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Inhibitory specificity and insecticidal selectivity of α-amylase inhibitor from *Phaseolus vulgaris*

Ivan Kluh [a], Martin Horn [a], Jana Hýblová [a,c], Jan Hubert [b], Lucie Dolečková-Marešová [a], Zdeněk Voburka [a], Iva Kudlíková [b], František Kocourek [b], Michael Mareš [a,*]

[a] *Department of Protein Biochemistry, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of The Czech Republic, Flemingovo nam. 2, 166 10, Praha 6, Czech Republic*
[b] *Research Institute of Crop Production, Drnovska 506, 16106 Praha, Czech Republic*
[c] *Czech University of Agriculture Prague, Kamycka 129, 16521 Praha, Czech Republic*

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Abstract

The primary structure and proteolytic processing of the α-amylase isoinhibitor αAI-1 from common bean (*Phaseolus vulgaris* cv. Magna) was determined by protein chemistry techniques. The inhibitory specificity of αAI-1 was screened with a panel of the digestive α-amylases from 30 species of insects, mites, gastropod, annelid worm, nematode and fungal phytopathogens with a focus on agricultural pests and important model species. This in vitro analysis showed a selective inhibition of α-amylases from three orders of insect (Coleoptera, Hymenoptera and Diptera) and an inhibition of α-amylases of the annelid worm. The inhibitory potential of αAI-1 against several α-amylases was found to be modulated by pH. To understand how αAI-1 discriminates among closely related α-amylases, the sequences of the α-amylases sensitive, respectively, insensitive to αAI-1 were compared, and the critical determinants were localized on the spatial α-amylase model. Based on the in vitro analysis of the inhibitory specificity of αAI-1, the in vivo activity of the ingested αAI-1 was demonstrated by suppression of the development of the insect larvae that expressed the sensitive digestive α-amylases. The first comprehensive mapping of αAI-1 specificity significantly broadens the spectrum of targets that can be regulated by α-amylase inhibitors of plant origin, and points to potential application of these protein insecticides in plant biotechnologies.

Keywords: α-Amylase inhibitor; αAI-1; *Phaseolus vulgaris*; Digestive enzyme; Enzyme inhibition; Insect; Annelid worm

1. Introduction

α-Amylase inhibitor αAI-1 belongs to the family of related defense proteins in seeds of common bean *Phaseolus vulgaris* and other species of the *Phaseolus* genus. This family includes also homologous phytohemaglutinins and arcelins, which genes evolved by duplication and divergence of a single ancestral gene (Mirkov et al., 1994; Finardi-Filho et al., 1996). In spite of the considerable sequence homology, the mode of action

Abbreviations: αAI-1, α-Amylase inhibitor from common bean (-*Phaseolus vulgaris*); BPTI, bovine pancreatic trypsin inhibitor; PDB, Protein Data Bank; RP-HPLC, reverse-phase high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +420 220183358; fax: +420 220183578.
E-mail address: mares@uochb.cas.cz (M. Mareš).

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of these proteins in protecting seeds is different. Phytohematoglutinins and arcelins are toxic to insects due to their binding to midgut epithelial cells (Harper et al., 1995; Paes et al., 2000). The bean α-amylase inhibitors were reported to interact with digestive α-amylases of bruchid beetles and mammals (Moreno and Chrispeels, 1989). In bruchids, the blockage of digestive tract results in suppression of larval growth by reducing carbohydrate assimilation and negatively influence their life span (Ishimoto and Kitamura, 1989; Pueyo et al., 1995). Two related α-amylase inhibitors, αAI-1 and αAI-2, were identified in common bean and in certain accessions of wild beans, respectively (Ishimoto and Chrispeels, 1996). The mutations in their primary structures with 78% homology are responsible for a different specificity pattern towards α-amylases (Grossi de Sa et al., 1997). αAI-1 inhibits mammalian α-amylases and the larval midgut amylases of Azuki bean weevil (*Callosobruchus chinensis*) and cowpea weevil (*Callosobruchus maculatus*) but not of Mexican bean weevil (*Zabrotes subfasciatus*). On the other hand, αAI-2 does not inhibit the α-amylases recognized by αAI-1 but inhibits the α-amylase of *Z. subfasciatus* (Ishimoto and Chrispeels, 1996).

The transfer of genes encoding the defense proteins from common bean to other crops by genetic engineering can be used to create pest resistant plants. The field experiments with the transgenic peas that express αAI-1 revealed a complete protection from pea weevil (*Bruchus pisorum*) (Morton et al., 2000). Introduction of transgenic plants eliminates the use of the broad-spectrum chemical insecticides and substitutes them with natural biopesticides of narrow specificities targeted against particular pests. A prerequisite for this approach is a detailed characterization of selectivity of the applied transgenic biomolecules and their possible cross-reactivity to nontarget species.

In this paper, we report an analysis of functional specificity of αAI-1 isoinhibitor from *P. vulgaris* cv. Magna. We determined its primary structure and compared it with the sequences of αAI-1 isoinhibitors from other cultivars. Further, we characterized in vitro the inhibitory spectrum of αAI-1 against the digestive α-amylases from a representative selection of species of relevant invertebrate taxa. These results were combined with an analysis of molecular recognition of αAI-1 as well as with in vivo demonstration of αAI-1 selectivity.

