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Protective Mechanism of the Mexican Bean Weevil against High Levels of α-Amylase Inhibitor in the Common Bean[1]

Masao Ishimoto[2,3]* and Maarten J. Chrispeels

Department of Biology 0116, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093–0116

α-Amylase inhibitor (αAI) protects seeds of the common bean (*Phaseolus vulgaris*) against predation by certain species of bruchids such as the cowpea weevil (*Callosobruchus maculatus*) and the azuki bean weevil (*Callosobruchus chinensis*), but not against predation by the bean weevil (*Acanthoscelides obtectus*) or the Mexican bean weevil (*Zabrotes subfasciatus*), insects that are common in the Americas. We characterized the interaction of αAI-1 present in seeds of the common bean, of a different isoform, αAI-2, present in seeds of wild common bean accessions, and of two homologs, αAI-Pa present in seeds of the tepary bean (*Phaseolus acutifolius*) and αAI-Pc in seeds of the scarlet runner bean (*Phaseolus coccineus*), with the midgut extracts of several bruchids. The extract of the *Z. subfasciatus* larvae rapidly digests and inactivates αAI-1 and αAI-Pc, but not αAI-2 or αAI-Pa. The digestion is caused by a serine protease. A single proteolytic cleavage in the β subunit of αAI-1 occurs at the active site of the protein. When degradation is prevented, αAI-1 and αAI-Pc do not inhibit the α-amylase of *Z. subfasciatus*, although they are effective against the α-amylase of *C. chinensis*. αAI-2 and αAI-Pa, on the other hand, do inhibit the α-amylase of *Z. subfasciatus*, suggesting that they are good candidates for genetic engineering to achieve resistance to *Z. subfasciatus*.

The seeds of starchy grain legumes are an important staple in many countries, but as with other crops, insect pests can cause considerable crop losses. The postharvest damage done by larvae of bruchids (Coleoptera:Bruchidae) can be quite extensive. Crops of the common bean (*Phaseolus vulgaris*), the scarlet runner bean (*Phaseolus coccineus*), and the tepary bean (*Phaseolus acutifolius*) can be severely damaged by the bean weevil (*Acanthoscelides obtectus*) and the Mexican bean weevil (*Zabrotes subfasciatus*) (Dobie et al., 1990). This damage occurs in spite of the presence in the seeds of a family of plant defense proteins that comprises PHA and αAI (see Chrispeels and Raikhel, 1991). The three *Phaseolus* species are not equally sensitive to the bruchids. For example, tepary bean is more resistant than common bean to the bean weevil (Shade et al., 1987). This may be related to the presence in these seeds of high levels of the *P. acutifolius* seed lectin (Pratt et al., 1990). Another member of this protein family, arcelin, is found only in wild accessions of the common bean, and the introgression of the arcelin gene(s) yields varieties that are resistant to *Z. subfasciatus* (Andreas et al., 1986; Osborn et al., 1986; Cardona et al., 1990). It appears that the larvae of *A. obtectus* and *Z. subfasciatus* are unaffected by the high levels (0.5%) of αAI present in bean seeds. The same protein imparts resistance to other species of bruchids, the cowpea weevil (*Callosobruchus maculatus*) and the azuki bean weevil (*Callosobruchus chinensis*) (Ishimoto and Kitamura, 1989, 1992; Huesing et al., 1991). Whether αAI is also involved in the resistance of tepary bean to *C. maculatus* and *C. chinensis* (Birch et al., 1985) is not known. Transfer of the cDNA encoding αAI-1 from the common bean to either pea (*Pisum sativum*) or azuki bean (*Vigna angularis*) makes these grain legumes resistant to *C. maculatus* and *C. chinensis* (Shade et al., 1994; Ishimoto et al., 1996). The success of these genetic engineering experiments has encouraged us to study in greater detail the interaction between αAIs from three species in the genus *Phaseolus* with bruchid larval enzymes.

αAI is not just a single protein, but exists in at least two, and possibly more, isoforms (Mirkov et al., 1994; Ishimoto et al., 1995). The best-characterized α-amylase inhibitor of the common bean, now termed αAI-1, has been studied in many laboratories since 1945 (Bowman, 1945), and its cDNA has been cloned (Hoffman et al., 1982; Moreno and Chrispeels, 1989). More recently, a new αAI, αAI-2, was purified from seeds of a wild accession of the common bean resistant to *Z. subfasciatus* and its cDNA has been cloned (Suzuki et al., 1993, 1994; Mirkov et al., 1994). αAI-1 and αAI-2 share 78% amino acid identity. αAI-2 is found in those wild accessions of *P. vulgaris* that also contain arcelin. However, arcelin rather than αAI-2 is the biochemical basis of the resistance (Cardona et al., 1990; Minney et al., 1990; Suzuki et al., 1995; Fory et al., 1996). The αAI present in the tepary bean, here called αAI-Pa, has not yet been characterized but its polypeptides are similar in size to those of αAI-1 and cross-react with antibodies to αAI-1, indicating a relatively high degree of homology (Pueyo et al., 1993).

In this paper we present evidence that αAI-1 is rapidly cleaved by a *Z. subfasciatus* protease at the active site of the

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[3] Present address: National Agriculture Research Center, Tsukuba 305, Japan.

* Corresponding author; e-mail ishimoto@narc.affrc.go.jp; fax 81–298–38–8515.

