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Characterization of Two *Acanthoscelides obtectus* α-Amylases and Their Inactivation by Wheat Inhibitors

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Wheat α-amylase inhibitors represent an important tool in engineering crop plants against bean bruchids. Because *Acanthoscelides obtectus* is a devastating storage bean insect-pest, we attempted to purify and characterize its gut α-amylases, to study their interaction with active proteinaceous inhibitors. Two digestives α-amylases (AoA1 and AoA2) were purified from gut larvae, showing molecular masses of 30 and 45 kDa for each one, respectively. The stoichiometry interaction between these α-amylases with two wheat inhibitors (0.19 and 0.53) showed a binding complex of 1:1 enzyme: inhibitor. *In vivo* activities of these inhibitors against *A. obtectus* were also evaluated using a rich ammonium sulfate inhibitor fraction (F_{20-40}) and purified inhibitors after reversed phase high-performance liquid chromatography columns. Incorporation of three different inhibitor concentrations (0.25, 0.5, and 1.0% w/w) into artificial seeds showed that addition of the purified 0.19 inhibitor at the highest concentration (1.0%) reduced the larval weight by 80%. Similar data were observed when 0.53 inhibitor was incorporated at 0.5%. When the concentration of purified 0.53 was enhanced to 1.0%, no larvae or adult emergence were observed. Our data suggest that these α-amylase inhibitors present great potential for use in *Phaseolus* genetic improvement programs.

KEYWORDS: α-Amylase inhibitors; wheat; bruchids; *Acanthoscelides obtectus*; weevils

INTRODUCTION

Common bean (*Phaseolus vulgaris*) and other starchy legume seeds are important food sources due their high level of protein, lipids, and carbohydrates storage. These characteristics are propitious for certain coleopteran insects, such as the bean weevils, that attack seeds in the field and in storage causing severe crop losses and great damage to resource poor farmers in different countries. Bruchid insect-pest larvae are extremely starch-dependent and utilize α-amylases (α-1,4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) to produce metabolic energy (*1*). These enzymes catalyze the initial hydrolysis of starch into shorter oligosaccharides, an important step that transforms starch into single carbohydrate units that can be assimilated by organisms. Weevils and other insects that feed on grain products during larval and/or adult life depend on their α-amylases for survival, which is particularly true for the yellow meal worm *Tenebrio molitor* (*2*), cowpea weevil *Callosobruchus maculatus* (*3*), and Mexican bean weevil *Zabrotes subfasciatus* (*4*).

Plant α-amylase inhibitors (α-AIs), particularly abundant in leguminosae (*5*−*9*) and cereals (*10*−*14*), have been extensively studied, in part due to their role as resistance factors against pests and pathogens (*9*, *15*). The involvement of α-AIs on bean plant defense and their specificities toward α-amylases have been demonstrated for two α-AI isoforms from common beans. While the inhibitor α-AI1 strongly inhibits PPA as well as the digestive α-amylases from *C. maculatus* and *C. chinensis*, the inhibitor α-AI2 inhibits the digestive α-amylase of *Z. subfasciatus*—ZSA (*6*, *16*). Nevertheless, neither α-AI1 nor α-AI2 were capable of inhibiting the α-amylases from bean weevil *A. obtectus*. Previous reports demonstrated the presence of inhibitors in wheat and rye kernels with high levels of inhibitory activity against digestive α-amylases from *A. obtectus* (*13*, *14*). Furthermore, these inhibitors also act against enzymes from yellow mealworm *T. molitor* (*17*), western corn rootworm *Diabrotiva virgifera* (*18*), and bean weevils, *Z. subfasciatus* and *C. maculatus* (*13*). For this reason, particular interest has been focused on the proteinaceous inhibitors of the Triticacea family, an expanding family of cereal α-AIs that contain members from different sources. In this report, two α-amylases from bean weevil *A. obtectus* were purified and their stoichoimetries in complex with the wheat α-AIs 0.19 and 0.53 were determined.

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The effects of both inhibitors on growth and development of bean weevil were also evaluated.

