

Protective Mechanism of the Mexican Bean Weevil against High Levels of α -Amylase Inhibitor in the Common Bean¹

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 (α AI) 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 α AI-1 present in seeds of the common bean, of a different isoform, α AI-2, present in seeds of wild common bean accessions, and of two homologs, α AI-Pa present in seeds of the tepary bean (*Phaseolus acutifolius*) and α AI-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 α AI-1 and α AI-Pc, but not α AI-2 or α AI-Pa. The digestion is caused by a serine protease. A single proteolytic cleavage in the β subunit of α AI-1 occurs at the active site of the protein. When degradation is prevented, α AI-1 and α AI-Pc do not inhibit the α -amylase of *Z. subfasciatus*, although they are effective against the α -amylase of *C. chinensis*. α AI-2 and α AI-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 α AI (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 α AI 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 α AI 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 α AI-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 α AIs from three species in the genus *Phaseolus* with bruchid larval enzymes.

α AI 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 α AI-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 α AI, α AI-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). α AI-1 and α AI-2 share 78% amino acid identity. α AI-2 is found in those wild accessions of *P. vulgaris* that also contain arcelin. However, arcelin rather than α AI-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 α AI present in the tepary bean, here called α AI-Pa, has not yet been characterized but its polypeptides are similar in size to those of α AI-1 and cross-react with antibodies to α AI-1, indicating a relatively high degree of homology (Pueyo et al., 1993).

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

¹ Supported by a grant from the U.S. Department of Energy-Energy Biosciences Program.

² M.I. was supported by a grant from the Science and Technology Agency, Japan.

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: α AI, α -amylase inhibitor; AEBSF, 4-(2-aminooethyl)-benzenesulfonyl fluoride hydrochloride; PHA, phytohemagglutinin; STI, soybean trypsin inhibitor.

protein. However, uncleaved α AI-1 does not inhibit *Z. subfasciatus* α -amylase. α AI-Pc is also cleaved by the protease and it also does not inhibit *Z. subfasciatus* α -amylase. α AI-2 is resistant to proteolytic digestion and it inhibits *Z. subfasciatus* α -amylase, but not *C. chinensis* α -amylase. α AI-Pa is also resistant to midgut proteases and it inhibits the α -amylases of both bruchid species. None of the α AI's 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 α AI's 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 Taisho-kintoki) 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 α AI-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 α AI-1 gene or a mutant α AI-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 15,000g for 10 min. Protein concentration was determined by the Lowry (1951) method using BSA as a standard.

α AI-1 Purification

α AI-1 was purified from the seeds of cultivar Taisho-kintoki 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 11,000g 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 concanava-

lin 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 α AI-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 10,000g 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 α AI 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- α AI serum for α AI-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 α AI-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-lysine 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 $\alpha\text{AI-1}$ to α -Amylase

$\alpha\text{AI-1}$ (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 (1500g 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 $\alpha\text{AI-1}$ 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 $\alpha\text{AI-1}$. 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, $\alpha\text{AI-1}$) and of *P. coccineus* (lane 4, $\alpha\text{AI-Pc}$) are the same. The polypeptide patterns of the seed extracts containing $\alpha\text{AI-2}$ and $\alpha\text{AI-Pa}$ are quite differ-

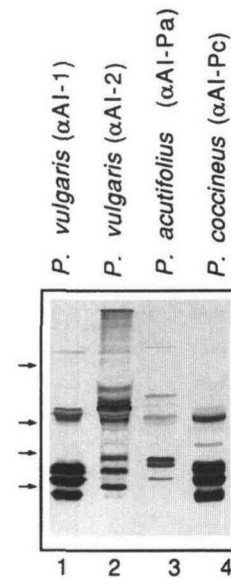


Figure 1. Immunoblot of seed extracts from different species in the genus *Phaseolus*. Lane 1, *P. vulgaris* (cv Taisho-kintoki, $\alpha\text{AI-1}$); lane 2, *P. vulgaris* (TAr4 line, $\alpha\text{AI-2}$); lane 3, *P. acutifolius* ($\alpha\text{AI-Pa}$); lane 4, *P. coccineus* ($\alpha\text{AI-Pc}$).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

ent from that of $\alpha\text{AI-1}/\alpha\text{AI-Pc}$, 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). $\alpha\text{AI-1}$ and $\alpha\text{AI-Pc}$ are more heavily stained than $\alpha\text{AI-2}$ or $\alpha\text{AI-Pa}$. 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 large polypeptides around 30 to 32 kD that reacted with the anti- $\alpha\text{AI-1}$ 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).