2. Results and discussion

2.1. Determination of primary structure of αAI-1

The α-amylase inhibitor αAI-1 has been isolated from seeds of the common bean *P. vulgaris* cv. Magna in the sequencing grade purity. The N-terminal sequencing of AI-1 revealed two parallel sequences (ATETS- and SAVGL-) corresponding to α- and β-subunit, respectively. These subunits were separated under denaturing condition, subjected to fragmentation, and the purified fragments were sequenced. The complete primary structure is shown in Fig. 1. This sequence has been compared with the available full-length cDNA sequences of α-amylase isoinhibitors from various cultivars of *P. vulgaris* and found identical with the accession P02873, and 96-99% homologous with the other nonredundant sequences (accessions CAD28835, AAT35809, AAB42071). Also, a high homology was found with the recently deposited set of partial nucleotide sequences

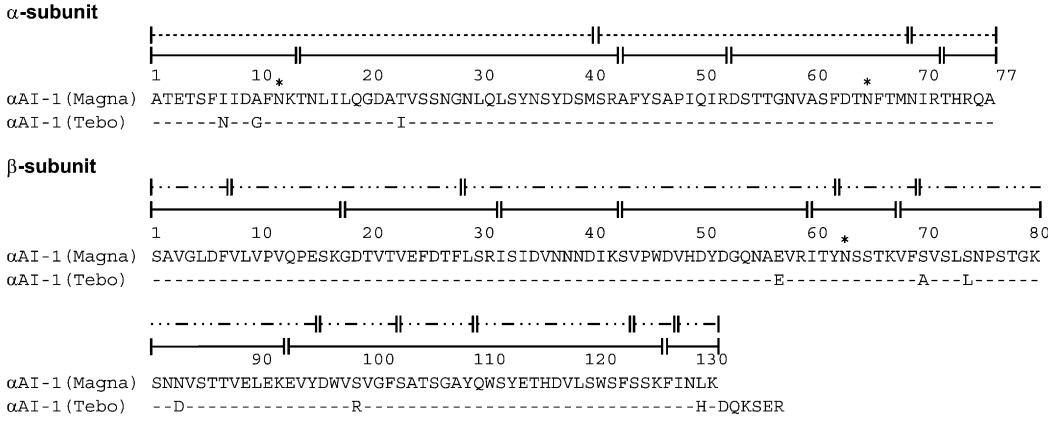

Fig. 1. The primary structure of the α-amylase inhibitor αAI-1 from *P. vulgaris* cv. Magna. The α- and β-subunit of αAI-1 were separated and their amino acid sequences were determined using the fragmentation approach. The peptide fragments were produced by digestion with trypsin (solid lines), chymotrypsin (dash-dotted lines) or cyanogen bromide (dotted lines), purified by RP-HPLC, and characterized by automated N-terminal sequencing. The *N*-glycosylation (*) was predicted according to low sequencing signal at Asn residue in consensus glycosylation sequence Asn–X–Ser/Thr. The sequence of the homologous αAI-1 from *P. vulgaris* cv. Tebo is aligned (Kasahara et al., 1996).

of five tens of AI-1 homologs from several *Phaseolus* species/cultivars (accessions AAG34442 to AAG34493).

The proteolytic processing of αAI-1 into α- and β-subunit was reported to be a crucial step in conversion of the inactive single-chain precursor to the mature αAI-1 molecule displaying the inhibitory activity (Pueyo et al., 1993). A comparison of the processing pattern of the isoinhibitor cv. Magna with the available data for two other isoinhibitors showed an analogous processing of the α-subunit (Kasahara et al., 1996; Young et al., 1999). A different trimming was found at C-terminus of β-subunit of the isoinhibitor cv. Magna, which was 6 and 8 amino acid residues shorter than C-terminus of isoinhibitor cv. Tebo and cv. Greensleeves, respectively (Fig. 1). This trimming, however, can not influence the inhibitory activity of αAI-1 as this part of molecule is not involved in interaction with α-amylase as inspected in the 3D structure of complexes of αAI-1 with α-amylase (accessions 1DHK, 1VIW). Further, we determined the molecular mass of 58 kDa for the isoinhibitor cv. Magna by sedimentation analysis, which indicated a formation of the dimer structure observed also in the 3D structures of αAI-1. This dimerization together with the proteolytic processing clearly revealed a correct maturation of structure of the investigated isoinhibitor. In conclusion, the αAI-1 isoinhibitor cv. Magna has high homology with isoinhibitors from other cultivars, and its function properties studied in this work can be generalized to other molecules of αAI-1 type. The determined primary structure was deposited in Swiss-Prot/TrEMBL database and merged under accession number P02873.

