JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Activity of α-Amylase Inhibitors from *Phaseolus coccineus* on Digestive α-Amylases of the Coffee Berry Borer

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Seeds of scarlet runner bean (*Phaseolus coccineus* L.) were analyzed for α-amylase inhibitor (α-AI) activity. Through the use of polyclonal antibodies raised against pure α-AI-1 from common bean (*Phaseolus vulgaris* L.), typical α-AI polypeptides (M_r 14−18 kDa) as well as a large polypeptide of M_r 32000 Da, usually referred to as "amylase inhibitor like", were detected. The inhibitor activity present in four accessions of *P. coccineus* was examined, both in semiquantitative zymograms allowing the separation of different isoforms and in quantitative assays against human salivary amylase, porcine pancreatic amylase, and coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae) amylase. Differential inhibition curves lead to the suggestion that the gene encoding one of the inhibitors in *P. coccineus* (in accession G35590) would be a good candidate for genetic engineering of coffee resistance toward the coffee berry borer. An in vitro proteolytic digestion treatment of pure α-AI-1 resulted in a rapid loss of the inhibitory activity, seriously affecting its natural capacity to interact with mammalian α-amylases.

KEYWORDS: α-Amylase inhibitor; scarlet runner bean; *Phaseolus coccineus*; coffee berry borer; *Hypothenemus hampei*

INTRODUCTION

Plant seeds are known to contain a variety of enzyme inhibitors that are thought to be involved in defense mechanisms against herbivores, insects, and pathogens. Among these proteins, which have been shown to be defense proteins, the α-amylase inhibitors (α-AIs) are found in several legume species (*1–5*). The α-AIs inhibit not only the enzymes of mammalian saliva and pancreas but also those present in the insect intestinal tracts of some insects such as bruchids and other storage product pests (*1, 3, 6–10*). It has been reported that the scarlet runner bean (*Phaseolus coccineus* L.) and other plant species belonging to the same genus, such as the common bean (*P. vulgaris* L.) and the tepary bean (*P. acutifolius* L.), contain a family of homologous plant defense proteins including phytohemagglutinins (PHA) and α-AIs (*11*). It is well-known that these three *Phaseolus* species are not equally sensitive to bruchid pest attack. For example, *P. vulgaris* is more sensitive than *P. acutifolius* to the bean weevil, *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae) (*12*). The sensitivity of these bean species could be explained by differences in the level and/or in the biological characteristics of their α-AIs.

The α-AIs of *Phaseolus* plants exist at least in two and possibly more isoforms (*13, 14*) and it has been shown that these isoforms have different biological specificities against their target enzymes. The well-characterized α-AI-1 of *P. vulgaris* inhibits porcine pancreatic amylase (PPA), human salivary amylase (HSA), and the α-amylases of the bruchids *Callosobruchus chinensis* L. (Coleoptera: Bruchidae) and *C. maculates* F. (Coleoptera: Bruchidae), but do not inhibit the α-amylase of the Mexican bean weevil, *Zabrotes subfasciatus* B. (Coleoptera: Bruchidae) (ZSA) (*7, 8, 15*). A related α-AI-2 found in some wild bean accessions from Mexico inhibits ZSA, but does not inhibit PPA (*16*).

The coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae) is a serious pest of coffee throughout the world. Endemic to Central Africa, it has now spread to most coffee-growing regions in the world, thereby reducing yields and/or lowering the quality of the final product (*10*). As part of a larger research project to create transgenic coffee plants that express amylase inhibitors, we examined different bean seeds as a gene source that could be used to create transgenic coffee plants.

