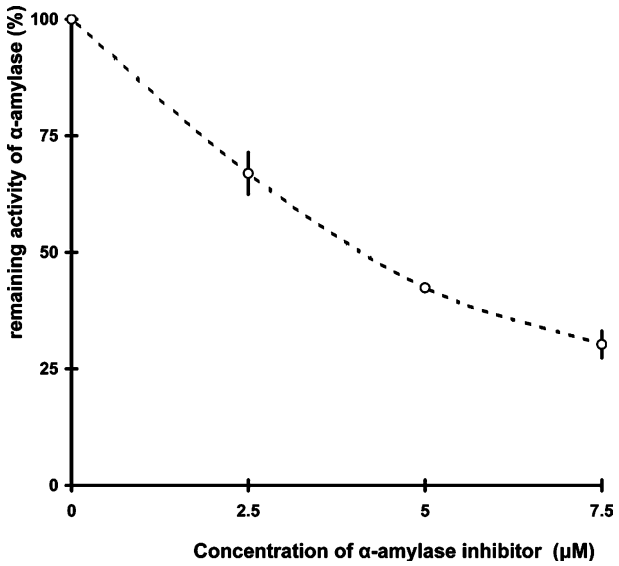


Figure 2. The inhibitory effect of α-amylase inhibitor on enzymatic activity of α-amylases from *Acarus siro*. The measurement on α-amylase activity was performed as described in Figure 1 at pH 6.0 in the presence of various concentrations of α-amylase inhibitor. The inhibition of α-amylase activity was expressed as relative remaining activity compared with the uninhibited control sample ($R = 0.99$; $y = 99.289e^{-0.1619x}$). Bars indicated standard deviations.

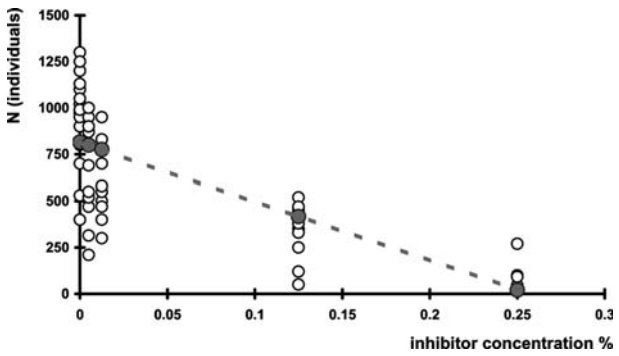
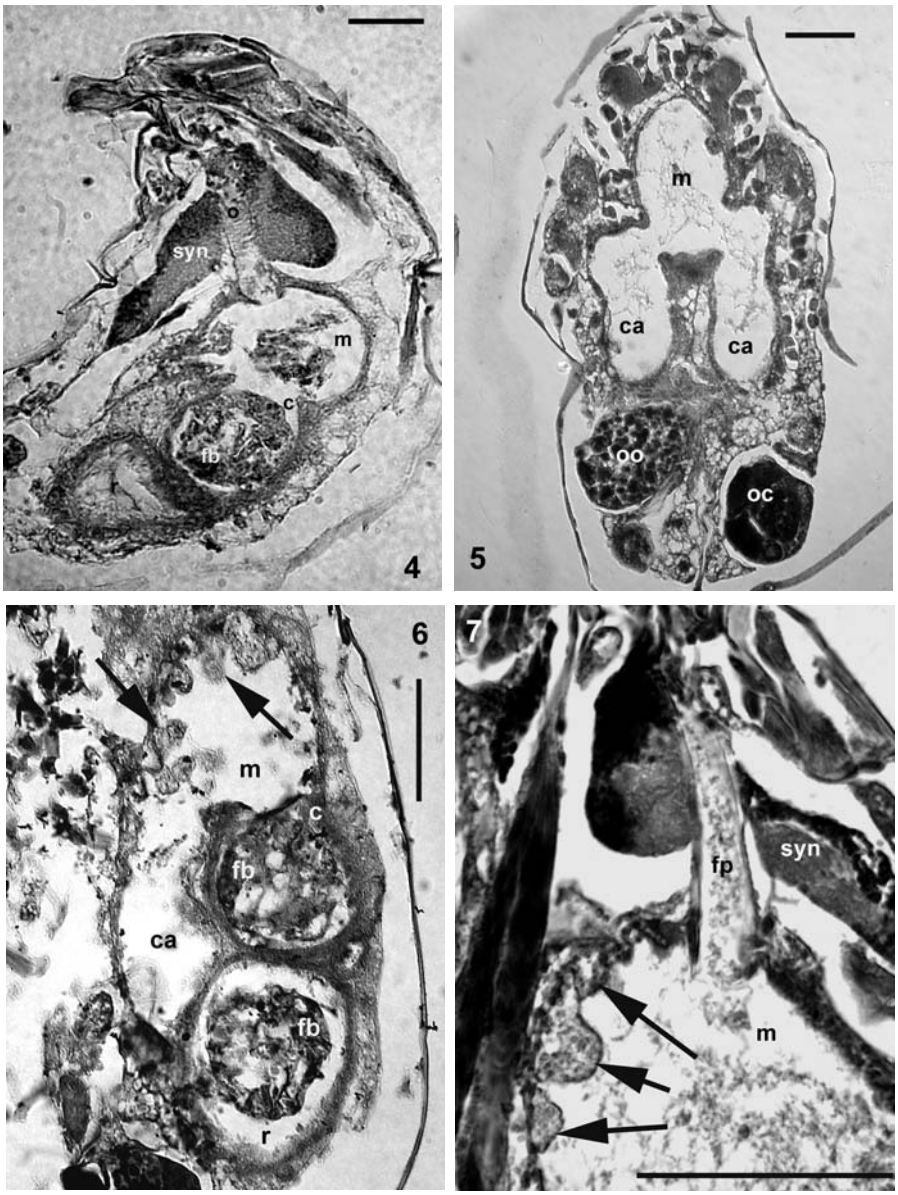