Abbreviations: αAI, α-amylase inhibitor; AEBSF, 4-(2-aminoethyl)-benzensulfonyl fluoride hydrochloride; PHA, phytohemagglutinin; STI, soybean trypsin inhibitor.

protein. However, uncleaved αAI-1 does not inhibit *Z. subfasciatus* α-amylase. αAI-Pc is also cleaved by the protease and it also does not inhibit *Z. subfasciatus* α-amylase. αAI-2 is resistant to proteolytic digestion and it inhibits *Z. subfasciatus* α-amylase, but not *C. chinensis* α-amylase. αAI-Pa is also resistant to midgut proteases and it inhibits the α-amylases of both bruchid species. None of the αAIs inhibit the α-amylase of *A. obtectus*, and the larval midgut proteases of *A. obtectus* do not digest the inhibitors. These results demonstrate that different αAIs differentially inhibit the α-amylases of different bruchids and help to explain why the cultivated common bean is not resistant to the bean weevil and the Mexican bean weevil.

MATERIALS AND METHODS

Insects and Seeds

Laboratory colonies of *Acanthoscelides obtectus*, *Callosobruchus chinensis*, and *Zabrotes subfasciatus* originated in the United States, Japan, and Colombia, respectively, and were maintained in Petri dishes in a 30°C room.

The common bean variety (*Phaseolus vulgaris* cv Taishokintoki) was obtained from the Hokkaido Tokachi Agriculture Experiment Station (Hokkaido, Japan). The TAr4 line was obtained by six recurrent backcrossings to a common bean cultivar (Taisho-kintoki) as a recurrent parent with selection for the presence of αAI-2 derived from a wild *P. vulgaris* accession G12953. *Phaseolus coccineus* (cv Tecomari) and *Phaseolus acutifolius* (cv White) were obtained from Native Seeds/Search (Tucson, AZ). Tobacco seeds were obtained from plants transformed with the αAI-1 gene or a mutant αAI-1 (WSY188-190GNV) gene as described (Mirkov et al., 1995).

Seed Protein Extraction

Proteins were extracted from seeds by grinding them in a mortar with 20 mM PBS, pH 6.7 (50 mg seeds/mL buffer), and standing them for 60 min at room temperature. The supernatant was obtained after centrifugation at 15,000*g* for 10 min. Protein concentration was determined by the Lowry (1951) method using BSA as a standard.

αAI-1 Purification

αAI-1 was purified from the seeds of cultivar Taishokintoki according to the procedure described previously by Ishimoto and Kitamura (1989) with some modifications as follows. Thirty grams of the seed flour were extracted with 600 mL of 20 mM PBS, pH 6.7, at 4°C for 4 h. After centrifugation at 11,000*g* for 20 min, the supernatant was subjected to ammonium sulfate fractionation. The precipitate obtained between 20 and 60% saturation was dissolved with 200 mL of 20 mM PBS and dialyzed against the same buffer. After dialysis for 24 h, the resulting precipitate was removed by centrifugation and the supernatant was applied to a column of DEAE-Sephacel (Pharmacia) equilibrated with 20 mM PBS. The column was washed with the same buffer. Then, the adsorbed proteins were eluted with 0.25 M PBS, pH.6.7, and applied to a column of concanavalin A-Sepharose 4B (Pharmacia) equilibrated with 20 mM PBS, pH 6.7, containing 0.5 M NaCl. Proteins adsorbed on concanavalin A-Sepharose 4B were eluted with the buffer solution containing 0.1 M methyl-D-mannopyranoside and dialyzed against 20 mM PBS, pH 6.7, for 24 h and applied to the DEAE-Sephacel column again. A linear gradient elution (0.02-0.25 M PBS, pH 6.7) was used and the eluate was monitored by measuring the A_{280}. The fraction containing αAI-1 was collected and applied to a column of Sephacryl-S200 (Pharmacia) equilibrated with 50 mM PBS, pH 6.7, containing 0.15 M NaCl. The purified inhibitor was dialyzed against distilled water and lyophilized.

Larval Extraction

Midguts dissected from the last instar larvae of *C. chinensis*, *Z. subfasciatus*, and *A. obtectus* were homogenized in 20 mM PBS, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$ (50 μL/midgut), and centrifuged at 10,000*g* for 20 min. The supernatant was filtered through a 0.2-μm nitrocellulose membrane filter to remove small, suspended particles and bacteria. The larval midgut extracts were used immediately.

In Vitro Proteolysis and Its Inhibition

Proteolysis of the αAI by larval enzymes was determined by incubating seed extract containing 500 μg of protein or 35 mg of purified inhibitor with larval extract containing 1 unit of α-amylase activity at 37°C for the times indicated. The reaction was terminated by adding the same amount of a twice-concentrated SDS sample buffer and boiling for 2 min. SDS-PAGE (13.5% acrylamide) was performed according to the method of Laemmli (1970). After separation by SDS-PAGE, the proteins in the gel were transferred onto a nitrocellulose membrane and immunostained as detailed in the Bio-Rad technical bulletin. The proteins were detected using a rabbit anti-αAI serum for αAI-1, an anti-PHA serum for PHA, or an anti-phaseolin serum for phaseolin. The sera were all made against chemically deglycosylated proteins. We used goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) as a secondary antibody.