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10.1021/jf0733564 CCC: $40.75
Published on Web 03/06/2008

In a recent work done by our group, a gene encoding an α-amylase inhibitor, named α-AI-Pc1, was isolated from cotyledons of *P. coccineus* seeds. The α-AI-Pc1 gene, constructed with the PHA-L phytohemaglutinin promoter, was introduced and expressed into tobacco plants (*17*). This recombinant protein could be related with *P. vulgaris* α-amylase proteinaceous inhibitors (α-AI-1 and α-AI-2). Despite the fact that two isoforms of α-amylase inhibitor (α-AI-1 and α-AI-2) have been well-characterized and cloned from *P. vulgaris* seeds, and partially characterized from *P. coccineus*, the totality and the biological potential of α-AI isoforms present in *P. coccineus*, here called α-AI-Pc, have not yet been characterized and identified. Therefore, the purpose of this study is to show electrophoretic evidence of the expression of multiple forms of α-amylase inhibitors in *P. coccineus* (α-AI-Pc) and their inhibition activity on some digestive α-amylases, as well zymographic evidence of the inhibition activity of a recombinant α-AI-1 expressed in tobacco seeds and its susceptibility to endogenous proteases of *H. hampei*.

MATERIALS AND METHODS

Insects and Seeds. *H. hampei* was obtained locally in Brazil and reared in an insectary at EMBRAPA-Cenargen, Brasilia, Brazil. Seeds of *P. coccineus* varieties (accessions G35514, G35590, G35018, and G35619) were used. Varieties of *P. coccineus* were obtained from the International Center for Tropical Agriculture (CIAT, Palmira, Colombia) and propagated in a greenhouse in Manizales, Colombia. Transgenic tobacco (*Nicotiana tabacum* L.) seeds expressing the recombinant α-AI-1 gene from common bean (*P. vulgaris*) donor were provided by Dr. Maarten J. Chrispeels (University of California).

Bean and Tobacco α-AI Extracts. α-Amylase inhibitors were extracted from seeds of transgenic tobacco and accessions of *P. coccineus* by homogenization of the powder with a hand-held homogenizer with 5 volumes of 100 mM NaCl for 3 h at 4 °C. The extract was centrifuged at 12000*g* for 20 min and the supernatant dialyzed against water to allow the globulins to precipitate. After centrifugation (12000*g* for 20 min), the supernatant was freeze-dried. The resulting powder was used as a source of α-AIs for zymogram and α-amylase inhibition assays.

α-AI-1 Purification. Purification of α-AI-1 was performed as described by Valencia et al. (*10*) including some small modifications. In this case, seeds (30 g) of the four accessions of *P. coccineus* were ground, and the powder was mixed with 350 mL of distilled water and stirred in the cold room for 6 h. The slurry was centrifuged for 30 min at 10000*g*, and the pH of the supernatant was adjusted to 4.0 by using a solution 1 M of HCl. Precipitated proteins were removed by centrifugation (20 min at 12000*g*), and the pH of the supernatant was adjusted to pH 7.0 with a solution 1 M of potassium phosphate. Any possible precipitate was removed again by a new centrifugation. Proteins in the clear supernatant were precipitated by the gradual addition of saturated ammonium sulfate (4.1 M) to a final concentration of 85% saturation. After standing on ice for 5 h, the proteins were collected by centrifugation for 20 min at 12000*g*. The pellet was resuspended in water and dialyzed against water for 36 h and then against 20 mM potassium phosphate (pH 7) for at least 12 h. The solution containing the protein was loaded on a DEAE cellulose column (120 × 16 mm) and the column equilibrated with 200 mL of the same phosphate buffer. The bound proteins were eluted with a linear NaCl gradient (0−100 mM), and absorbance at 280 nm and α-AI activity were measured in the eluted fractions.

Immunoblotting. SDS-PAGE was performed according to the method of Laemmli (*18*). Electrophoresis of both *P. coccineus* accessions (G35514, G35590, G35018, and G35619) and transgenic tobacco seeds was performed by using a PhastSystem instrument (GE Healthcare Life Sciences) operating with a gradient Phastgel Media 8–25%, following the directions of the manufacturer. Gradient electrophoresis was performed on a thermostatic plate maintaining the temperature of 15 °C. After transfer to a nitrocellulose membrane, proteins were detected using a rabbit anti α-AI-1 serum obtained as described by Moreno and Chrispeels (*19*). We used a goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase (Bio-Rad) as a secondary antibody.