2.2. In vitro inhibitory activity of αAI-1

So far, the inhibitory activity of αAI-1 was demonstrated with α-amylases of mammals (both pancreatic and salivary enzymes) (Kotaru et al., 1987; Pueyo et al., 1993) and α-amylases of several gramnivorous beetles (Pueyo et al., 1995; Ishimoto and Chrispeels, 1996). We screened the inhibitory specificity of αAI-1 against a large set of α-amylases from 30 species, which cover the main insect taxa, model invertebrates and phytopathogenic fungi (Table 1). This selection contains the important agricultural pests. In the first step, the pH optimum of the tested α-amylases was determined. Accordingly, the representative pH values 4.5, 6.0 and 10.0, respectively, were chosen for screening the sensitivity of particular α-amylases to αAI-1 inhibition. The IC_{50} values were determined for the submicromolar inhibition to focus on physiologically important interactions of αAI-1 with the target enzymes (Table 1).

For the insect taxa, the results showed a significant discrimination of αAI-1 inhibition among the insect orders and a weaker discrimination among species within an order. The αAI-1 selectively inhibited α-amylases of the model species from three orders – Coleoptera, Diptera and Hymenoptera. The species from five other insect orders expressed the α-amylases that were insensitive to αAI-1 inhibition. There were differences up to three orders of magnitude in IC_{50} values for the sensitive insect α-amylases. The lowest inhibitory potential was found for two beetle species *Acanthoscelides obtectus* and *Sitophilus granarius*. The former was reported to be evolutionary adapted to feeding on legume seeds (Ishimoto and Chrispeels, 1996), and some *Sitophilus* strains were able to feed on legume seeds (Subramanyan and Hagstrum, 1996). These gramnivors can express digestive α-amylases insensitive to αAI-1 similar to that characterized in *Z. subfasciatus* (Grossi de Sa et al., 1997). Further, we tested the inhibition of α-amylases from both adults and larvae for selected beetle species (Table 1). This comparison suggests that, in some species, the adults and larvae express different isoenzymes of α-amylases having a different sensitivity to AI-1. A switch in isoenzyme pattern of the digestive enzymes during insect ontogeny was already reported (Pope et al., 1986).

Outside the insect taxa, the αAI-1 was screened in stored mites and dust mites that produce the allergenic α-amylases (Lake et al., 1991; Mills et al., 1999). These digestive enzymes were reported to interact with the α-amylase inhibitors of the cereal class (Sanchez-Monge et al., 1996), however, they were not recognized by αAI-1. In other invertebrates tested, the αAI-1 inhibition was weak for α-amylases of mollusk and nematode but was significant for that of the annelid earthworm. Two serious soil borne fungal phytopathogens (*Fusarium* spp. and *Sclerotinia* spp.) were cultured in medium containing starch, which elicited secretion of the digestive amylolytic enzymes. Their screening with αAI-1 did not reveal any inhibition although the inhibition of α-amylases from *Fusarium* spp. by the α-amylase inhibitor from corn seeds has been recently published (Figueira et al., 2003).

The inhibitory potential of αAI-1 against the tested α-amylases can be modulated by pH. The IC_{50} values determined at pH 4.5 and 6.0, respectively, significantly differed for several α-amylases (Table 1). The pH dependence was reported for mammalian α-amylases that formed the complex with αAI-1 optimally at pH 4.5 or 5.5 contrary to the unfavorable pH 6.9 that is, however, optimal for their enzymatic activity (Kotaru et al., 1987; Le Berre-Anton et al., 1997). We investigated this phenomenon in the experiment with a pH shift where the αAI-1 complex was preformed at acidic pH and then transferred and analyzed at higher pH, and compared it with an analogous experiment without an acidic preincubation (Figs. 2 and 3). This analysis with selected insect α-amylases revealed that the acidic preincubation can importantly improve the inhibition by αAI-1 (Fig. 3) although it had an individual efficiency among the

Table 1
Screening of αAI-1 on inhibition of enzymatic activity of α-amylases from various species