MATERIAL AND METHODS

Purification of α-Amylases from *A. obtectus* Larvae Midgut. *A. obtectus* larval guts of 17−20 day old larvae were dissected in ice-cold 25 mM NaCl, homogenized, and centrifuged at 4000*g* for 20 min at 4 °C to remove the gut walls and cellular debris. The supernatant from 250 dissected guts (1.5 mL) was applied onto an ionic exchange column CM-Cellulose (1.0 cm × 15 cm) equilibrated with 0.05 M acetate buffer, pH 5.6, at 4 °C. The flow through was washed off with equilibration buffer while the adsorbed compounds were eluted from the column using a linear gradient of 0−0.5 M NaCl. Fractions (1.0 mL) were collected at a flow rate of 25 mL h^{-1} and used to measure the α-amylolitic activity as described below. Fractions containing enzymatic activity were pooled and dialyzed overnight against distilled water. This fraction was applied onto an affinity chromatography Sepharose-6B conjugated with *β*-cyclodextrin equilibrated with 0.1 M phosphate buffer, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$. The adsorbed proteins were eluted with one single step of 20 mM *β*-cyclodextrin. Fractions (2.0 mL) were collected at a flow rate of 28 mL h^{-1} and used to measure amylolitic activity. Retained fractions were pooled, dialyzed for 48 h against distilled water, and concentrated. The retained fraction was applied to a high-performance liquid chromatography (HPLC) size exclusion column equilibrated with 0.05 M sodium phosphate buffer, pH 6.0 (Shimadzu), at a flow rate of 0.5 mL min^{-1}. The material eluted in individual peaks was collected, lyophilized, and stored at −20 °C.

Purification of Wheat α-AIs. BR35 wheat (*Triticum aestivum*) kernels were obtained from the Embrapa Trigo (Passo Fundo-RS). Kernels were ground into flour, and the flour was extracted with 0.15 M NaCl (1:5 w/v, meal-to-buffer ratio) with continuous stirring for 5 h at 4 °C. The material was then centrifuged at 10,000*g*, 4 °C, for 30 min. The precipitate was discarded, and the supernatant was submitted to fractionation with ammonium sulfate. The fraction obtained between 20 and 40% saturation (F_{20-40}) was applied to a HPLC reverse phase analytical column (Vydac 218 C-18TP) at a flow rate of 1.0 mL min^{-1}. The material eluted in individual peaks was collected, lyophilized, and stored at −20 °C.

α-Amylase and α-Amylase Inhibitory Assays. α-Amylase and α-amylase inhibitory activities were measured by the Bernfeld method (*19*) in 0.1 M phosphate buffer, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$. Each assay contained 6.0 units of α-amylase activity, and inhibitors were tested at several standard concentrations, as determined by the Bradford method (*20*). One α-amylase unit (1 UI) was defined as the amount of enzyme that increased the absorbance at 530 nm by 0.1 OD during 25 min of the assay. Assays were carried out in triplicate, using a range of α-AI concentrations of 12.5−75 *μ*M. Triplicate inhibition values differed by no more than 10%.

Polyacrylamide Gel Electrophoresis and Western Blotting. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was conducted as described by Laemmli (*21*) at room temperature. Protein molecular weight markers were from Pharmacia. After SDS−PAGE, the protein bands were transferred to a nitrocellulose membrane according to Towbin et al. (*22*). The specific antibody, raised against ZSA, was diluted 1:2000. The reaction was performed by using horseradish peroxidase goat anti-rabbit IgG.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Analyses. α-Amylases and their cognate inhibitors were analyzed by mass spectrometry in agreement with Franco et al. (*13*). Freeze-dried samples of the peaks from HPLC were prepared for MALDI-TOF on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA). Samples were dissolved in trifluoracetic acid (TFA) 1.0% and α-cyan (a satured solution dissolved in acetonitrile/0.1% TFA 1:1, v/v) from Sigma Chemicals. The solution was then vortex mixed, and aliquots of 1.0 mL were applied onto the Voyager Bioworkstation sample plate. Samples were air-dried at room temperature. The spectrometer, equipped with a delayed extraction system, was operated in linear mode. Sample ions were evaporated by irradiation with a N_2 laser at a wavelength of 337 nm and accelerated at 23 kV potential in the ion source with a delay of 150 ns. Samples were ionized with 100−200 shots of a 3 ns pulse width laser. The signal was digitalized at a rate of 500 MHz, and averaged data were presented to a standard Voyager data system for manipulation. MALDI-TOF was calibrated using a Saquazyme calibration mixture (Applied Biosystems) consisting of bovine insulin (5734 Da), *Escherichia coli* thioredoxin (11674 Da), and horse apomyoglobin (16952Da)