α-AIs Separation by Electrophoresis in Native and Isoelectrofocusing (IEF) Gels. Proteins from seeds of transgenic *N. tabacum* and *P. coccineus* accessions that were obtained as previously described were separated by using native electrophoresis (8–25% gradient Phastgel) and isoelectrofocusing (IEF 3–9 Phastgel). For the inhibitor zymograms, gradient and IEF gels were incubated with 1.5% soluble starch dissolved in 50 mM sodium succinate buffer (pH 5.0) containing 10 mM NaCl and 20 mM $CaCl_2$ for 1 h at 4 °C. Then, the starch−Phastgel was incubated at 30 °C for 20 min with human salivary amylase, dissolved in the same buffer, rinsed with water, and stained with a KI−I_2 solution (0.5% I_2 and 5% KI) for 10 min. According to this method, the amylase digests the starch everywhere on the gel except where the inhibitor is located. This treatment produced dark blue bands on a light background.

Effect of Temperature on α-Amylase Inhibitor Activity. The biological activity of the α-AIs present in *P. coccineus* accessions was assayed by preincubating the inhibitory extracts for 5, 10, 15, 30, and 60 min at 80 °C. Aliquots (50 μL) were then immediately cooled in an ice bath before the residual inhibitory activity was measured. Each assay was performed in triplicate.

Amylase and Amylase Inhibitory Activity. Porcine pancreas α-amylase was purchased from Sigma (Sigma A3176). *H. hampei* α-amylase was a crude extract prepared by homogenization of insect intestinal tracts with a hand-held homogenizer in a 10 mM NaCl solution containing 20 mM $CaCl_2$, followed by centrifugation at 10000*g* for 30 min at 4 °C. The supernatant was freeze-dried and the resulting powder used as a source of enzyme. For measurements of α-amylase activity was used the method described by Valencia-Jimenez et al. (*10*). In this case, an aliquot (50 μL) of the crude enzyme extract was incubated for 15 min at 30 °C with 650 μL of succinate buffer (50 mM; pH 5.0) containing 10 mM NaCl and 20 mM $CaCl_2$ and 500 μL of a solution of 0.125% soluble starch (Sigma S2004) in 50 mM succinate (pH 5.0). The reaction was stopped by the addition of 5 mL of iodine/iodide (0.5% I_2 and 5% KI), and the absorbance of the solution was determined at 580 nm by using a spectrophotometer Unicam UV2. Amylase inhibition was determined by preincubating the enzyme with the α-AI extract for 15 min at 30 °C in activity buffer [50 mM succinate buffer (pH 5.0) containing 10 mM NaCl and 20 mM $CaCl_2$]. The results were compared with the activity of the same amount of enzyme without α-AI.

Extraction Procedures of Insect Proteases. Twenty intestinal tracts of coffee berry borer were carefully separated with the aid of a dissection microscope and homogenized with a hand-held homogenizer in a microcentrifugue tube with 20 μL of 0.1 M citrate buffer (pH 3.0) and then centrifuged at 15000*g* for 20 min at 4 °C. Only the soluble components present in the supernatant were used as a source of protease activity. It is important to point out that whole-insect extracts were used due the difficulty to obtain sufficient gut α-amylases for all of the assays. However, we have shown that all of the enzyme activity in a whole insect extract of *H. hampei* is due to the gut α-amylase activity (*10*).

Protease Activity Assays. Total proteolytic activity from the intestinal tracts of *H. hampei* was assayed according to the method of Lenney (*20*) and Blanco Labra et al. (*21*). Briefly, the bovine hemoglobin was prepared in water and denatured with a 6.0 N HCl solution (pH 1.0). A final hemoglobin solution was prepared at 0.083% by using 0.1 M citrate buffer (pH 3.0). One enzymatic activity unit was defined as a decrement of 0.001 in absorbance at 280 nm.