Taxon	Species[a]	IC_{50} (nM) pH 4.5[b]	IC_{50} (nM) pH 6.0[b]	pH optimum[c]
Insects				
Coleoptera	*Acanthoscelides obtectus* (ad) bean weevil	>1000	448.0	7.0–7.5
	Cryptolestes ferrugineus (ad) rusty grain beetle	10.9	4.2	5.0–5.5
	Cryptolestes pusillus (ad)	88.6	N.I.	5.0–6.0
	C. pusillus (la) flat grain beetle	359.9	N.D.	5.0
	Oryzaephilus surinamensis (ad)	44.9	>1000	4.5–5.0
	O. surinamensis (la) sawtoothed grain beetle	134.1	695.6	5.5
	Sitophilus granarius (ad) grain weevil	>1000	>1000	4.5
	Tribolium castaneum (ad)	2.4	4.8	4.5–5.0
	T. castaneum (la) red flour beetle	4.5	7.5	4.5–5.0
Diptera	*Drosophila melanogaster* (ad) fruit fly	N.A.	14.0	7.0–8.0
	Sarcophaga bullata (ad) fleshfly	N.A.	82.0	7.0
	Aedes aegypti (adm) yellow fever mosquito	N.A.	41.1	7.0
Hymenoptera	*Monomorium pharaonis* (ad) pharaoh ant	N.D.	29.6	5.0–5.5
	Apis mellifica (ad) honeybee	33.8	39.4	5.0–5.5
	Venturia canescens (ad) wasp	N.A.	22.7	5.0–5.5
Lepidoptera	*Ephestia cautella* (la) almond moth	N.A. (4.5; 6.0), N.I. (10.0)		10.0–11.0
	Ephestia elutella (la) cacao moth	N.A. (4.5; 6.0), N.I. (10.0)		9.0–10.0
	Ephestia kuehniella (la) mill moth	N.A. (4.5; 6.0), N.I. (10.0)		9.5
	Manduca sexta (la) tobacco hornworm	N.A. (4.5; 6.0), N.I. (10.0)		10.0
	Ostrinia nubilalis (la) European corn borer	N.A. (4.5; 6.0), N.I. (10.0)		11.0
Blattodea	*Blattella germanica* (ad) German cockroach	N.A.	N.I.	6.0
Psocoptera	*Liposcelis decolor* (mi) cereal psocid	N.A.	N.I. (6.0; 7.5)	7.5
Orhoptera	*Acheta domesticus* (mi) house cricket	N.I.	N.I.	5.5–6.5
Hemiptera	*Eurydema oleracea* (mi) Brassica bug	N.I.	N.I.	7.0–8.0
	Graphosoma lineatum (mi) shield bug	>1000	>1000	6.0
Others				
Acari	*Acarus siro* (mi) flour mite	N.I.	N.I.	6.0
	Dermatophagoides pteronyssinus (mi) European house dust mite	N.A.	N.I. (6.0; 7.5)	7.5
	Lepidoglyphus destructor (mi) long-haired mite	N.A.	N.I.	6.5
Gastropoda	*Helix pomatia* (ad) Roman snail	N.I.	>1000	6.0
Annelida	*Dendrobaena mrazeki* (ad) earthworm	84.1	>1000	7.0
Nematoda	*Caenorhabditis elegans* (ad)	N.A.	802.1	6.5–7.5
Fungi	*Fusarium oxysporum* (cm)	N.A.	N.I. (6.0; 7.0)	6.5–7.5
	Sclerotinia sclerotiorum (cm)	N.A.	N.I. (6.0; 7.0)	5.5–6.5

[a] (ad), adults; (adm); adult males, (la), larvae; (cm), culture medium; (mi), mixture of instars.
[b] N.A., no activity of free α-amylase (at the indicated pH); N.I., no inhibition of α-amylase activity by 1 μM αAI-1 (at the indicated pH); N.D., not determined.
[c] pH optimum for α-amylase activity.

tested enzymes (Fig. 2). The α-amylases insensitive to αAI-1 did not acquire any sensitivity after preincubation at various pH (data not showed). We suppose that the effect of pH shift has a physiologic relevance in those insect species, which gut contains a gradient pH with the acidic to alkaline pH profile found in some coleopterans (e.g. Tenebrionidae, Curculionidae, Cucujidae) (see Terra and Ferreira, 1994). The αAI-1 complex preformed optimally in the anterior acidic part of the midgut where the α-amylases are secreted (Cristofoletti et al., 2001) would be stable along the whole digestive tract and sustain the insecticidal action of αAI-1.

2.3. Molecular analysis of αAI-1 specificity

The analysis of inhibitory specificity of αAI-1 revealed a striking pattern that discriminates among closely evolutionary related α-amylases (Table 1). We investigated what are the structural features of α-amylases controlling their sensitivity to the αAI-1 inhibition. The structural determinants responsible for the interaction of α-amylases with αAI-1 were analyzed with the use of amino acid sequences available for several of the screened α-amylases and for the α-amylases with previously reported sensitivity to αAI-1. This set included 7 sensitive and 4 insensitive α-amylases (see Section 4). Their sequences were compared and aligned according to their homology. A general homology of 45–60% was found among sequences of the mammalian sensitive, invertebrate sensitive and invertebrate insensitive α-amylases. There was found no relationship between the sensitivity and the local insertions/deletions in the sequences.

Further, the sequences were analyzed with the help of spatial model of α-amylase molecule with the focus on that part, which is involved in interaction with αAI-1.

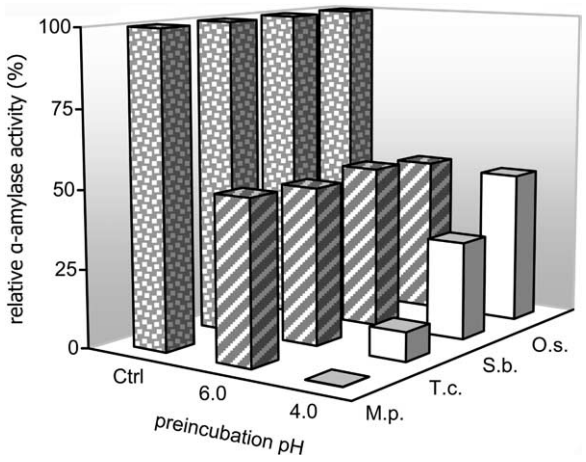

Fig. 2. The effect of pH on inhibitory potential of αAI-1 against different insect α-amylases. The α-amylase extract was preincubated with αAI-1 at pH 4.0 or 6.0 (as indicated in the figure). Afterwards, the inhibition of α-amylase activity was determined at pH 6.0 and expressed as remaining activity (%) against the corresponding uninhibited control (Ctrl). The amount of αAI-1 applied corresponds to IC_{50} determined at pH 6.0. The α-amylases tested were from *Monomorium pharaonis* adults (M.p.), *T. castaneum* larvae (T.c.), *Sarcophaga bullata* adults (S.b.) and *Oryzaephilus surinamensis* larvae (O.s.).