Feeding Tests. The effects of 0.19 and 0.53 α-AIs on insect development were examined using artificial seeds made of *P. vulgaris* flour and three different concentrations of inhibitor (0.25, 0.5, and 1.0%). The freeze-dried inhibitor added to the bean meal was thoroughly mixed in a mortar to ensure a uniform distribution. Each artificial bean was made into a columnar shape of 300 mg (10 mm diameter) with a hand compressor. The artificial beans were placed in a plastic dish, and three *A. obtectus* eggs per seed were manually introduced. The plastic dishes were kept in a chamber at 28 °C and about 80% relative humidity. At the 20th day, the survivors' larvae weights were measured and after 45 days the adult insects were counted. Negative controls (distilled water) were used in which the artificial beans lacked α-AIs. Each treatment was done in triplicate, and each sample contained six infected seeds.

RESULTS AND DISCUSSION

Purification of Digestive α-Amylases from Bean Weevil *A. obtectus.* Several insects, especially those similar to seed weevils that feed on starchy seeds during larval and/or adult stages, depend on their α-amylases for survival. Studies on insect starch digestion as a target for the control of starch-dependent insects were stimulated in recent years. Aiming to understand the *A. obtectus* starch digestion, 250 guts were extracted and loaded into an ionic exchange CM-Cellulose column in order to purify digestive target α-amylases. Retained proteins were eluted with a NaCl gradient, and only the retained peak showed amylolitic activity (**Figure 1A**, dashed line). After dialysis, the eluted fractions were applied onto an Epoxi-Sepharose 6B (**Figure 1B**) and the adsorbed material was eluted using a single step of *β*-cyclodextrin. This compound interacts with the catalytic site, mimicking the presence of the substrate. The retained peak with high amylolytic activity (**Figure 1B**, dashed line) was dialyzed by using an HPLC size exclusion column (Shimadzu). Six fractions (I−VI) were obtained, and only two of them (IV and V) showed amylolytic activity (**Figure 1C**). Furthermore, **Table 1** indicates the purification improvement of both α-amylases. SDS−PAGE analysis of peaks IV and V (**Figure 2A**), stained with silver, showed a single band in each lane of approximately 30 and 45 kDa. A Western blot was also carried out in order to confirm the identity of α-amylases. The antibodies used, raised against ZSA (**Figure 2B**,**C**), were capable of recognizing both proteins, named AoA1 and AoA2, confirming the protein nature (*6*). The degrees of purification of α-AIs and α-amylases were determined by MALDI-TOF, which demonstrated a major peak of 13433.9 Da to inhibitor 0.19 and 13283.7 Da to inhibitor 0.53, with small amounts of dimers. Furthermore, single monomeric peaks of 32194.6 Da to AoA1 and 46990.8 Da to AoA2 were also observed by MALDI-TOF analyses (data not shown). A similar AoA2 molecular mass (47.0 kDa) was demonstrated for two other insect α-amylases, TMA (*2*, *23*, *24*) and ZSA (*4*). Nevertheless, only one α-amylase, from cowpea weevil *C. maculatus*, demonstrates a similar molecular mass to AoA1, with approximately 35.0 kDa (*25*). It's also remarkable that different molecular masses of insect α-amylases were observed before, when compared to AoA1 and AoA2, as the minor α-amylases