Figure 3. The suppression effect of α-amylase inhibitor on the population growth of *Acarus siro*. The starting population of 50 individuals in 10 replicates per tested diet was counted after 21 days after separation of mites in the Berlese-Tullgren apparatus. The experimental diets (0.05 g) were derived from the control diet by homogenously incorporated α-amylase inhibitor in the defined concentrations. The regression model $y = -1590x + 815$ was significant ($R = 0.77$; $F_{(4.00)} = 84.58$, $p > 0.001$) and explained about 60% of total variability. Legends: ○- observed population, ●- fit population.



Figures 4–7. The comparison of gut from *Acarus siro* fed on diet with 0.005% α-amylase inhibitor (Figures 4 and 6) and on control diet (Figures 5 and 7): (4) parasagittal section of the gut. (5) horizontal section of midgut. (6) parasagittal section of midgut, arrows point to proliferated mesenteral cells. (7) food ingestion into mesenteron, arrows point to proliferated mesenteral cells. Scales: 0.02 mm. Abbreviation used: c – colon, ca – caecum, fb – food bolus, fp – food particles, m – mesenteron o – oesophagus, oo – egg, oc – oocyte, ph – pharynx, r – rectum, syn – synganglion.

In vivo *analysis*

In vivo experiments showed that the α-amylase inhibitor supplied in the experimental diet to *A. siro* suppressed the mite population during 3 weeks of the biotest. The population growth correlated negatively with the inhibitor content in the treated diet ($R = 0.77$; $F_{(4.00)} = 84.58$, $p < 0.001$) in concentration range of 0.005–0.25% inhibitor (Figure 3). The inhibitor concentration about 0.125% ($\sim 1.9\mu$mol acarbose g^{-1} diet) decreased the population growth of *A. siro* to 50% in comparison with the control experiment. The highest concentration of the inhibitor tested (0.25%) led to a high suppression of population growth (to 2.5% of control population) but not complete extinction of the experimental population.