Proteolytic Digestion Assay of Pure α-Amylase Inhibitor (α-AI-1). To study the effect of proteases from the digestive fluid of *H. hampei* on the activity of α-AI-1, 1 μL of the pure α-AI-1 (2 μg/μL) was incubated with 1 μL of crude proteolytic extract containing 4.0, 2.0. and 0 enzymatic activity units for 2 h at 36 °C and then stored at −80 °C. Thereafter, 4 μL of this solution containing the digested α-AI was isoelectrofocused in an IEF PhastGel (*3–9*) and assayed for residual inhibitory activity. Zymography was performed according to the protocol of Valencia-Jimenez et al. (*22*) and described previously.

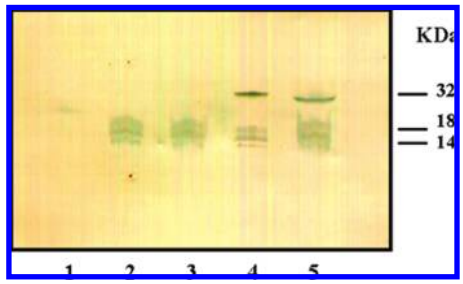

Figure 1. Immunoblot of extracts from seeds of four *P. coccineus* accessions using polyclonal antibodies raised against *P. vulgaris* α-AI1: lane 1, transgenic tobacco seed extract containing recombinant α-AI1; lane 2, *P. coccineus* G35514; lane 3, *P. coccineus* G35590; lane 4, *P. coccineus* G35018; lane 5, *P. coccineus* G35619. Arrows on the left indicate approximate molecular masses of 14, 18, and 32 kDa from bottom to top. Electrophoretic separation was performed on gradient Phastgel 8–25%.

RESULTS

Immunoblot Analysis. To study if the *P. coccineus* and transgenic tobacco seeds contain some polypeptides that cross-react with the α-AI-1 antiserum, seed extracts from four accessions of *P. coccineus* and from transgenic tobacco expressing the recombinant α-AI-1 gene from *P. vulgaris* were analyzed. Seed extracts were separated by SDS-PAGE and immunoblotted with antibodies that were produced against α-AI-1. In this assay the recombinant α-AI-1 protein from tobacco transgenic seeds was recognized as showing the specificity of the antiserum (**Figure 1**). The α-AI-1 antibodies react with small polypeptides in the 14–18 kDa molecular mass range. The seed extract of accession G35018 of *P. coccineus* contained a large molecular mass polypeptide of 32 kDa that reacts with the α-AI-1 antibodies.

Zymograms of α-Amylase Inhibitors in *P. coccineus* Seeds and Transgenic Tobacco Seeds. To evidence different isoforms of α-AIs in *P. coccineus* accessions and if they differ from the α-AI-1, protein extracts were analyzed in native-PAGE (gradient 8–25%) and by isoelectrofocusing (IEF 3–9) gels. This procedure allowed us to separate the inhibitors on the basis of charge and then to detect their activity in the gel. The zymogram shows that among the four accessions of *P. coccineus* analyzed, the two accessions G35590 and G35018 exhibited more clearly different inhibitory bands with apparent differences in their protein expression level (**Figure 2**). Therefore, only those two accessions will be used for the subsequent analyses. The zymogram that was obtained after separation of the proteins present in *P. coccineus* (G35590 and G35018) extracts on an IEF Phastgel (see **Figure 3**) showed the presence of different inhibitory bands in each accession, with the same qualitative differences in their inhibitory activity as found in the previous experiment done by using native PAGE.