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* ($\alpha\text{AI-1}$) and *P. coccineus* ($\alpha\text{AI-Pc}$) inhibited both *C. chinensis* α -amylase and porcine α -amylase but not *A. obtectus* or *Z. subfasciatus* α -amylase. Seed extracts containing $\alpha\text{AI-2}$ and $\alpha\text{AI-Pa}$ inhibited *Z. subfasciatus* α -amylase. The seed extract of *P. vulgaris* containing $\alpha\text{AI-2}$ 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* ($\alpha\text{AI-Pa}$) inhib-

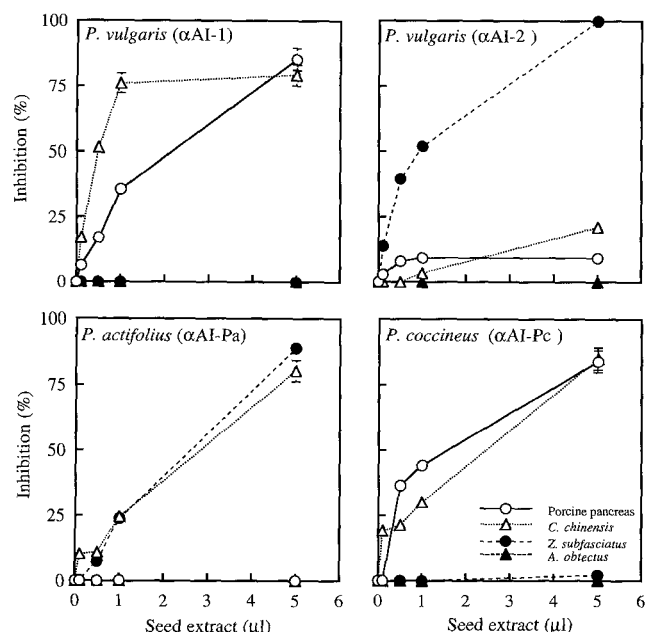


Figure 2. Effects of seed extracts containing α AI-1, α AI-2, α AI-Pa, or α AI-Pc on α -amylases from four different sources. Inhibition (%) is relative to 1 unit of α -amylase activity without addition of seed extract. Bars represent \pm SD.

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 α AI by Larval Enzymes

Our previous work showed that *Z. subfasciatus* larval enzyme(s) can cleave α AI-1 to smaller molecules (Ishimoto and Kitamura, 1992). To study the cleavage of α AI by larval proteases, seed extracts were incubated with larval midgut extracts of *Z. subfasciatus*, *A. obtectus*, and *C. chinensis*, and the resulting α AI 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 α AI-1 and α AI-Pc, as shown by a diminution in the staining intensity of the α AI 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, α AI-2 and α AI-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 α AI-1 and α AI-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 α AI.

Prevention of Proteolysis by Protease Inhibitors

To characterize the proteolytic activity responsible for the digestion of α AI-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 α AI-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 α AI-1 and α AI-Pc with AEBSF made it possible to determine whether these α AI actually inhibit *Z. subfasciatus* larval α -amylase. After all, the lack of inhibition described in Figure 2 probably resulted from α AI-1 and α AI-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, α AI-1 and α AI-Pc still did not inhibit *Z. subfasciatus* α -amylase (data not shown). Thus, this bruchid species has double protection from the α AI found in cultivated beans: its α -amylase is not inhibited and it has the protease able to digest the α AI.

Inactivation of α AI-1 by Larval Proteases

Larval protease(s) are able to digest α AI-1 to smaller polypeptides, and the polypeptide pattern before and after incubation of purified α AI-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 α AI-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 α AI-1 with a larval midgut extract

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

Sources of α -Amylase	Type of α AI			
	α AI-1	α AI-2	α AI-Pa	α AI-Pc
Porcine pancreas	+	-	-	+
<i>C. chinensis</i>	+	-	+	+
<i>Z. subfasciatus</i>	-	+	+	-
<i>A. obtectus</i>	-	-	-	-