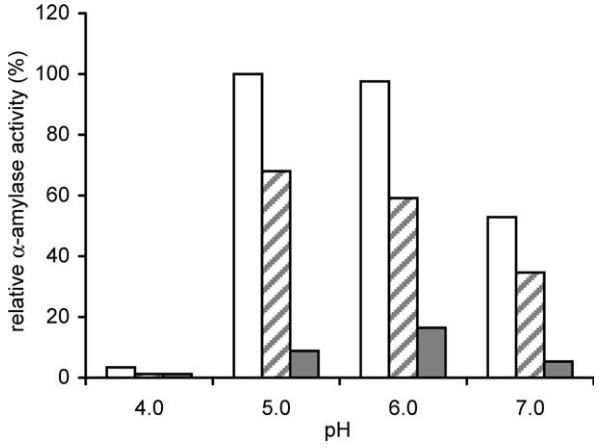

Fig. 3. The pH profile of inhibitory potential of αAI-1 against *T. castaneum*. The α-amylase activity in extract from *T. castaneum* larvae was determined at the pH indicated. The uninhibited controls (white bars) are compared with the samples inhibited by 5 μM αAI-1. Before the measurement of the residual activity, the inhibitor was preincubated with the extract at pH 4.0 (black bars) or at the same pH as is the final pH of the assay (hatched bars). The α-amylase activity of the control at pH optimum 5.0 is set as 100%.

This contact region is defined in the X-ray structures of the complex of αAI-1 with porcine pancreatic α-amylase, respectively, α-amylase from *Tenebrio molitor* (Bompard-Gilles et al., 1996; Nahoum et al., 1999). It represents a large area of ca. 1500 $Å^2$ on the surface of α-amylase with 50 amino acid residues responsible for interaction with αAI-1. The overall homology of the analyzed set of α-amylase sequences implicated their common spatial fold and topology of the contact residues. We inspected the mutations that occurred in positions of the potential contact residues. First, the variability of amino acid residues found in particular contact positions was different, ranging from fully conservative to highly variable positions. The variable contact positions were generally mutated in the group of sensitive α-amylases as well as in that of insensitive α-amylases. It indicates that the sensitive α-amylases can receive some structural changes and still retain the ability to interact with αAI-1. This plasticity is evidently due to an extremely high number of residues that contribute to the interaction and can compensate for some mutations, which are not productive in the interaction. Second, some amino acid residues in the contact positions, however, were found only in the sensitive, respectively, insensitive α-amylases. The mutations present specifically in the insensitive α-amylases were determined for particular contact position as putative mutations incompatible with the sensitivity (see Section 4 for details). A frequency of these mutations was expressed in a color representation on the 3D structure of the representative α-amylase (Fig. 4). The obtained pattern shows that the central part of the contact region containing the catalytic residues is evolutionary conservative and similar in sensitive and insensitive α-amylases. The incompatible mutations that prevent the

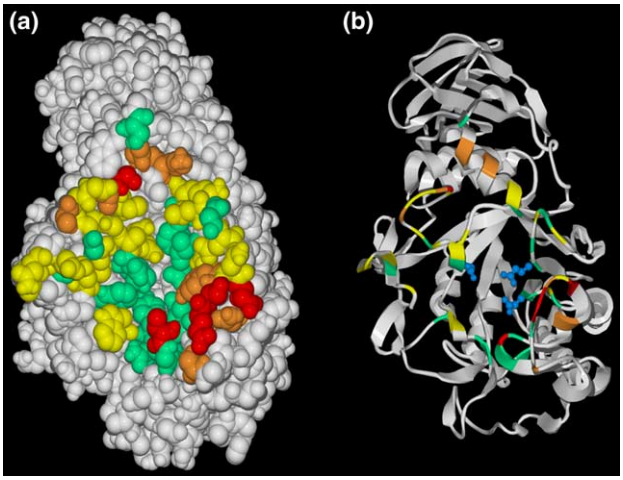

Fig. 4. Analysis of the interaction region on α-amylase molecule for binding to αAI-1. The α-amylase molecule in the same orientation is shown in surface representation (a) and ribbon representation (b), respectively. The amino acid residues of α-amylase that interact with αAI-1 are colored. The color coding differentiates between the mutations found in the sequences of α-amylases sensitive, respectively, insensitive to αAI-1 inhibition. The gradient green–yellow–orange–red represents an increased frequency of the mutations incompatible with the αAI-1 binding. The side chains of the conserved amino acid residues essential for catalytic activity of α-amylases (the triad Asp/Glu/Asp) are depicted in blue stics (see b). The model is based on the comparison of 11 α-amylase sequences and PDB coordinates for complex of pig pancreatic α-amylase with αAI-1 (accession 1DHK) (see Section 4).