Inhibition of Porcine, Human Salivary and Coffee Berry Borer α-Amylases. To evidence the influence of different inhibitor isoforms on α-amylases, we assayed the effect of two *P. coccineus* accessions exhibiting different inhibitor isoforms on the α-amylase activity of porcine pancreas, human salivary, and coffee berry borer (*H. hampei*) α-amylase activity. Pure pancreatic porcine and human salivary α-amylase and whole-insect extract were tested for amylase activity after incubation with increasing amounts of a bean α-AI extract. It was found that the amylase inhibitor from *P. coccineus* seeds (α-AI-Pc) inhibited both mammalian α-amylases and *H. hampei* α-amylase. Addition of 50 μg/μL of the seed protein of crude extract of *P. coccineus* (G35590) was sufficient to completely inhibit

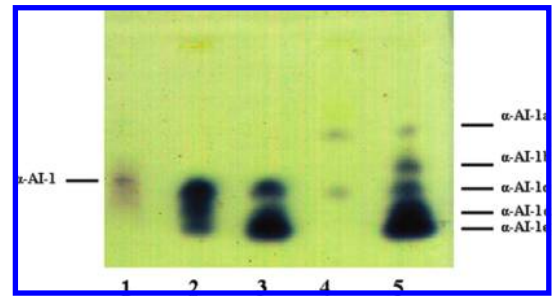

Figure 2. Electrophoretic separation of α-amylase inhibitors from seed extracts of four *P. coccineus* accessions and from tobacco transgenic seed extract: lane 1, transgenic tobacco seed extract containing recombinant α-AI1; lane 2, *P. coccineus* G35514; lane 3, *P. coccineus* G35590; lane 4, *P. coccineus* G35018; lane 5, *P. coccineus* G35619. Electrophoretic separation was performed on native gradient Phastgel 8–25%. After native electrophoresis, the Phastgel was incubated in 1% starch solution for 60 min, followed by incubation in salivary amylase solution to 30 °C for 15 min. Finally, the gel was washed with water and stained with iodine solution to detect α-AI activity bands.

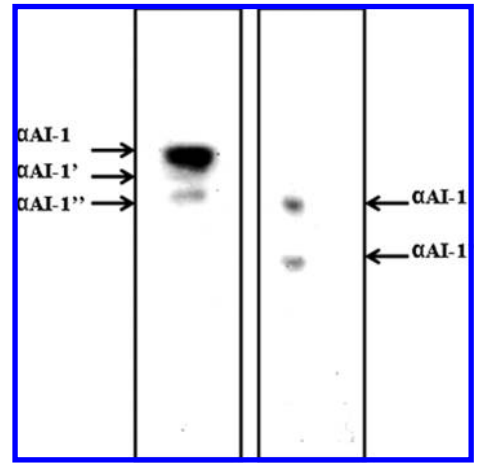

Figure 3. Isoelectrofocusing of α-amylase inhibitors of seed extracts of two accessions of *P. coccineus*: lane 1, *P. coccineus* G35590; lane 2, *P. coccineus* G35018. Electrophoresis was performed on Phastgel IEF 3–9. After electrophoresis, the IEF Phastgel was incubated in 1% starch solution for 60 min, followed by incubation in salivary amylase solution to 30 °C for 15 min. Finally, the gel was washed with water and stained with iodine solution for α-AI activity bands.

mammalian α-amylases under the conditions of our assay (**Figure 4a**,**b**), whereas the same quantity of α-AI (expressed as crude protein) inhibited the *H. hampei* amylase activity by only 85% (**Figure 41c**). Interestingly differences between the inhibitors became evident at the lower protein concentrations.

When used at 20 μg/μL, the seed protein of crude extract of *P. coccineus* (G35018) seeds inhibited porcine pancreatic and human salivary amylases by 20% (**Figure 4a**,**b**) and the *H. hampei* amylase by 60% (**Figure 4c**). At the same concentration, the inhibitor from *P. coccineus* (G35590) seeds inhibited the two mammalian amylases differentially (30% for salivary and 60% for porcine pancreatic amylase) and *H. hampei* amylase by 75%. At lower protein concentrations (10 mg/mL) the trend was the same, but the differences were more pronounced. At this concentration extracts of *P. coccineus* (G35590) seeds barely inhibited porcine pancreatic amylase and inhibited *H. hampei* amylase by 68% (**Figure 4c**).