To determine the type of protease involved in αAI-1 cleavage by the *Z. subfasciatus* larval extract, we treated the extract with a variety of protease inhibitors, including PMSF, AEBSF, leupeptin, aprotinin, *N*-α-tosyl-L-lysin chloromethyl ketone, lima bean trypsin inhibitor, STI, tosyl-L-Phe chloromethyl ketone, chymostatin, *p*-chloromercuribenzoic acid, *N*-ethyl maleimide, *N*-[*n*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine, pepstatin A, and EDTA before the addition of seed extract.

α-Amylase and Inhibitory Activity

α-Amylase activity was measured by a modification of the Bernfeld method (1955). Porcine pancreatic α-amylase was purchased from Sigma. The larval midgut extract with 1 unit of α-amylase activity in 100 μL was preincubated with seed extract at 37°C for 15 min prior to the addition of 250 μL of substrate solution (1% soluble potato starch

[Sigma] solution in 0.1 M PBS, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$). After 10 min the reaction was terminated by the addition of 500 μL of 3,5-dinitrosalicylic acid reagent, followed by boiling for 10 min in a water bath. After addition of 5 mL of water, the solution was mixed and allowed to stand at room temperature for 15 min, and then A_{546} was measured. A standard curve was prepared using maltose. One α-amylase enzyme unit is defined as the amount of enzyme that liberates 1 μmol of maltose in 10 min at 37°C.

To prevent αAI from being cleaved by the *Z. subfasciatus* larval extracts, the larval extract was treated with 4 mM AEBSF for 15 min before the addition of the seed extract. Then the activity was measured as described above.

Determination of Binding Ability of Cleaved αAI-1 to α-Amylase

αAI-1 (100 μg) was dissolved in 150 μL of 15 mM succinate buffer (pH 5.6) containing 20 mM $CaCl_2$ and 0.5 M NaCl, and it was incubated with 50 μL of the *Z. subfasciatus* larval extract at 37°C for 3 h. Porcine pancreas α-amylase (Sigma) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions. The beads with immobilized enzyme were equilibrated with the succinate buffer, and the incubated inhibitor solution was added to 1 mL of beads. The mixture was tumbled at 37°C for 1 h, and then the beads were washed three times with 5 mL of the buffer. The unadsorbed fractions were collected and pooled. The bound inhibitor was released by tumbling for 30 min with 0.12 M sodium citrate (pH 3.0) (1 mL/mL beads). Then the suspension was centrifuged (1500*g* for 15 min) and the supernatant was saved. The citrate washing was repeated, and both supernatants were pooled as the adsorbed fraction. Both fractions were filtered through sintered glass funnels. Fractions were concentrated by precipitation with TCA (25% final concentration). Precipitates were dissolved in 200 μL of SDS sample buffer and analyzed by SDS-PAGE.

N-Terminal Amino Acid Sequencing

Tricine SDS-PAGE (15% acrylamide) for separation of digested αAI-1 was performed according to the method of Schagger and von Jagow (1987). After they were transferred to a PVDF membrane (Millipore), the bands corresponding to the peptides were cut out and the amino acid sequences were determined.

RESULTS

Specificity of αAI to Larval α-Amylases

Proteins from the seed extracts of *P. vulgaris*, *P. coccineus*, and *P. acutifolius* were separated by SDS-PAGE and immunoblotted with antibodies against αAI-1. The αAI polypeptides are in the 14.3- to 18.4-kD region of the immunoblot (Fig. 1), and the polypeptide patterns of the cultivated variety of *P. vulgaris* (lane 1, αAI-1) and of *P. coccineus* (lane 4, αAI-Pc) are the same. The polypeptide patterns of the seed extracts containing αAI-2 and αAI-Pa are quite different from that of αAI-1/αAI-Pc, and this difference may reflect greater evolutionary divergence. This difference in the polypeptide patterns has been observed previously (Pueyo et al., 1993; Suzuki et al., 1993). αAI-1 and αAI-Pc are more heavily stained than αAI-2 or αAI-Pa. It is not clear whether this less-intense staining reflects their lower abundance in the seeds or the evolutionary distance between them, and therefore a decrease of immunological cross-reactivity. These seed extracts also contained larger polypeptides around 30 to 32 kD that reacted with the anti-αAI-1 serum. These probably represent proteins in the same family, including uncleaved αAI precursor (Moreno et al., 1990), lectins, lectin-like proteins, or arcelins (Mirkov et al., 1994). Although this serum does not cross-react with PHA, it may cross-react with the lectins of the wild bean accessions and the tepary bean. The serum also reacts with αAI-like proteins that have an amino acid sequence that is closely related to αAI (Finardi-Filho et al., 1996).

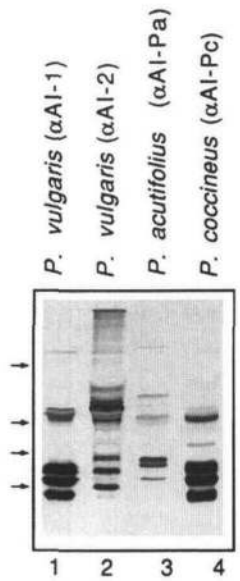

Figure 1. Immunoblot of seed extracts from different species in the genus *Phaseolus*. Lane 1, *P. vulgaris* (cv Taisho-kintoki, αAI-1); lane 2, *P. vulgaris* (TAr4 line, αAI-2); lane 3, *P. acutifolius* (αAI-Pa); lane 4, *P. coccineus* (αAI-Pc). The blot was developed with an antiserum against deglycosylated αAI-1. Arrows on the left indicate approximate molecular masses of 14.3, 18.4, 29, and 43 kD from bottom to top.