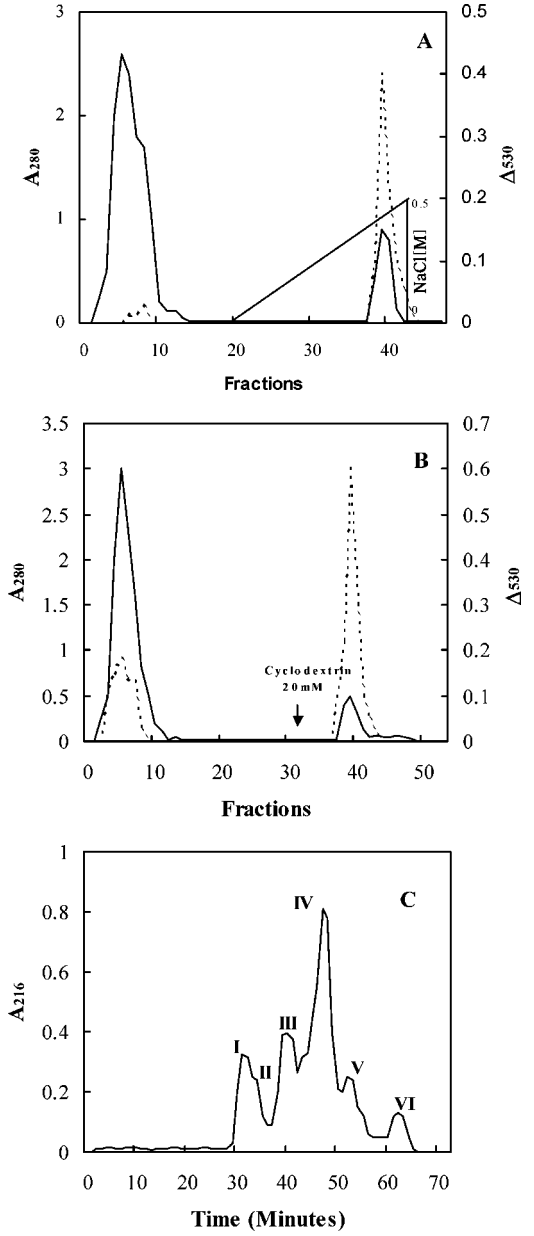

Figure 1. Purification steps of α-amylases from *A. obtectus*. Chromatographic profiles obtained (**A**) during the CM-Cellulose chromatography equilibrated with 0.05 M acetate buffer, pH 5.6. The bold line indicates the linear gradient of 0.0–0.5 M NaCl, and the dashed line indicates the amylolitic activity measured by the Bernfeld method (*19*) (**B**) during the Epoxi-Sepharose 6B chromatography equilibrated with 0.1 M phosphate buffer, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$. The black arrow indicates the single step application of 20 mM β-cyclodextrin dissolved in the same buffer and (**C**) during the size exclusion HPLC chromatography equilibrated 0.05 M sodium phosphate buffer, pH 6.0 (Shimadzu) at a flow rate of 0.5 mL min^{-1}. The material eluted in individual peaks was collected, lyophilized, and stored at –20 °C.

isoforms (65.0 and 81.3 kDa) from *Z. subfasciatus* (*25*) and the major α-amylase (60.2 kDa) from *Prostephanus truncates* Horn. (*26*). In this report, only two different α-amylases were found. Nevertheless, we cannot discard the possibility of *A. obtectus* larvae to produce more α-amylase isoforms in response to the presence of entomotoxic plant proteins, which were not observed in this report but were detected in other bean weevils in response to antimetabolic proteins such as α-AIs (*25*, *27*–*29*).

Table 1. Purification Improved of Two Digestive *A. obtectus* α-Amylases[a]

purification step	protein (μg)	activity (U)	yield (%)	specific activity (U μg^{-1})	purification fold
crude extract	5000	6000	100	1.2	1
CM-Cellulose	980	5400	90.0	5.5	4.6
Epoxi-Sepharose	98	2500	41.7	25.5	21.3
HPLC size exclusion, AoA1	44	1610	26.8	36.6	30.5
HPLC size exclusion, AoA2	12	942	15.7	78.5	65.4

[a] The activity was determined by the Bernfeld method (*19*) as described in the text, using starch as a substrate. Accordingly, one enzyme unit is defined as the amount of enzyme necessary to increase 0.1 in optical density at 530 nm.

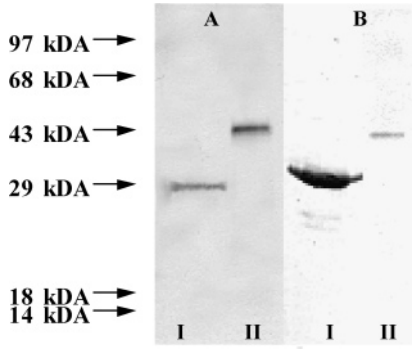

Figure 2. (**A**) SDS–PAGE 15% analysis of fraction IV (AoA1, I) and V (AoA2, II), stained with silver. (**B**) Western blot of AoA1 (I) and AoA2 (II) in nitrocellulose membrane. AoAs was reacted against antibodies raised against *Z. subfasciatus* α-amylase (*4*).