Heat Inactivation and Proteolytic Digestion Assay of α-Amylase Inhibitor (α-AI-1). The effect of heat treatment on α-amylase inhibitory activity was done using two accessions

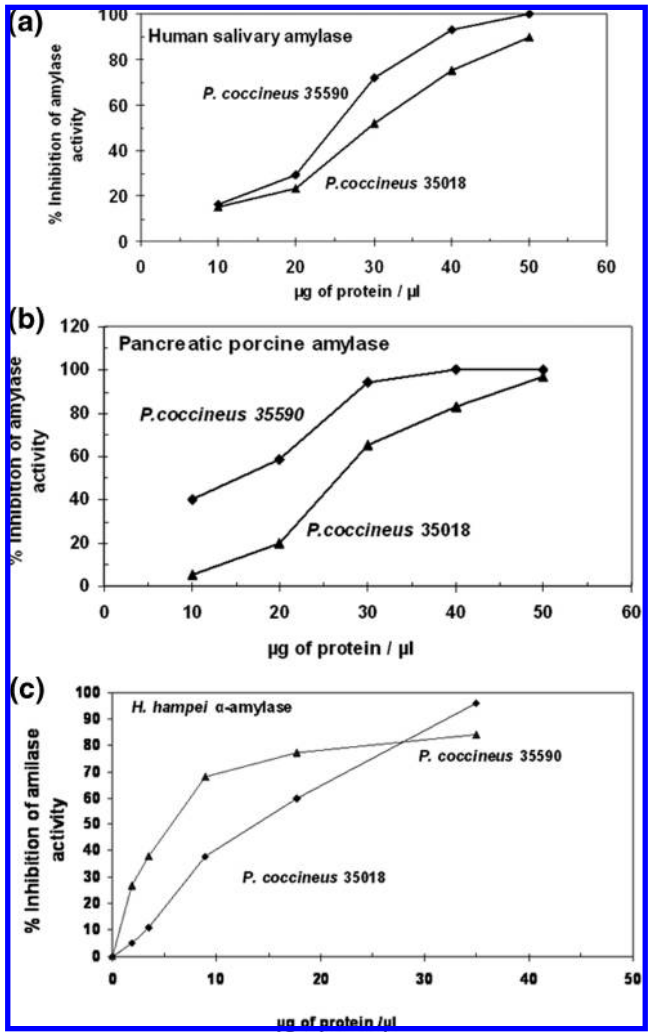

Figure 4. Effect of two crude inhibitor preparations of *P. coccineus* seed extracts containing α-AI-Pc on α-amylases from three different sources: (**a**) inhibition of human salivary α-amylase; (**b**) inhibition of pancreatic porcine α-amylase; (**c**) inhibition of *H. hampei* α-amylase. Enzyme and inhibitors were preincubated for 15 min prior to the addition of starch to measure enzyme activity. Inhibition (percent) is relative to the control assay (without seed extract). Each point is the average of three measurements.

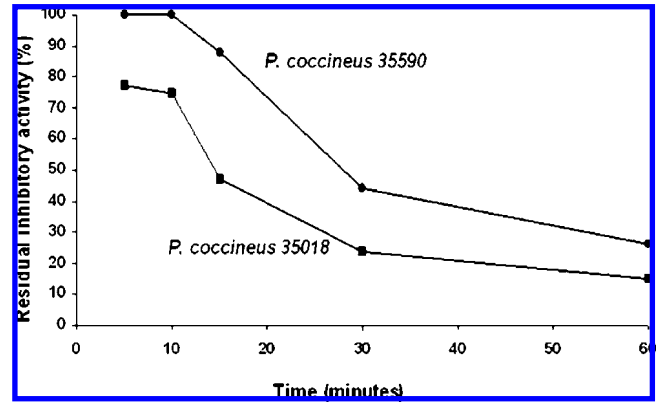

Figure 5. Heat inactivation of α-AI activity in the seed extracts of two *P. coccineus* accessions as a function of incubation time at 80 °C. Thermal stability was determined by measuring the residual α-amylase inhibitory activity after 60 min. Each point is the average of three measurements.