To study the specificity of these four αAIs to α-amylase enzymes, the seed extracts were tested for α-amylase inhibitory activity with α-amylases from larval midguts of three species of bruchids and α-amylase from porcine pancreas (Fig. 2). Seed extracts of *P. vulgaris* (αAI-1) and *P. coccineus* (αAI-Pc) inhibited both *C. chinensis* α-amylase and porcine α-amylase but not *A. obtectus* or *Z. subfasciatus* α-amylase. Seed extracts containing αAI-2 and αAI-Pa inhibited *Z. subfasciatus* α-amylase. The seed extract of *P. vulgaris* containing αAI-2 inhibited *Z. subfasciatus* α-amylase but not the other three α-amylases, as previously described (Ishimoto and Kitamura, 1993; Suzuki et al., 1993). The seed extract of *P. acutifolius* (αAI-Pa) inhib-

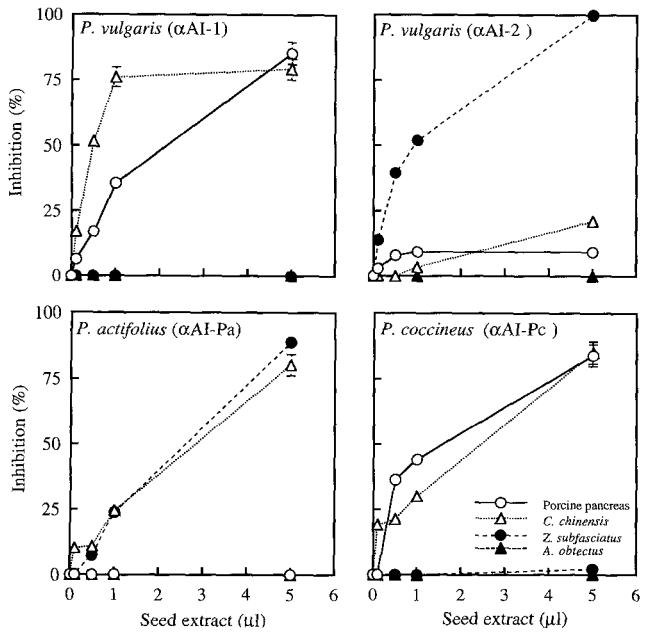

Figure 2. Effects of seed extracts containing αAI-1, αAI-2, αAI-Pa, or αAI-Pc on α-amylases from four different sources. Inhibition (%) is relative to 1 unit of α-amylase activity without addition of seed extract. Bars represent ±SD.

ited α-amylases from both *C. chinensis* and *Z. subfasciatus* but not *A. obtectus* or porcine α-amylase. Because of the complexity of the interaction, these results are summarized in Table I. This table makes it clear that the α-amylase of *A. obtectus* is not affected by any of the inhibitors, whereas porcine pancreatic α-amylase and *Z. subfasciatus* α-amylases have opposite specificity for inhibition by these four inhibitors. Together, these results show the variability that exists in both the α-amylases and the inhibitors.

Proteolysis of αAI by Larval Enzymes

Our previous work showed that *Z. subfasciatus* larval enzyme(s) can cleave αAI-1 to smaller molecules (Ishimoto and Kitamura, 1992). To study the cleavage of αAI by larval proteases, seed extracts were incubated with larval midgut extracts of *Z. subfasciatus*, *A. obtectus*, and *C. chinensis*, and the resulting αAI polypeptides were visualized on an immunoblot after SDS-PAGE. Incubation of seed extracts with *Z. subfasciatus* extract for 15 min resulted in the digestion of αAI-1 and αAI-Pc, as shown by a diminution in the staining intensity of the αAI polypeptides and by the appearance of a smaller polypeptide (see asterisks in Fig. 3). Thus, the two inhibitors that fail to inhibit *Z. subfasciatus* α-amylase (see Fig. 2) appear to be digested by midgut proteases. The two other inhibitors, αAI-2 and αAI-Pa, were not digested by midgut proteases of *Z. subfasciatus*. Extracts of *C. chinensis* and *A. obtectus* were unable to digest any of the four inhibitors tested. The specific digestion of αAI-1 and αAI-Pc stands in contrast to the lack of digestion of the major seed proteins phaseolin and PHA. The polypeptides retained their intensity of staining (Fig. 3), and after this short (15 min) incubation there were no breakdown products (data not shown). These results indicate that *Z. subfasciatus* has a protease that can quickly digest certain types of αAI.

Prevention of Proteolysis by Protease Inhibitors

To characterize the proteolytic activity responsible for the digestion of αAI-1, we repeated the incubation in the presence of 11 known protease inhibitors. These inhibitors inhibit the four major classes of proteases: Ser proteases, thiol proteases, aspartic proteases, and metalloproteases. Only 2 of the 11 inhibitors tested, AEBSF at 4 mM and PMSF at 1 mM, inhibited the proteolysis of αAI-1 (Fig. 4A). These two protease inhibitors are known to inhibit a broad range of Ser proteases (Gold, 1967; Mintz, 1993). Having defined the active protease as a Ser protease, we also tried the protease inhibitors tosyl-L-Phe chloromethyl ketone, STI, and chymostatin (Fig. 4B). Of these inhibitors, only STI at 1% (w/v) was effective, and it was more effective than AEBSF (compare lane 6 with lane 3 in Fig. 4B). STI inhibits trypsin-like as well as chymotrypsin-like enzymes of various species (Kassell, 1970).