Stoichiometry of AoA Inhibition by 0.19 and 0.53. For the stoichiometry studies, both inhibitors 0.19 and 0.53 were purified from wheat kernels as described by Franco et al. (*13*). **Figure 3** indicates that a complex of 1:1 could be formed between both AoAs and 0.19. A similar result was obtained with 0.53 (**Figure 3B**), suggesting that both α-AIs tested are univalent inhibitors acting as monomers, in which both inhibitors could inhibit AoA1 and AoA2. Additionally, stoichiometry data could be correlated with the results above, in which 50% of the AoAs enzymes were inhibited at 0.5 molar rate inhibitor/α-amylase. Similar results were found to *T. molitor* α-amylase, which binds to 0.28 and 0.19 α-AIs from wheat in a proportion of 1:1 enzyme: inhibitor (*30*). The same stoichiometry of 1:1 was also observed in the complex of α-amylase/subtilisin inhibitor, BASI with the α-amylase from barley seeds, AMY2 (*31*). In all three cases, the inhibitors showed a monomeric or homodimeric structure (*13*, *32*, *33*), which was different that observed to α-AI1 that shows a tetrameric structure (*34*). One α-AI1 molecule, a tetrameric $2\alpha2\beta$ inhibitor from white kidney beans, could inhibit two molecules of PPA in a stoichiometry of 2:1 enzyme: inhibitor. These results could be correlated with the stoichiometry presented in **Figure 3**, in which monomeric or homodimeric inhibitors follow a expected ratio 1:1 (enzyme:inhibitor) and probably only tetrameric inhibitors may act in 2:1 (enzyme: inhibitor) proportions.

The ability of wheat inhibitors to inhibit both AoA1 and AoA2 is particularly interesting in crop protection, since they showed an enhanced activity against different enzymes from the same source. Otherwise, it is also important to remember that *in vitro* conditions were insufficient to indicate if an inhibitor is efficient toward a pest or not. In vivo conditions may crucially modulate α-amylase specificity. The acidic pH for inhibition may be responsible for certain amylase inhibition

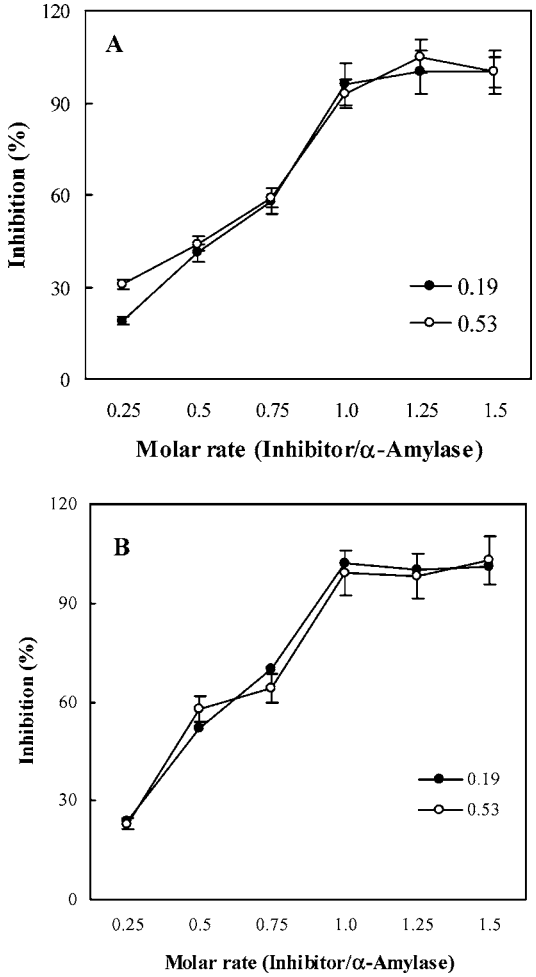

Figure 3. Inhibition of purified α-amylases AoA1 (**A**) and AoA2 (**B**) by 0.19 (black dots) and 0.53 (white dots). AoAs (5 μM) were incubated with increasing amounts of wheat inhibitors in 0.1 M phosphate buffer, pH 5.8, containing 20 mM NaCl and 0.1 mM $CaCl_2$ at 37 °C for 30 min. The protein concentration was calculated using Bradford (*20*) assays, and data and molecular weights were calculated by MALDI-TOF.