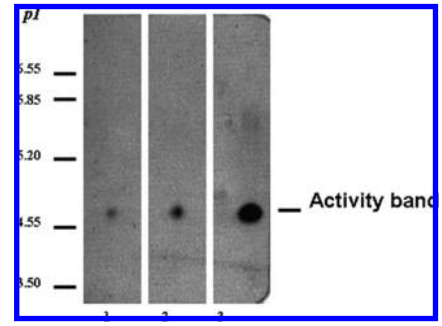

Figure 6. Proteolytic digestion treatment of the α-AI from *P. vulgaris* with different concentrations of acidic proteases from intestinal tracts of *H. hampei* larvae: lane 1, 4.0 protease units/μL; lane 2, 2.0 protease units/μL; lane 3, 0 protease units/μL. After this digestion treatment, 1 μL of the samples containing the amylase inhibitor was applied to each of three lanes on PhastGel IEF 3−9. Finally, the gel was washed with water and stained with iodine solution to detect the α-AI activity bands.

of *P. coccineus* (G35590 and G35018). It was found that the α-AIs of both accessions are stable at 80 °C for 10 min (**Figure 5**). When the pure α-AI-1 was submitted to proteolytic digestion, it was found that digestive proteases from *H. hampei* intestinal tracts rapidly inactivate the pure α-AI-1, showing an almost complete abolition of amylase inhibitory activity of this inhibitor, after 2 h at 36 °C, when 4.0 protease activity units/μL was used in the digestion test (**Figure 6**).

DISCUSSION

Immunoblot Analysis. The large protein (32 kDa) that reacts with the α-AI-1 antibodies probably belongs to a lectin-like protein commonly found in these bean seeds or an other unprocessed molecule in this protein family (*23, 24*). Proteins in this molecular mass range also have plant defense properties (e.g., the arcelins), and they could be responsible for the differential sensitivity of *P. coccineus* to insects attacks.

Zymograms of α-Amylase Inhibitors in *P. coccineus* Seeds and Transgenic Tobacco Seeds. Analysis of the α-amylase inhibitor proteins present in *P. coccineus* (G35590 and G35018) extracts on an IEF Phastgel showed the presence of different inhibitory bands, with the same semiquantitative differences in their inhibitory activity as found in native PAGE zymography. These differences in the electrophoretic migration of the isoinhibitors may reflect differences in the primary amino acid sequence and could reflect different in situ and in vitro inhibitory properties of the isoinhibitors (*10*). The results indicate that those two accessions of *P. coccineus* contain several α-amylase isoinhibitors, which could be used to produce insect-resistant crops through biotechnology. Using the same two approaches, in gel inhibition and spectrophotometric assay, we were unable to detect α-amylase inhibitory activity in extracts of lima bean (*Phaseolus lunatus*) (data not shown).

Previous research work done by our group showed that seeds of common bean (*P. vulgaris*) contained only one isoform of α-AI (*10*), which corresponds to the band seen in **Figure 1**, lane 1 (transgenic tobacco seeds). In contrast, *Cajanus cajan* (Fabaceae) seeds contain at least four isoforms of α-AIs (*2*).

Inhibition of Porcine, Human Salivary, and Coffee Berry Borer α-Amylases. The formation of the enzymatic α-AI complex depends on a large number of amino acids at the interface of the two proteins (*25*) and is pH-dependent with an optimum ranging between 4 and 5 (*15, 26*). Previously, we have reported that the pH in the midgut of coffee berry borer (*H. hampei*) is between 4.5 and 5.2 (*10*). Therefore, a buffer solution of pH 5.0 was selected to perform both the α-amylase and