The ability to prevent the degradation of αAI-1 and αAI-Pc with AEBSF made it possible to determine whether these αAIs actually inhibit *Z. subfasciatus* larval α-amylase. After all, the lack of inhibition described in Figure 2 probably resulted from αAI-1 and αAI-Pc degradation. For these experiments, we used the water-soluble AEBSF, which at 4 mM does not inhibit bruchid α-amylase (data not shown). When the experiments shown in Figure 2 were repeated in the presence of AEBSF, αAI-1 and αAI-Pc still did not inhibit *Z. subfasciatus* α-amylase (data not shown). Thus, this bruchid species has double protection from the αAI found in cultivated beans: its α-amylase is not inhibited and it has the protease able to digest the αAI.

Inactivation of αAI-1 by Larval Proteases

Larval protease(s) are able to digest αAI-1 to smaller polypeptides, and the polypeptide pattern before and after incubation of purified αAI-1 with a larval midgut extract from *Z. subfasciatus*, as shown in Figure 5, lanes 1 and 2, respectively. To find out if this proteolytic cleavage inactivated αAI-1, we passed the mixture after digestion over an affinity column consisting of porcine pancreatic α-amylase coupled to Sepharose 4B and stained the polypeptides in the bound (and eluted) fraction and the unbound fraction with Coomassie brilliant blue (Fig. 5, lanes 3-6). As a control, we treated purified αAI-1 with a larval midgut extract

Table I. *Specificity of inhibition of four different α-amylases by four αAIs from different sources*

Sources of α-Amylase	Type of αAI			
	αAI-1	αAI-2	αAI-Pa	αAI-Pc
Porcine pancreas	+	−	−	+
C. chinensis	+	−	+	+
Z. subfasciatus	−	+	+	−
A. obtectus	−	−	−	−

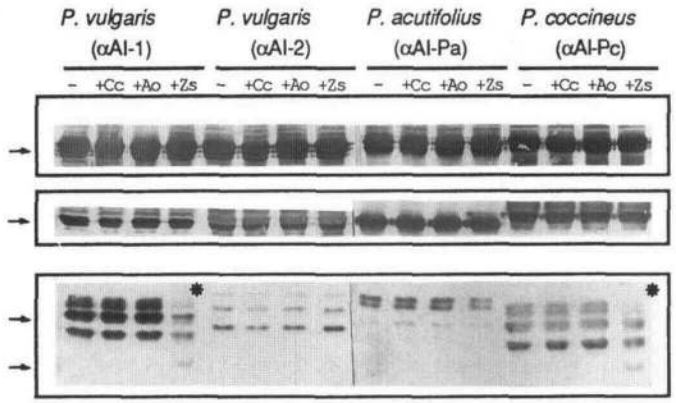

Figure 3. Digestion of αAI, PHA, and phaseolin by larval midgut extracts from different bruchid species. Proteins from seed extracts containing αAI-1, αAI-2, αAI-Pa, or αAI-Pc were incubated with larval extracts from *C. chinensis* (+Cc), *A. obtectus* (+Ao), *Z. subfasciatus* (+Zs), or PBS (−) and immunoblotted with antibodies against phaseolin, PHA, and αAI. Asterisks indicate proteolysis products of αAI. Arrows on the left indicate approximate molecular masses of 6.2, 14.3, 29, and 43 kD from bottom to top.

from *C. chinensis*. In this case, αAI-1 polypeptides were present in the bound fraction (lane 6) but not in the unbound fraction (lane 5). The opposite was the case after treatment with larval midgut extracts from *Z. subfasciatus*. In that case, all of the polypeptides were in the unbound fraction (lane 3) rather than in the bound fraction (lane 4). Thus, after αAI-1 was cleaved by the midgut protease(s) it failed to bind the α-amylase column, suggesting that it had been inactivated.