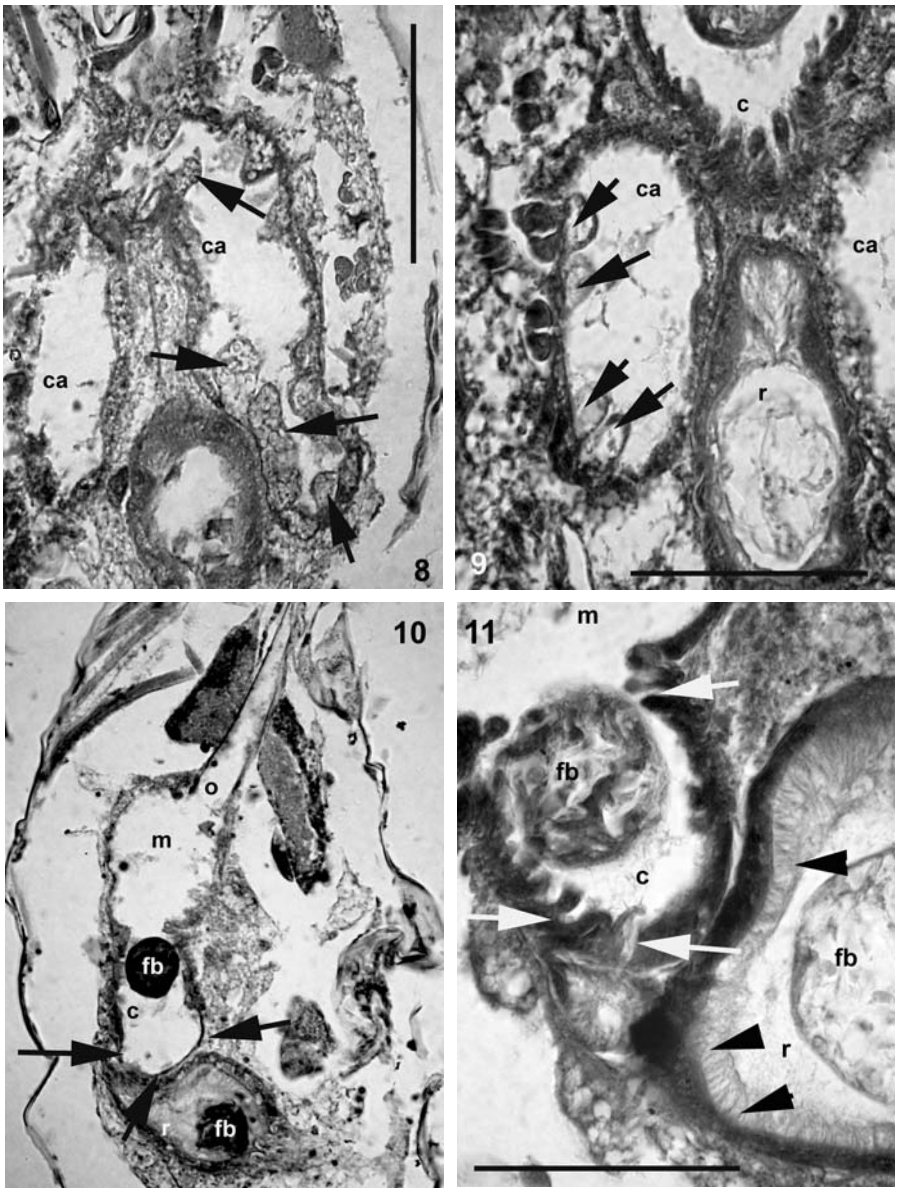
Microanatomy of digestive tract

The gut content and anatomy were compared for individuals reared on the control diet and the experimental diet with a low concentration of the inhibitor (0.005%). The colon cells showed the main differences between the inhibitor treated and untreated specimens. Their size was reduced and the cells looked flat in the treated mites (Figure 10), while the vacuolized cells of various sizes were observed in the control mites (Figure 11). No other differences in gut structures were found. The food boli were present in all part of digestive tract (i.e. mesenteron, colon and rectum) (Figures 4, 6 and 11). All mesenteral cells were filled by granulae, and they proliferated into mesenteral lumen in anterior part (Figures 6 and 7). The caecal cells were vacuolized and intensively proliferated into caecal lumen (Figures 8 and 9). The rectal cells contained well-developed microvilli (Figures 10 and 11). An inspection of fat body in the control mite revealed the glycogenous inclusions, which were absent in the inhibitor treated mites.

Discussion

Our finding of the high level of activity of the digestive α-amylases in the whole-body extract from *A. siro* is similar to results found for several other species of stored-product mites (Bowman and Child 1982; Bowman 1984). It indicates a general adaptation of the digestive apparatus of these species to starch digestion and utilization. The pH optimum identified for the α-amylase activity corresponds to pH conditions in the mesenteron and caeca of *A. siro* (see Akimov and Barabanova 1976), which points to its gut localization and digestive function.

The high activity of α-amylases in the stored-product mites enabled us to experimentally test the inhibition of the digestive amylolytic system as a tool



Figures 8–11. The comparison of gut from *Acarus siro* fed on diet with 0.005% α-amylase inhibitor (Figures 8 and 10) and on control diet (Figures 9 and 11): (8–9) horizontal section of caecum, arrows point to proliferated cells. (10) parasagittal section of midgut, arrows point to reduced cells of colon. (11) colon and rectum, arrows point to highly developed cells of colon, arrowheads to rectal microvilli. Scales: 0.02 mm. Abbreviation used: c – colon, ca – caecum, fb – food bolus, o – oesophagus, r – rectum, syn – synganglion.