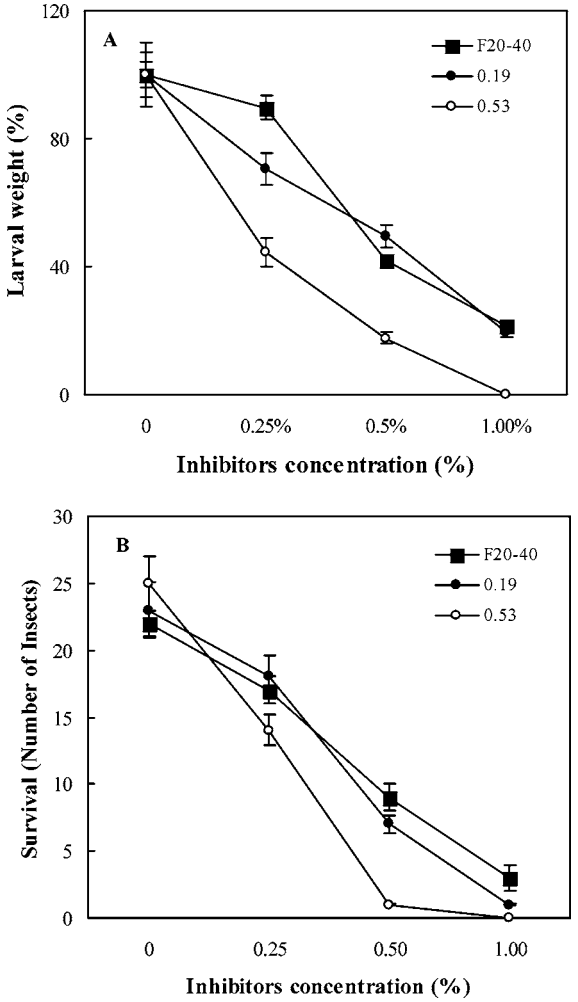

Figure 4. Effects of F_{20-40} (black squares), 0.19 (black dots), and 0.53 inhibitors (white dots) on (**A**) larval weight and on (**B**) survival of *A. obtectus*. Bruchid survival was calculated based on insect number, surviving pupae, and larvae toward hatched eggs on artificial beans 40 days after oviposition. Each point is a mean of four replicates, and each replicate does not differ more than 10%.

degrees in Coleopteran, whose intestinal contents are acidic, but not of α-amylases from Lepidoptera, where the intestinal contents are alkaline (*35*). Furthermore, the cleavage of inhibitors by insect digestive proteinases may interfere with pest control by using α-AIs and could explain the low in vivo efficiency of some inhibitors against insect pests (*36*). The α-amylase diversity found in a single insect indicates that unless an α-AI has reasonably broad specificity, being able to inhibit all of the α-amylase isoforms produced by the insect, its incorporation in artificial seeds or its expression in transgenic plants would probably have no impact on starch digestion and therefore would not constitute a deterrent against predation of these seeds.

Biological Activity of 0.19 and 0.53 Inhibitors. Different plant sources containing α-AIs have been screened for *A. obtectus* α-amylases before, but success was only found with Triticacea family, where two α-AIs (0.19 and 0.53) isolated from wheat kernels showed higher inhibition toward *A. obtectus* larvae α-amylases at low concentrations (**Figure 3**; *13*). Similar data were observed to other α-AI from rye seeds (*14*), and no other inhibitor showed this capability. Until this date, no α-AI was able to influence the *A. obtectus* development, and for this reason, the wheat α-AIs *in vivo* effectiveness toward bean weevil *A. obtectus* was determined in this report by using a rich ammonium sulfate α-AIs fraction (F_{20-40}) and purified inhibitors 0.19 and 0.53. They were incorporated into artificial seeds at different concentrations (0.25, 0.5, and 1.0% w/w), and all three treatments strongly inhibited larval growth (**Figure 4**). The addition of F_{20-40} and 0.19 at highest concentration (1.0%) reduced the larval weight in approximately 80%. Similar results were observed with an incorporation of 0.53 at 0.5%, where no larvae were observed at 1.0% concentration (**Figure 4A**). F_{20-40} was not more active than the purified inhibitors, showing that, in this specific case, several inhibitors at low concentration were less efficient than a unique inhibitor at a higher concentration. **Figure 5** clearly shows the reduction in larvae development caused by inhibitor antinutritional effects. Similar results were observed in bioassays using α-AI1 against *C. maculatus* and *C. chinensis* when 0.2% strongly influenced the larval development, and at 1.0%, no survivors were found (*28*).