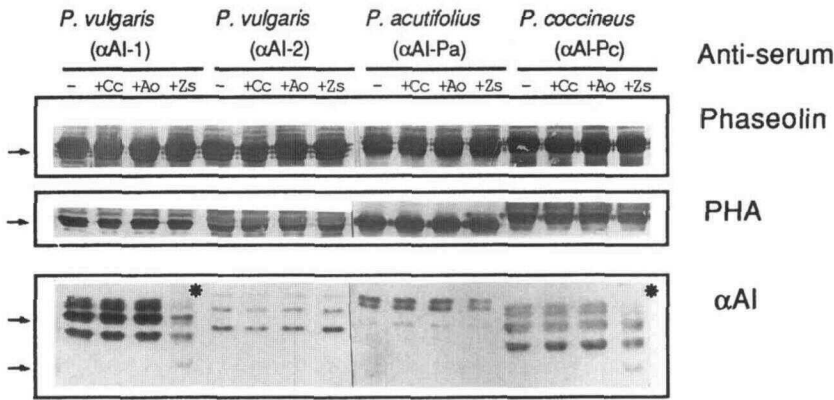


Figure 3. Digestion of α AI, PHA, and phaseolin by larval midgut extracts from different bruchid species. Proteins from seed extracts containing α AI-1, α AI-2, α AI-Pa, or α AI-Pc were incubated with larval extracts from *C. chinensis* (+Cc), *A. obtectus* (+Ao), *Z. subfasciatus* (+Zs), or PBS (-) and immunoblotted with antibodies against phaseolin, PHA, and α AI. Asterisks indicate proteolysis products of α AI. 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, α AI-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 α AI-1 was cleaved by the midgut protease(s) it failed to bind the α -amylase column, suggesting that it had been inactivated.

Cleavage of α AI-1 at Its Active Site by Larval Protease

To determine the site at which α AI-1 is cleaved by *Z. subfasciatus* larval midgut protease, we analyzed the amino acid sequences of the N termini of the new peptides. Purified α AI-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. α AI-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 α AI-1 (Fig. 7). The active site of α AI-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 α AI-2 has a different motif at this position: TrpSerTyr (WSY) is replaced by TyrSerPhe (YSF).

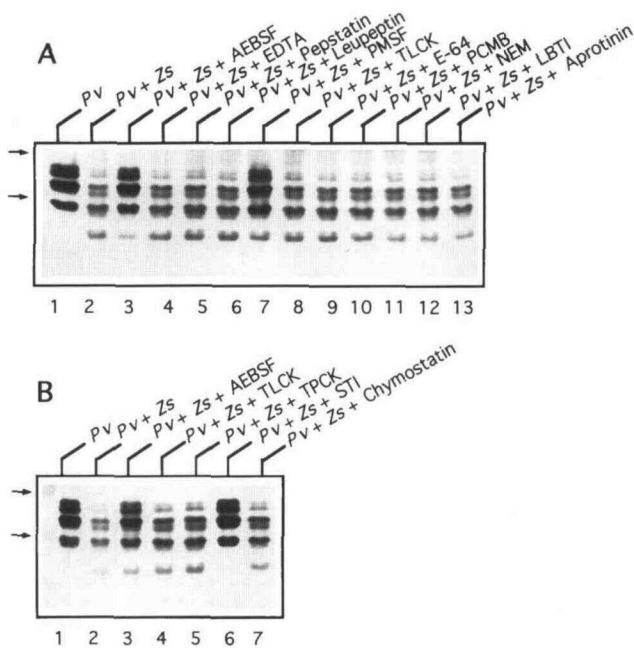


Figure 4. Effect of protease inhibitors on the digestion of α AI-1 by larval gut extracts. Proteins from seed extracts (Pv) containing α AI-1 were incubated with larval extracts from *Z. subfasciatus* (Zs) in the presence of 1 of 11 (A) or 5 (B) different protease inhibitors and immunoblotted with antibodies against α AI. Arrows on the left indicate approximate molecular masses of 14.3 and 18.4 kD from bottom to top. TLCK, *N*- α -tosyl-L-lysine 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.

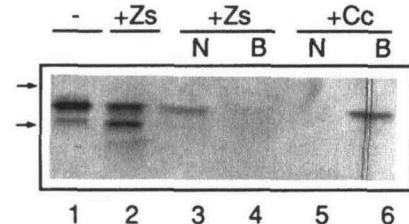


Figure 5. Inactivation of α AI-1 by *Z. subfasciatus* larval protease(s). Purified α AI-1 was incubated with larval midgut extracts from *Z. subfasciatus* (+Zs) or *C. chinensis* (+Cc) and fractionated on porcine pancreatic α -amylase immobilized to Sepharose 4B into a bound (B) fraction and an unbound (N) fraction. Untreated (-) α AI-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.