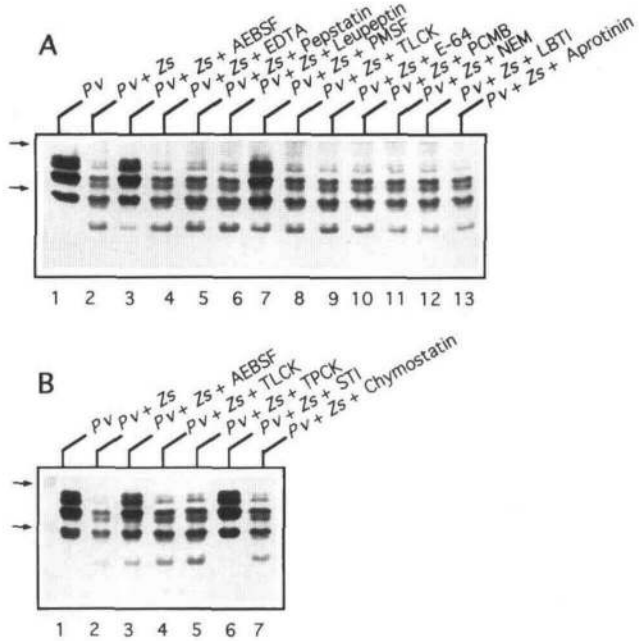

Figure 4. Effect of protease inhibitors on the digestion of αAI-1 by larval gut extracts. Proteins from seed extracts (*Pv*) containing αAI-1 were incubated with larval extracts from *Z. subfasciatus* (*Zs*) in the presence of 1 of 11 (A) or 5 (B) different protease inhibitors and immunoblotted with antibodies against αAI. Arrows on the left indicate approximate molecular masses of 14.3 and 18.4 kD from bottom to top. TLCK, *N*-α-tosyl-L-lysin chloromethyl ketone; E-64, *N*-[*n*-(L-3-*trans*-carboxirane-2-carbonyl)-L-leucyl]-agmatine; PCMB, *p*-chloromercuribenzoic acid; NEM, *N*-ethyl maleimide; LBTI, lima bean trypsin inhibitor; TPCK, tosyl-L-Phe chloromethyl ketone.

Cleavage of αAI-1 at Its Active Site by Larval Protease

To determine the site at which αAI-1 is cleaved by *Z. subfasciatus* larval midgut protease, we analyzed the amino acid sequences of the N termini of the new peptides. Purified αAI-1 was incubated with a larval midgut extract of *Z. subfasciatus*, and the resulting polypeptides were separated by Tricine SDS-PAGE and transferred onto a PVDF membrane. The amino acid sequences at the N termini of the polypeptides were determined. αAI-1 normally has two different subunits, α and β, with N termini corresponding to ATETSF and SAVGLD, respectively (Moreno and Chrispeels, 1989). Among the cleavage products, we found an additional polypeptide that starts with SAVGLD and is somewhat smaller than the normal β subunit and a 3-kD polypeptide that has SYETHDVL at its amino terminus (Fig. 6, d and e). The latter sequence is found close to the C terminus of the β subunit and starts within the active site of αAI-1 (Fig. 7). The active site of αAI-1 is composed of an Arg residue in the α subunit and Trp and Tyr residues in the β subunit that are part of a TrpSerTyr motif (Mirkov et al., 1995). The larval protease cuts between the Trp and the SerTyr in this motif. It is interesting to note in this respect that αAI-2 has a different motif at this position: TrpSerTyr (WSY) is replaced by TyrSerPhe (YSF).

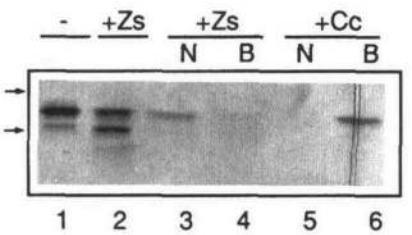

Figure 5. Inactivation of αAI-1 by *Z. subfasciatus* larval protease(s). Purified αAI-1 was incubated with larval midgut extracts from *Z. subfasciatus* (+Zs) or *C. chinensis* (+Cc) and fractionated on porcine pancreatic α-amylase immobilized to Sepharose 4B into a bound (B) fraction and an unbound (N) fraction. Untreated (−) αAI-1 is shown for comparison (lane 1). Arrows on the left indicate approximate molecular masses of 14.3 and 18.4 kD from bottom to top.

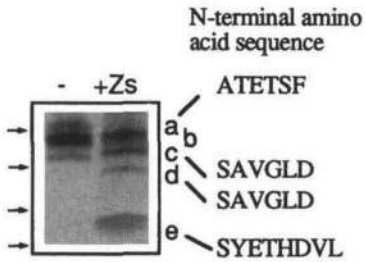

Figure 6. Tricine SDS-PAGE analysis of αAI-1 digested by *Z. subfasciatus* larval protease(s) (+Zs) and the N-terminal amino acid sequences obtained by sequencing the polypeptides of resulting polypeptides. Arrows on the left indicate approximate molecular masses of 3, 6.2, 14.3, and 18.4 kD from bottom to top.

Prevention of the Proteolytic Cleavage by a Simple Mutation at the Active Site

As part of our previous work to determine the active site of αAI-1 (Mirkov et al., 1995), the WSY motif was replaced with GNV, the tripeptide found in PHA-L at this equivalent position (Fig. 7). When this αAI-1 mutant, here called αAI-1ΔWSY, was expressed in tobacco, it was found to be inactive as an inhibitor. When αAI-1 and the mutant αAI-1ΔWSY are expressed in tobacco, the polypeptides are processed somewhat differently compared to the processing in common beans, but the αAI-1 polypeptides form an active αAI-1 protein (Altabella and Chrispeels, 1990). Treatment of tobacco seed extracts with larval midgut proteases of *Z. subfasciatus* altered the polypeptide pattern of αAI-1 (Fig. 8, lane 4) but did not affect the polypeptide pattern of the mutant (lanes 5 and 6). Thus, this simple mutation is sufficient to make the αAI-1 protein resistant to the *Z. subfasciatus* protease.

DISCUSSION

The results in this paper provide further insight into the coevolution of insects and their host plants. The data presented here lead to two important conclusions. First, there is considerable specificity and variation in the inhibitory activities of bean αAIs with respect to the α-amylases of different bruchids. Second, the Mexican bean weevil has a double protection against αAI-1 and αAI-Pc: its midgut α-amylase is not inhibited by these inhibitors and a larval midgut Ser protease can cleave the inhibitor.