A great number of genes conferring pest resistance have been incorporated into crops (*37*, *38*) including α-AIs, which are thought to inhibit larval growth by slowing down the digestion of the plant material ingested by insect pests, thus reducing carbohydrate assimilation. Transgenic peas and azuki beans expressing *P. vulgaris* α-AI1 have enhanced resistance to certain species of Bruchidae whose digestive α-amylases are inhibited

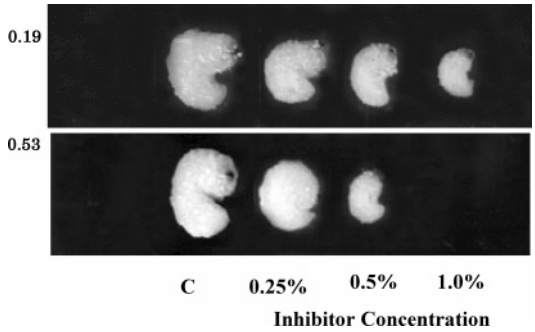

Figure 5. Effects of wheat α-AIs on the *A. obtectus* larval development. Larvae were reared in artificial bean seeds containing three different concentrations (0.25, 0.5, and 1.0%) of 0.19 and 0.53 inhibitors. Negative control (C) was done rearing the larvae in artificial seeds without the presence of α-AIs.

by this molecule (*39*−*41*). When cDNA α-AI2 was expressed in peas, a partial protection was obtained against bruchids. However, the α-AI2 gene could be used to extend the time before the weevil damage reaches the break-even cost of spraying and thus remove the necessity of chemical pesticides in the crop and fumigation during storage (*41*). The transgenic plants expressing the insecticidal bean α-AI1 demonstrated minimal detrimental effects on the nutritional value of pea fed to rats until 30% of the diet (*42*). Few studies were done in order to produce transgenic plants expressing wheat inhibitors. In this report, we found that two α-AIs (0.19 and 0.53) were capable to inhibit α-amylases of *A. obtectus* and control this important pest in artificial conditions. Furthermore, it is also important to keep in mind that they also inhibit α-amylases from cowpea weevil *C. maculatus* and Mexican bean weevil *Z. subfasciatus* (*13*), making it possible to have protection for these two weevils too. Another remarkable occurrence is that they differ in their intrinsic specificity against mammalian α-amylases. While 0.19 inhibits PPA, 0.53 did not show any activity to this enzyme (*13*). Just as important as the proof of protection of transgenic crops against insect-pests is the demonstration that the new crops present no health risk to consumers, as reviewed by Payan (*43*). A high specificity of 0.53 to insect α-amylases, added to a possible proteinaceous inhibitor denaturation by seed cooking (*13*), could give some advances to safety insect resistant transgenic plants containing α-AIs. Because 0.53 α-AI showed an increased specificity to insect α-amylases (*13*) and showed more in vivo effectiveness against common bean weevil when compared to 0.19 inhibitor, it seems to be a good tool to obtain transgenic bean plants with enhanced resistance to the common bean weevil *A. obtectus*.

ABBREVIATIONS USED

0.19 and 0.53, α-amylase inhibitor purified from wheat; α-AI1, α-amylase inhibitor 1 from cultivated *Phaseolus vulgaris*; α-AI2, α-amylase inhibitor 2 from wild *P. vulgaris*; AoA1, *Acanthoscelides obtectus* α-amylase 1; AoA2, *A. obtectus* α-amylase 2; F_{20-40}, wheat inhibitors rich fraction precipitated with ammonium sulfate in a range of 20−40; PPA, porcine pancreatic α-amylase; TMA, *Tenebrio molitor* α-amylase; ZSA, *Zabrotes subfasciatus* α-amylase.

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