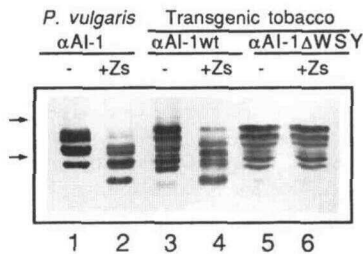


Figure 8. Resistance of a mutant α AI-1 expressed in tobacco seeds to *Z. subfasciatus* larval protease(s). Proteins from seed extracts of *P. vulgaris* (α AI-1) and transgenic tobacco with the wild-type gene for α AI-1 (α AI-1wt) and the WSY \rightarrow GNV mutant gene (α AI-1 Δ WSY) were incubated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

caused by the presence of α AI of common beans or if these bruchids simply invaded new territory and took advantage of a food source that had an ineffective α AI. 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 α AI-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 α AI-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 α AI-1 and α AI-2 (M. Ishimoto, unpublished results), suggesting that α AI-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 α AI-1 in the seeds, because α AI-1 does not inhibit its α -amylase.

Why Does α AI 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 α AI-2 in the same locus (Suzuki et al., 1995). Thus, the introgression of these arcelin genotypes into cultivars results in the replacement of α AI-1 by α AI-2. Yet, it appears that α AI-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 α AI, the level of α AI-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 α AI-2 to 1% resulted in the inhibition of larval development of *Z. subfasciatus* (Suzuki et al., 1993). This suggests that high levels of α AI-2 can inhibit the development of these larvae and that α AI-2 and α AI-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 α AI variant that could be used for genetic engineering of resistance in grain legumes to *A. obtectus*. None of the α AI tested was effective against *A. obtectus* α -amylase.

The Structures of α AI-1 and α AI-2 and Their Relationship to the Resistance Mechanism against the Mexican Bean Weevil Larval Protease

α AI-1 and α AI-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 α AI-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). α AI-2 also has an Arg residue (R72) corresponding to the R74 of α AI-1, but a YSF motif replaces the WSY motif of α AI-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 α AI 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 α AI-1 may bind to neighboring subsites, mimicking the Glc residues of the substrate (Mirkov et al., 1995). The corresponding Tyr and Phe residues in α AI-2 may also bind to neighboring subsites, but of an α -amylase with a somewhat different amino acid sequence.

Our findings that α AI-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 α AI-1 by the midgut protease suggest that this protease is highly sequence specific. The failure of the midgut protease to cleave α AI-2 is probably related to the replacement of the WSY motif of α AI-1 by YSF in α AI-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.

ACKNOWLEDGMENT

We thank T. Erik Mirkov for making the Δ WSY mutant of α AI-1 in which WSY is substituted for by GNV.

Received November 3, 1995; accepted February 28, 1996.
Copyright Clearance Center: 0032-0889/96/111/0393/09.