How Do Bruchids Tolerate High Levels of αAI in Beans?

Proteinaceous α-amylase inhibitors occur not only in some legumes in the genus *Phaseolus*, but also in the starchy plant parts of other species. These inhibitors have been most extensively studied in wheat (*Triticum aestivum*) grains and other cereals. Wheat inhibitors inhibit the α-amylases of the insects that cause damage to the grains and to the wheat products in storage (Silano et al., 1975; Yetter et al., 1978; Gutierrez et al., 1990). However, storage pests that normally attack wheat have a markedly higher level of α-amylase in their digestive tracts than insects that do not develop on wheat, and it is precisely because of these high α-amylase levels that the pests can overcome the αAIs present in wheat and other cereals. It appears that bruchids that infest common beans have overcome the αAI problem in a different way. Two bruchid species in the Americas, *A. obtectus* and *Z. subfasciatus*, develop on cultivated common beans in spite of the presence of high levels of αAI-1. These two bruchids have midgut α-amylases that are not inhibited by αAI-1, and in addition, *Z. subfasciatus* has a larval midgut protease that can cleave the inhibitor at the active site to inactivate it. This cleavage removes a small C-terminal peptide and makes the inhibitor ineffective against pancreatic α-amylase. Cys proteases rather than Ser proteases are abundant in larval midguts of bruchids and may be primarily responsible for protein digestion (Kitch and Murdock, 1986; Campos et al., 1989). However, prevention of proteolysis by protease inhibitors shows that the midgut protease responsible for the cleavage of αAI-1 is a Ser protease.

αAI-1 effectively inhibits the development of other bruchid species (*C. chinensis*, *Callosobruchus maculatus*, and *Bruchus pisorum*) because it inhibits their midgut α-amylase, and it is obviously present at high enough levels to prevent larval development (Shade et al., 1994; Schroeder et al., 1995; Ishimoto et al., 1996). However, these are all Old World bruchids that did not evolve in the same location as the common bean, a New World plant. We do not know if the α-amylase gene(s) of *Z. subfasciatus* and *A. obtectus* evolved in response to the selection pressure

```
          1        10        20 ∇      30        40        50
αAI-1     MASSKLLSLALFLALLSHANSATETSFIIDAFNKTNLILQGDATVSSNGN
αAI-2     MASSNLLTLALFLVLLTHANSASDTSFNFYSFNETNLILQGDATVSSKGY
PHA-L     MASSKFFTV-LFLVLLTHANSSNDIYFNFQRFNETNLILQRDASVSSSGQ

                  60        70        80        90        100
αAI-1     LQLS------YNSYDSMSRAFYSPPIQIRDSTTGNVASFDTNFTMNIRTH
αAI-2     LQL--------HTVDSMCSAFYSAPIQIRDSTTGNVASFDTNFTMNITTQ
PHA-L     LRLTNLNGNGEPRVGSLGRAFYSAPIQIWDNTTGTVASFATSFTFNIQVV

             ∇  110       120       130       140       150
αAI-1     RQANSAVGLDFVLVPV--QPESKG---------------DTVTVEF----
αAI-2     REANSVIGLDFALVPV--QPKSKG---------------HTVTVQF----
PHA-L     NNAGPADGLAFALVPVGSQPKDKGGFLGLFDGSNSNFHT--VAVEFDTLV

               160       170       180       190       200
αAI-1     --DTFLSRISIDVNNN-DIKSVPWDVHDYDGQNAEVRITYNSSTKVFSVS
αAI-2     --DTFRSRISIDVNNN-DIKSVPWDEQDYDGQNAKVRITYNSSTKVLAVS
PHA-L     NKDWDPTERHIGIDVNSIRSIKTTRWDFVNGENAEVLITYDSSTNLLVAS

                  210       220       230       ↓         250
αAI-1     LSNPPTGKSNNVSTTVELEKEVYNWVSVGFSATSGAYQWSYETHDVLSWS
αAI-2     LSNPSTGKSNEVSARMEVEKELDDWVRVGFSAISGVHEYSFETRDVLSWS
PHA-L     LVYPSQKTSFIVSDTVDLKSVLPEWVSVGFSATTGINKGNVETNDVLSWS

               260       270
αAI-1     FSSKFINLKDQKSER-SNIVLNKIL
αAI-2     FSSKFSQ--HTTSER-SNILLNNIL
PHA-L     FASKLSDGTTSEGLNLANLVLNKIL
```

Figure 7. Amino acid sequence comparison of αAI-1, αAI-2, and the PHA-L isolectin. Triangles indicate the N termini of the α and β subunits of mature αAI-1 and αAI-2. Double underlines represent the N-terminal amino acid sequences obtained by sequencing the polypeptides of digested αAI-1 protein. Boldface characters show the residues that form the active site of αAI-1. The arrow indicates the cleavage position of αAI-1 by larval gut protease(s) of *Z. subfasciatus*.