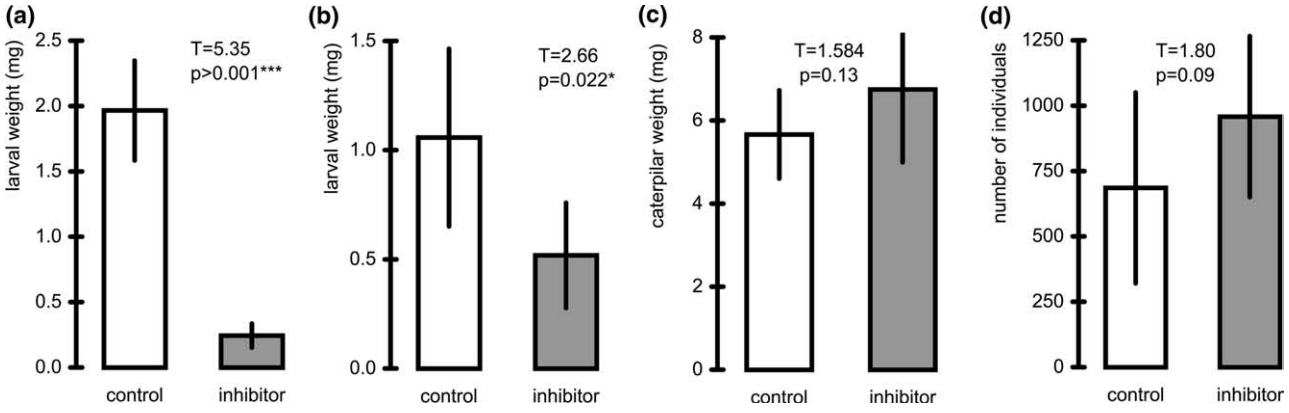

Fig. 5. Biological activity of αAI-1. The feeding bioassay was performed with 1% αAI-1 (w/w) incorporated in the diet of the following arthropods: (a) *T. castaneum*, (b) *D. melanogaster* (c) *E. kuehniella*, and (d) *A. siro*. The animals fed on the diet supplemented with αAI-1 were compared with those on control diet. The monitored biological parameters were the weight of insect larvae (*T. castaneum*, *D. melanogaster*, and *E. kuehniella*) and the population growth (*A. siro*). The statistics was calculated by *T*-test with the significant probability $p < 0.05$. The mean (column) and standard deviation (bar) is indicated.

interaction of the insensitive α-amylases with αAI-1 are cumulated in two areas localized at the side of the contact region, and they surround and directly interact with two main interacting loops of αAI-1. These mutations cover a spectrum of various structural changes that influence a charge, hydrophobicity or residue volume. In conclusion, the analysis suggests that αAI-1 interacts with a sensitive enzyme through a complex net of contacts, and this interaction is not established with an insensitive enzyme due to the contribution of multiple incompatible structural changes rather than a single critical mutation. This result is in accord with the recent modeling study on inhibitory specificity of related molecule of αAI-2 (Da Silva et al., 2000).

2.4. In vivo inhibitory activity of αAI-1

The inhibitory specificity of αAI-1 determined in vitro was compared with the selectivity of its activity in vivo. The αAI-1 was incorporated in the feeding diet of four species, and the effect of the ingested αAI-1 was monitored by biological parameters: (i) weight of insect larvae (*Tribolium castaneum*, *Drosophila melanogaster*, *Ephestia kuehniella*), and (ii) population growth (*Acarus siro*). The obtained results showed an important suppression of development of *T. castaneum* and *D. melanogaster* contrary to insignificant changes in development of *E. kuehniella* and *A. siro* (Fig. 5). These results on biological activity of αAI-1 are in accord with the in vitro specificity of αAI-1 as *T. castaneum* and *D. melanogaster* expressed the digestive α-amylases sensitive to αAI-1 inhibition, and *E. kuehniella* and *A. siro* expressed the insensitive enzymes (Fig. 6 and Table 1). The strong suppressive effect in *T. castaneum* correlates with the lowest IC_{50} value. The 1% dose of αAI-1 applied in the bioassay is comparable with the effective doses reported previously for larvae of several bruchid beetles and with the natural content of αAI-1 in *Phaseolus* seeds (Pueyo et al., 1995; Ishimoto and Chrispeels, 1996; Morton et al., 2000).

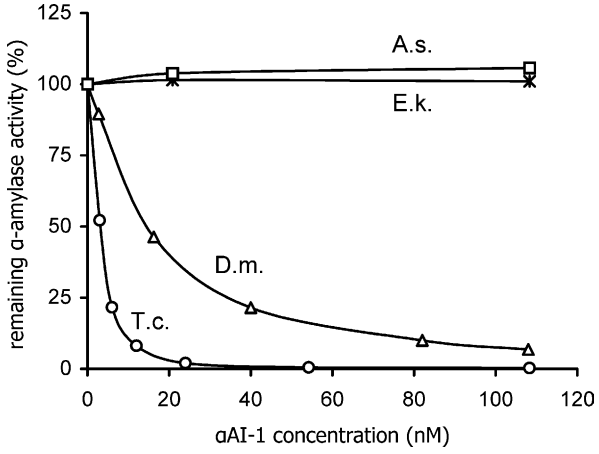

Fig. 6. Biochemical activity of αAI-1. The in vitro inhibitory effect of αAI-1 on enzymatic activity of α-amylases from the following arthropods: *T. castaneum* larvae (T.c.), *D. melanogaster* larvae (D.m.), *E. kuehniella* larvae (E.k.), and *A. siro* mixed instars (A.s.). The measurement on α-amylase activity was performed in the presence of various concentrations of αAI-1 at pH 4.5 (*T. castaneum*), 6.0 (*D. melanogaster*, *A. siro*) and 10.0 (*E. kuehniella*). The inhibition of α-amylase activity is expressed as relative remaining activity (%) compared with the uninhibited control sample.