LITERATURE CITED

- Altabella T, Chrispeels MJ (1990) Tobacco plants transformed with the bean α i gene express an inhibitor of insect α -amylase in their seeds. *Plant Physiol* **93**: 805–810
- Andreas JR, Yandell BS, Bliss FA (1986) Bean arcelin 1. Inheritance of a novel seed protein of *Phaseolus vulgaris* L. and its effect on seed composition. *Theor Appl Genet* **72**: 123–128
- Bernfeld P (1955) Amylases, α and β . *Methods Enzymol* **1**: 149–158
- Birch N, Southgate BJ, Fellows LE (1985) Plants for arid lands. In GE Wickens, JR Goodin, DV Field, eds, *Royal Botanic Gardens*, Kew. George Allen and Unwin, London, 303–318
- Bowman DE (1945) Amylase inhibitor of navy bean. *Science* **102**: 358–359
- Campos FAP, Xavier-Filho J, Silva CP, Ary MB (1989) Resolution and partial characterization of proteinases and α -amylase from midguts of larvae of the bruchid beetle *Callosobruchus maculatus* (F.). *Comp Biochem Physiol* **92B**: 51–57
- Cardona C, Kornegay J, Posso CE, Morales F, Ramirez H (1990) Comparative value of four arcelin variants in the development of dry bean lines resistant to the Mexican bean weevil. *Entomol Exp Appl* **56**: 197–206
- Chrispeels MJ, Raikhel NV (1991) Lectins, lectin genes and their role in plant defense. *Plant Cell* **3**: 1–19
- Dobie P, Dendy J, Sherman C, Padgham J, Wood A, Gatehouse AMR (1990) New sources of resistance to *Acanthoscelides obtectus* (Say) and *Zabrotes subfasciatus* Boheman (Coleoptera:Bruchidae) in mature seeds of five species of *Phaseolus*. *J Stored Prod Res* **26**: 177–186
- Finardi-Filho F, Mirkov TE, Chrispeels MJ (1996) A putative precursor protein in the evolution of the bean α -amylase inhibitor. *Phytochemistry* (in press)
- Fory LF, Finardi-Filho F, Quintero CM, Osborn TC, Cardona C, Chrispeels M, Mayer JE (1996) α -Amylase inhibitors in resistance of common beans to the Mexican bean weevil and the bean weevil (Coleoptera:Bruchidae). *J Econ Entomol* **89**: 204–210
- Gold AM (1967) Sulfonylation with sulfonyl halides. In CHW Hirs, ed, *Enzyme Structure*, Vol 11. Academic Press, New York, pp 706–711
- Gutierrez C, Sanchez-Monge R, Gomez L, Ruiz-Tapiador M, Castanera P, Salcedo G (1990) α -Amylase activities of agriculture insect pests are specifically affected by different inhibitor preparations from wheat and barley endosperms. *Plant Sci* **72**: 37–44
- Hoffman LM, Ma Y, Barker RF (1982) Molecular cloning of *Phaseolus vulgaris* lectin mRNA and use of cDNA as a probe to estimate lectin transcript levels in various tissues. *Nucleic Acids Res* **10**: 7819–7828
- Huesing JE, Shade RE, Chrispeels MJ, Murdock LL (1991) α -Amylase inhibitor, not phytohemagglutinin, explains the resistance of common bean seeds to cowpea weevil. *Plant Physiol* **96**: 993–996
- Ishimoto M, Kitamura K (1989) Growth inhibitory effects of an α -amylase inhibitor from kidney bean, *Phaseolus vulgaris* (L.) on three species of bruchids (Coleoptera:Bruchidae). *Appl Entomol Zool* **24**: 281–286
- Ishimoto M, Kitamura K (1992) Tolerance to the seed α -amylase inhibitor by the 2 insect pests of the common bean, *Zabrotes subfasciatus* and *Acanthoscelides obtectus* (Coleoptera,Bruchidae). *Appl Entomol Zool* **27**: 243–251
- Ishimoto M, Kitamura K (1993) Specific inhibitory activity and inheritance of an α -amylase inhibitor in a wild common bean accession resistant to the Mexican bean weevil. *Jpn J Breed* **43**: 69–73
- Ishimoto M, Sato T, Chrispeels MJ, Kitamura K (1996) Bruchid resistance of transgenic azuki bean expressing α -amylase inhibitor of common beans. *Entomol Exp Appl* (in press)
- Ishimoto M, Suzuki K, Iwanaga M, Kikuchi F, Kitamura K (1995) Variation of seed α -amylase inhibitors in the common bean. *Theor Appl Genet* **90**: 425–429
- Janzen DH (1980) When is it coevolution? *Evolution* **34**: 611–612
- Kassell B (1970) Trypsin and chymotrypsin inhibitors from soybeans. *Methods Enzymol* **19**: 853–862
- Kitch LW, Murdock LL (1986) Partial characterization of a major gut thiol proteinase from larvae of *Callosobruchus maculatus* F. *Insect Biochem Physiol* **3**: 561–575
- Kornegay J, Cardona C, Posso CE (1993) Inheritance of resistance to the Mexican bean weevil in common bean, determined by bioassay and biochemical tests. *Crop Sci* **33**: 589–594