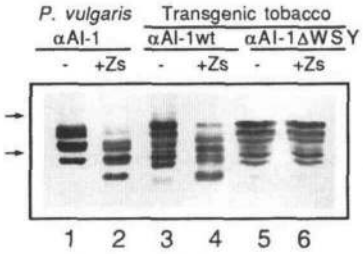

Figure 8. Resistance of a mutant αAI-1 expressed in tobacco seeds to *Z. subfasciatus* larval protease(s). Proteins from seed extracts of *P. vulgaris* (αAI-1) and transgenic tobacco with the wild-type gene for αAI-1 (αAI-1wt) and the WSY→GNV mutant gene (αAI-1ΔWSY) were incubated with larval extracts from *Z. subfasciatus* (+Zs) or PBS (−) and immunoblotted with antibodies against αAI. Arrows on the left indicate approximate molecular masses of 14.3 and 18.4 kD from bottom to top.

caused by the presence of αAI of common beans or if these bruchids simply invaded new territory and took advantage of a food source that had an ineffective αAI. Coevolution is defined as "an evolutionary change in a trait of the individuals in one population in response to a trait of the individuals of a second population, followed by an evolutionary response by the second population to the change in the first" (Janzen, 1980). If the evolution of the α-amylase genes in *Z. subfasciatus* and *A. obtectus* and/or the protease in *Z. subfasciatus* that cleaves αAI-1 made it possible for these bruchids to use common beans as a food source, then the emergence of a bean population that carries the gene for αAI-2 could be viewed as a case of coevolution. However, tepary beans also evolved with these two bruchids and they are more resistant to these bruchids than common beans (Shade et al., 1987). Certain wild accessions and some cultivars of common beans have both αAI-1 and αAI-2 (M. Ishimoto, unpublished results), suggesting that αAI-2 may have arisen by duplication or recombination of genes at this locus. The presence of the Ser protease in *Z. subfasciatus* cannot be considered a response of this bruchid to the αAI-1 in the seeds, because αAI-1 does not inhibit its α-amylase.

Why Does αAI Not Play a Role in the Resistance of Introgressed Varieties?

Certain wild accessions of common beans are resistant to *Z. subfasciatus* and several groups have introgressed this resistance into cultivars by selecting for arcelin (Schoonhoven et al., 1983; Osborn et al., 1988; Cardona et al., 1990). Arcelin is not a single protein, but is classified as six electrophoretic variants (Osborn et al., 1986; Suzuki et al., 1995). Some of the arcelin genotypes, Arc3, Arc4, and Arc6, also contain αAI-2 in the same locus (Suzuki et al., 1995). Thus, the introgression of these arcelin genotypes into cultivars results in the replacement of αAI-1 by αAI-2. Yet, it appears that αAI-2 is not an important factor in resistance to *Z. subfasciatus*, and resistance is ascribed to the high levels of arcelin (Minney et al., 1990; Fory et al., 1996). As discussed above for wheat αAI, the level of αAI-2 in these introgressed varieties may not be high enough to play a role in bruchid resistance. Feeding tests showed that increasing the content of αAI-2 to 1% resulted in the inhibition of larval development of *Z. subfasciatus* (Suzuki et al., 1993). This suggests that high levels of αAI-2 can inhibit the development of these larvae and that αAI-2 and αAI-Pa could be significant tools for genetic engineering not only for beans, but also for other grain legumes that are damaged by *Z. subfasciatus*.

Resistance to *A. obtectus* is also found in some wild accessions of *P. vulgaris* that contain arcelins (Schoonhoven et al., 1983). However, introgression of this locus into cultivated common beans did not yield resistant varieties (Kornegay et al., 1993). We did not find a candidate αAI variant that could be used for genetic engineering of resistance in grain legumes to *A. obtectus*. None of the αAIs tested was effective against *A. obtectus* α-amylase.

The Structures of αAIs and Their Relationship to the Resistance Mechanism against the Mexican Bean Weevil Larval Protease

αAI-1 and αAI-2 share 78% amino acid sequence identity, indicating considerable evolutionary divergence of these proteins within the same species (Mirkov et al., 1994; Suzuki et al., 1994). In earlier work the active site of αAI-1 was determined as consisting of R74 in the α subunit and the WSY motif near the C terminus of the β subunit (Mirkov et al.,1995). αAI-2 also has an Arg residue (R72) corresponding to the R74 of αAI-1, but a YSF motif replaces the WSY motif of αAI-1. It is possible that this replacement changes the specificity of the inhibitor and its resistance to the *Z. subfasciatus* larval protease at the same time. It was suggested that the Arg residue in αAI could interact with the Asp residue at the active site of α-amylase (Qian et al., 1993), whereas the flanking Trp and Tyr residues in αAI-1 may bind to neighboring subsites, mimicking the Glc residues of the substrate (Mirkov et al., 1995). The corresponding Tyr and Phe residues in αAI-2 may also bind to neighboring subsites, but of an α-amylase with a somewhat different amino acid sequence.

Our findings that αAI-1 is cleaved at a unique position by the *Z. subfasciatus* larval protease and that the simple mutation with GNV replacing the WSY motif prevents the cleavage of αAI-1 by the midgut protease suggest that this protease is highly sequence specific. The failure of the midgut protease to cleave αAI-2 is probably related to the replacement of the WSY motif of αAI-1 by YSF in αAI-2. However, we cannot rule out that other factors, such as the higher-order structure of the protein or the placement of glycan, are also important in this resistance to proteolytic digestion as well as to the specificity against α-amylases.

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