3. Concluding remarks

We present an analysis of αAI-1 specificity that defines the spectrum of species that can be potentially regulated by this protein insecticide. The gramnivorous specialists like *A. obtectus* evolved the molecular

adaptation to feeding on legume seeds, and express the digestive α-amylases with low sensitivity to αAI-1. The other coleopteran species being generalists or not co-evolved with *Phaseolus* plants were sensitive to αAI-1 inhibition, which makes them potential targets for the suppression strategies. As this group contains serious stored grain pests, the transgenic crops producing αAI-1 can diminish considerable losses during pre-harvest and post-harvest period. This approach becomes reasonably safe as defense proteins of beans have been present in the human food chain for a very long time without any detrimental effect on human population. The αAI-1 was even clinically tested to improve postprandial carbohydrate tolerance in patients with diabetes mellitus (Layer et al., 1986). We further demonstrated that physiology of members of Hymenoptera and Diptera orders can be potentially affected by the ingested αAI-1. This fact should help assess the possible risk for nontarget species interacting with transgenic crops including the beneficial insect species like honeybee, and points to the importance of seed-specific expression of the αAI-1 transgene to minimize such exposition.

4. Experimental

4.1. Purification of αAI-1

Seeds of common bean (*P. vulgaris*, cv. Magna) were obtained from the Research Institute of Crop Production, Praha. The isolation procedure was analogous to that described by Marshall and Lauda (1975). It included acid extraction of seed flour, ammonium sulfate fractionation (35–65% saturation), chromatography on DEAE cellulose (Amersham Biosciences, Uppsala, Sweden) and gel chromatography on Biogel P-100 (Bio-Rad, Hercules, CA).

4.2. Protein chemistry methods

For separation of αAI-1 subunits, the purified αAI-1 was dissolved (3 mg/ml) in 50 mM Na-phosphate, pH 8 containing 6 M guanidine.HCl, heated for 30 min at 50 °C, and dialyzed in Spectraphore 3 membrane (Spectrum Medical Industries, Houston, TX) against 50 mM Tris–HCl, pH 8 containing 8 M urea. The mixture was chromatographed on DEAE-Sephacel column (Amersham Biosciences, Uppsala, Sweden) in 50 mM Tris–HCl, pH 8 containing 8 M urea with a gradient of 0–1 M NaCl. The materials corresponding to the separated subunits were desalted on Sephadex G-25 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 0.4% NH_4HCO_3. The β-subunits was further purified on Superdex 200 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 50 mM Na-phosphate, 50 mM H_3BO_3, pH 7.5. Purity of the prepared subunits was verified by SDS–PAGE and by N-terminal sequencing. The subunits were digested by trypsin, respectively, chymotrypsin in 0.2% NH_4HCO_3, pH 8.3 with enzyme-to-substrate ratio 1:50 (w/w) for 4 h at 37 °C. The fragmentation with cyanogen bromide was performed with protein-to-CNBr ratio 1:1 (w/w) in 70% trifluoracetic acid for 16 h at room temperature. The mixtures of peptidic fragments were chromatographed by RP-HPLC on C18 column (218TP54, Vydac, Hesperia, CA) equilibrated in 0.1% trifluoracetic acid with a gradient of 0–90% acetonitrile. The purified peptides were characterized by automated N-terminal sequencing on AB Procise sequencer (Foster City, CA) and by amino acid analysis on Biochrom 20 (Amersham Biosciences, Uppsala, Sweden). The molecular weight of the native αAI-1 was determined by the method of sedimentation equilibrium on Beckman-Spinco Ultracentrifuge Model E (Palo Alto, CA) in 50 mM Na-acetate, pH 5.5 containing 150 mM NaCl, 1 mM $CaCl_2$.

4.3. Biological materials

The experimental animals and fungi used for in vitro and in vivo studies were obtained from collections and standardized rearing facilities at the following institutions: Research Institute of Crop Production, Praha (insects, mites, fungi, *Helix pomatia*), Institute of Inherited Metabolic Disorders, Praha (*Caenorhabditis elegans*), Institute of Soil Zoology, České Budějovice (*Dendrobaena mrazeki*), National Institute of Public Health, Praha (*Aedes aegypti*). For in vitro analysis, three types of materials were prepared from biological specimens: (i) the conditioned culture medium was collected by centrifugation after four days of cultivation of fungi (at 26 °C) in the modified liquid Czapek–Dox medium (Pitt and Hocking, 1997) containing 1% starch instead sucrose, (ii) the extract from dissected guts (*Manduca sexta*, *Blattella germanica*, *D. mrazeki*, *H. pomatia*), (iii) the whole body extract (all other species). The animals were starved for one day before the specimen was collected.