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275
- Minney BHP, Gatehouse AMR, Dobie P, Dendy J, Cardona C, Gatehouse JA (1990) Biochemical bases of seed resistance to *Zabrotes subfasciatus* (bean weevil) in *Phaseolus vulgaris* (common bean): a mechanism for arcelin toxicity. *J Insect Physiol* **36**: 757–767
- Mintz GR (1993) An irreversible serine protease inhibitor. *Biopharmacology* **6**: 34–37
- Mirkov TE, Evans SV, Wahlstrom J, Gomez L, Young NM, Chrispeels MJ (1995) Location of the active site of the bean α -amylase inhibitor and involvement of a Trp, Arg, Tyr triad. *Glycobiology* **5**: 45–50
- Mirkov TE, Wahlstrom JE, Hagiwara K, Finardi-Filho F, Kjemtrup S, Chrispeels MJ (1994) Evolutionary relationships among proteins in the phytohemagglutinin-arcelin- α -amylase inhibitor family of the common bean and its relatives. *Plant Mol Biol* **26**: 1103–1113
- Moreno J, Altabella T, Chrispeels MJ (1990) Characterization of α -amylase-inhibitor, a lectin-like protein in the seeds of *Phaseolus vulgaris*. *Plant Physiol* **92**: 703–709
- Moreno J, Chrispeels MJ (1989) A lectin gene encodes the α -amylase inhibitor of the common bean. *Proc Natl Acad Sci USA* **86**: 7885–7889
- Osborn TC, Alexander DC, Sun SSM, Cardona C, Bliss FA (1988) Insecticidal activity and lectin homology of arcelin seed protein. *Science* **240**: 207–210
- Osborn TC, Blake T, Gepts P, Bliss FA (1986) Bean arcelin. 2. Genetic variation, inheritance and linkage relationships of a novel seed protein of *Phaseolus vulgaris* L. *Theor Appl Genet* **71**: 847–855
- Pratt RC, Singh NK, Shade RE, Murdock LL, Bressan RA (1990) Isolation and partial characterization of a seed lectin from tepary bean that delays bruchid beetle development. *Plant Physiol* **93**: 1453–1459
- Pueyo JJ, Hunt DC, Chrispeels MJ (1993) Activation of bean α -amylase inhibitor requires proteolytic processing of the pro-protein. *Plant Physiol* **101**: 1341–1348
- Qian M, Haser R, Payan F (1993) Structure and molecular model refinement of pig pancreatic α -amylase at 2.1 Å resolution. *J Mol Biol* **231**: 785–799
- Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* **166**: 368–379
- Schoonhoven AV, Cardona C, Valor J (1983) Resistance to the bean weevil and the Mexican bean weevil (Coleoptera:Bruchidae) in noncultivated common bean accessions. *J Econ Entomol* **76**: 1255–1259
- Schroeder HE, Gollasch S, Moore A, Tabe LM, Craig S, Hardie D, Chrispeels MJ, Spencer D, Higgins TJV (1995) Bean α -amylase inhibitor confers resistance to the pea weevil, *Bruchus pisorum*, in genetically engineered peas (*Pisum sativum* L.). *Plant Physiol* **107**: 1233–1239
- Shade RE, Pratt RC, Pomeroy MA (1987) Development and mortality of the bean weevil, *Acanthoscelides obtectus* (Coleoptera:Bruchidae), on mature seeds of tepary beans, *Phaseolus acutifolius*, and common beans, *Phaseolus vulgaris*. *Environ Entomol* **16**: 1067–1070
- Shade RE, Schroeder HE, Pueyo JJ, Tabe LM, Murdock LL, Higgins TJV, Chrispeels MJ (1994) Transgenic peas expressing the α -amylase inhibitor of the common bean are resistant to bruchid beetles. *Bio/Technology* **12**: 793–796

- Silano V, Furia M, Gianfreda L, Macrei A, Palescandola R, Rab A, Scardi V, Stella E, Valfre F** (1975) Inhibition of amylases from different origins by albumins from the wheat kernel. *Biochim Biophys Acta* **391**: 170–178
- Suzuki K, Ishimoto M, Iwanaga M, Kikuchi F, Kitamura K** (1995) Inheritance of seed α -amylase inhibitor in the common bean and genetic relationship to arcelin. *Theor Appl Genet* **90**: 762–766
- Suzuki K, Ishimoto M, Kikuchi F, Kitamura K** (1993) Growth inhibitory effect of an α -amylase inhibitor from the wild common bean resistant to the Mexican bean weevil (*Zabrotes subfasciatus*). *Jpn J Breed* **43**: 257–265
- Suzuki K, Ishimoto M, Kitamura K** (1994) cDNA sequence and deduced primary structure of an α -amylase inhibitor from a bruchid-resistant wild common bean. *Biochim Biophys Acta* **1206**: 289–291
- Yetter MA, Saunders RM, Boles HP** (1978) α -Amylase inhibitors from wheat kernels as factors in resistance to postharvest insects. *Cereal Chem* **56**: 243–244