for suppression of the mite metabolism. As potential candidates, natural α-amylase inhibitors belong to several families of molecules having protein or saccharide character. For example, the proteinaceous α-amylase inhibitors of plant origin were previously reported to be *in vitro* inhibitors of α-amylases of house-dust mites (see Sánchez-Monge et al. 1996). We focused on the saccharidic inhibitor acarbose that belongs to trestatin family of α-amylase inhibitors produced by *Actinomycetes* (Gilles et al. 1996).

α-Amylases catalyze the initial hydrolysis of starch into oligosaccharides, an important step towards transforming sugar polymers into simple units that can be assimilated by the organism. We demonstrated *in vitro*, that acarbose in micromolar concentrations efficiently blocks the activity of α-amylases from *A. siro*. This potency is comparable with inhibition of the insect digestive α-amylases by acarbose (Hubert et al. 2002b). The previous experiments with insects showed that α-amylase inhibitors generally block the whole starch metabolisms, and nutrition of the organisms is impaired causing shortness in energy (Puyeo et al. 1995). Our microanatomy analysis of *A. siro* treated with acarbose showed that the food is present in all part of the gut. Also, the high secretory activity of mesenteral and caecal cells indicated an intensive feeding of the mites. However, the mites were starved as demonstrated by the absence of glycogenous inclusions in fat body and by reduction of the size of the colon cells responsible for food absorption (cf. Hubert and Šustr 2001; Alberti et al. 2003). In conclusion, the α-amylase inhibitor applied at even low concentration was able to mimick the nutrient deficiency. This deficit leads to the lower reproduction rate, possibly associated with a lower egg production, and the final suppression of population growth (Figure 3).

Although the population of *A. siro* decreased dramatically with increasing acarbose concentration (Figure 3) the ingested inhibitor did not cause extinction of the pest population. The experiments based on combination of the inhibitors with other strategies including the predatory mites (see Žďárková 1998) are currently in progress. Our results demonstrate the general potential of α-amylase inhibitors for post-harvest protection of crops against the stored-product mites. These natural compounds represent promising acaricides with potential application in plant biotechnologies.

Acknowledgements

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