4.4. Preparation of enzymatically active extracts

The biological samples (50 mg of fresh weight) were homogenized in 400 μl of 50 mM MES, pH 6.0 containing 100 M NaCl, 5 mM $CaCl_2$, 25% glycerol, 0.02% Na azide and protease inhibitors (3 μM E-64, 0.3 μM BPTI) and extracted for 30 min on ice. The homogenate was centrifuged (10,000*g* for 10 min at 4 °C) and the supernatant was filtrated with Micropure-0.22 Separator (Millipore, Bedford, MA). The content of proteins in the extract was quantified according to the Bradford method (Bradford, 1976). The culturing media of fungi after separation of cells were filtrated as above. The extracts and media displaying the α-amylase activity were stored at −70 °C.

4.5. 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, Buchs, Switzerland). The aliquot of the extract or medium was incubated with 0.3% RBB-Starch in 0.1 M Britton–Robinson buffer with the indicated pH for 20 min at 26 °C. The reaction was stopped by 150 mM NaOH, and the mixture was centrifuged. The absorbance of the supernatant was measured at 620 nm against the corresponding control sample prepared without the extract. Defined unit of α-amylase activity liberates the chromolytic product with $A_{620} = 1$ under these conditions. Typically 0.35 unit of α-amylase activity at pH optimum was used in assay. The assay on inhibition of α-amylase activity was performed analogously as above with an initial preincubation step: the extract/medium was preincubated with αAI-1 (up to 1 μM) for 20 min at 26 °C and, afterwards, the reaction was started by adding the RBB-Starch. The inhibition was expressed as remaining α-amylase activity (%) compared with the corresponding uninhibited sample, and the IC_{50} value was calculated. For measurement of inhibition of α-amylase activity with pH shift, the preincubation step with αAI-1 in IC_{50} concentration (determined at pH 6.0) was performed in 5 mM Britton–Robinson buffer pH 4.0 or 6.0. Afterwards, the reaction was started by adding the RBB-Starch and 0.1 M Britton–Robinson buffer pH 6.0.

4.6. In silico analysis

The amino acid sequences of the investigated α-amylases were searched in the protein database of NCBI (http://www.ncbi.nlm.nih.gov) and included the following accessions (a) α-amylases sensitive to αAI-1: P04746, human pancreatic; P00690, porcine pancreatic; P56634, *T. molitor*; P09107, *T. castaneum*; P08144, *D. melanogaster*; AAM20738, *Apis mellifera*; P53354, *A. aegypti* and (b) α-amylases insensitive to αAI-1: AAF73435, *Z. subfasciatus*; AAD38942, *Dermatophagoides pteronyssinus*; AAA03715, *Ostrinia nubilalis*; NP_506303, *C. elegans*. The amino acid sequences were aligned according to their homology with Clustal W program at EMBL-EBI (http://www.ebi.ac.uk/clustalw). The amino acid residues that interact with αAI-1 were selected in the "template" sequences of porcine pancreatic α-amylase and α-amylase from *T. molitor*. These contact residues were determined in X-ray studies of complexes of αAI-1 with porcine pancreatic α-amylase (accessions 1DHK), respectively, α-amylase from *T. molitor* (accessions 1VIW), and were listed previously (Bompard-Gilles et al., 1996; Nahoum et al., 1999; Da Silva et al., 2000). Based on the sequence alignment, the contact residues in the "template" sequences defined the positions of the putative contact residues in the other homologous sequences. The mutations incompatible with the AI-1 binding were analyzed for each contact position in the alignment: (1) the number of amino acid mutations found in the "insensitive" sequences (e.g. sequences of α-amylases insensitive to αAI-1) but not in the "sensitive" sequences was determined, (2) this score gradually increased from a conserved position (with no mutations specific for "insensitive" sequences) to a position with total mutation variability in the "insensitive" sequences, (3) the score range was proportionally divided into four gradual categories corresponding to color coding used in Fig. 4. The assigned color coding of the contact positions was demonstrated on 3D structure of porcine pancreatic α-amylase (accession 1DHK) using molecular graphics software Deep View version 3.7 (http://www.expasy.org/spdbv).

4.7. Bioassay

The diet was composed of wheat germ and oak flakes (1:1) for the stored-product arthropods, and wheat powdered porridge for *D. melanogaster*. The experimental and control diet was derived by addition of 1% (w/w) αAI-1 or casein, respectively. The bioassay was performed in 10 replicates in darkness at 65% RH and 25 °C. For *T. castaneum*, the eggs were placed individually in chambers with 50 mg of the diet, and the larvae were weighted on the 14th day of breeding. For *E. kuehniella*, the 14 days old caterpillars were kept on 100 mg of the diets and weighted on the 21st day. For *D. melanogaster*, the newly hatched larvae were kept on 50 mg of the diet and weighted on the 5th day. For *A. siro*, the chambers contained 50 mg of the diet and 50 adults of mites, and the final population was recorded on the 21st day after separation of mites in the Berlese–Tullgren apparatus. The monitored biological parameters showed normal distribution and were compared by *T*-test with 0.05 probability level.

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