inhibitory activity assays, mimicking the natural pH condition found in the insect gut. We postulate that the difference observed in the inhibitory effects of the partially purified bean extracts are caused by differences in affinity between inhibitor and amylase at this pH. The evident differences that were observed between accessions G35018 and G35090 in the zymogram assays in contrast to the quantitative spectrophotometric test reflect the semiquantitative nature of the zymograms. Purified inhibitor and insect enzyme are needed to test the affinity between the two polypeptides. The extracts of *P. coccineus* seed have been reported to inhibit both *Callosobruchus chinensis* (Coleoptera: Bruchidae) amylase and pancreatic porcine amylase but not *Acanthoscelides obtectus* (Coleoptera: Bruchidae) or *Zabrotes subfasciatus* (Coleoptera: Bruchidae) amylases (*3*). In addition, *P. coccineus* had been reported to contain α-amylase inhibitors that can inhibit both insect and mammalian α-amylases (*3*).

On the basis of our results, *P. coccineus* seeds seem to contain different isoforms of α-AIs, which express a high inhibitory potency against *H. hampei*. Our previous work showed that the common bean inhibitor (wild α-AI-Pc1) is more effective at inhibiting the salivary amylase than the *H. hampei* amylase (*10*). When the recombinant α-AI-Pc1 was expressed in tobacco plants, it was able to inhibit 65% of enzyme activity of digestive *H. hampei* α-amylases (*17*). It is possible that the differences in activity between both inhibitors (wild and recombinant α-AI-Pc1) are a consequence of a low expression of the recombinant α-AI-Pc1 in the tobacco seeds or that the cloned inhibitor does not represent the most active isoform of this α-amylase inhibitor.

Heat Inactivation and Proteolytic Digestion Assay of α-Amylase Inhibitor (α-AI-1). Our results indicated that the α-AIs of both accessions of *P. coccineus* are stable at 80 °C for 10 min. Similarly, it has been shown that heating of pigeon pea (*C. cajan*) α-AIs under the same conditions tends to destroy the inhibitory activity (*2*). In general, it was found that the α-AIs from *P. coccineus* seeds are heat labile. However, a notable resistance to heat treatment was observed. After being held for 1 h at these experimental conditions, the inhibitory extracts retained between 20 and 30% of their inhibitory activity.

The proteolytic digestion assays of the pure α-AI-1 did not generate new active α-amylase inhibitor fragments as found in similar experiments. In contrast to our result, longer protease treatments of α-AIs from *C. cajan* seeds extracts resulted in the formation of new active α-amylase inhibitor fragments, with a notable resistance to proteases (*27*). This noticeable difference could be a consequence of the different susceptibilities of the two α-amylase inhibitors to endogenous digestive proteases from different insect sources. Our results indicate that the proteolytic digestion treatment induces a rapid loss of the activity of the α-amylase inhibitor, seriously affecting its natural capacity to interact with mammalian α-amylases. The natural interaction of α-AIs with endogenous proteases from insect intestinal tracts must be assayed under differential concentration conditions of both α-amylase inhibitor and the intestinal protease. According to this, the complete inhibition of an insect amylase depends not only on the inhibitor potency but also on its natural resistance to the insect intestinal proteases.

The data presented here lead to four important conclusions. First, pure isoforms of amylase inhibitors (α-AIs) from *P. coccineus* show specificity and variation in their inhibitory activities in relation to the different sources of α-amylases that were tested. Second, because of the inhibitory specificities of these α-AI isoforms against the mammalian amylase activity, probably they correspond to the α-AI-1 type of *P. vulgaris*. Third, it appeared that *P. coccineus* (G35590) seeds are more promising sources of amylase inhibitor genes than *P. coccineus* (G35018) seeds for the production of coffee plants with resistance to *H. hampei*. Fourth, the biological activity of any α-AI-1 could be seriously compromised if it could be digested by an endogenous protease activity occurring under similar biological and physiological conditions as in the insect intestinal tract.

ACKNOWLEDGMENT

We thank Dr. Maarten J. Chrispeels for providing the protein extract of transgenic tobacco seeds, for a critical review of the manuscript, and for helpful suggestions and comments on the work.

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Received for review November 16, 2007. Revised manuscript received January 10, 2008. Accepted January 19, 2008.

JF0733564