Journal of Biochemistry and Molecular Biology, Vol. 40, No. 4, July 2007, pp. 494-500

JOURNAL OF Biochemistry and Molecular Biology

Molecular Identification of Four Different α-amylase Inhibitors from Baru (*Dipteryx alata*) Seeds with Activity Toward Insect Enzymes

Krishna B. Bonavides[1], Patrícia B. Pelegrini[1], Raúl A. Laumann[2], Maria F. Grossi-de-Sá[2], Carlos Bloch Jr.[2], Jorge A.T. Melo[2], Betania F. Quirino[1], Eliane F. Noronha[1] and Octávio L. Franco[1,3,*]

[1]Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Distrito Federal, Brazil

[2]Embrapa-Cenargen, Brasília, Distrito Federal, Brazil

[3]Departamento de Biologia, Universidade Federal de Juiz de Fora, Juiz de Fora MG, Brazil

Received 13 November 2006, Accepted 24 January 2007

The endophytic bruchid pest *Callosobruchus maculatus* causes severe damage to storage cowpea seeds, leading to economical losses. For this reason the use of α-amylase inhibitors to interfere with the pest digestion process has been an interesting alternative to control bruchids. With this aim, α-amylase inhibitors from baru seeds (*Dipteryx alata*) were isolated by affinity chromatographic procedures, causing enhanced inhibition of *C. maculatus* and *Anthonomus grandis* α-amylases. To attempt further purification, this fraction was applied onto a reversed-phase HPLC column, generating four peaks with remarkable inhibition toward *C. maculatus* α-amylases. SDS-PAGE and MALDI-ToF analysis identified major proteins of approximately 5.0, 11.0, 20.0 and 55 kDa that showed α-amylase inhibition. Results of *in vivo* bioassays using artificial seeds containing 1.0% (w/w) of baru crude extract revealed 40% cowpea weevil larvae mortality. These results provide evidence that several α-amylase inhibitors classes, with biotechnological potential, can be isolated from a single plant species.

Keywords: α-amylase inhibitors, Bean weevils, *Callosobruchus maculates, Dipteryx alata*

Abbreviations: AAI, *Amaranthus* α-amylase inhibitor; α-AI1 and α-AI2, α-amylase inhibitors from common bean; AgA, *Anthonomus grandis* α-amylase; AoA, *Acanthoscelides obtectus* α-amylase; BASI, *Bacillus* α-amylase/subtilisin inhibitor; CmA, *Callosobruchus maculatus α-amylase*; DaRP, *Dipteryx alata* retained peak; DaNRP, *Dipteryx alata* non retained peak; HPLC, high performance liquid chromatography; MALDI-ToF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometer; PPA, porcine pancreatic α-amylase; RBI, Ragi bifunctional inhibitor; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; SIα1, SIα2 and SI1α3, *Sorghum* α-amylase inhibitors; ZSA, *Zabrotes subfasciatus* α-amylase.

*To whom correspondence should be addressed.
Tel: 55-61-3348-7220; Fax: 55-61-3347-4797
E-mail: ocfranco@pos.ucb.br

Introduction

Cowpea weevil *Callosobruchus maculatus* is one of the most studied storage grain insect-pests (Franco *et al.*, 2000). During its endophytic larval stage, it burrows into starchy seeds to feed and develop, causing severe economic losses to subsistence farmers. In order to obtain metabolic energy, the cowpea weevil relies on a hydrolytic enzyme known as α-amylase (α-1,4-glucan-4-glucanohydrolases). This protein belongs to a selective group of enzymes that occur in a wide variety of organisms, and is able to catalyze the hydrolysis of α-1,4 glycosidic bonds, transforming polysaccharides into mono- and disaccharides (Grossi-de-Sá and Chrispells, 1997; Franco *et al.*, 2002; Pelegrini *et al.*, 2006).

A strategy to combat insect pests is the use of α-amylase inhibitors to reduce insect growth by interfering with carbohydrate absorption (Yamada *et al.*, 2001). Leguminous seeds are known as rich sources of proteinaceous α-amylase inhibitors (α-AIs) (Payan, 2004). These α-amylase inhibitors can be classified according to their tertiary structure in six different classes, namely, lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thaumatin-like (Richardson, 1990; Franco *et al.*, 2002). α-Amylase inhibitors can be found in seeds of several plant species and have been shown to act by different mechanisms. Lectin-like α-amylases inhibitors have been purified and characterized from different varieties of common bean (*Phaseolus vulgaris*). These inhibitors have two variants with a high degree of sequence homology (Suzuki *et al.*, 1994) and remarkable contrasting specificities. One of them is α-AI1, which inhibits porcine pancreatic α-amylase (PPA) as well as digestive α-amylases from *C. maculatus* and *C. chinensis*. α-AI1 shows no inhibitory effect against the Mexican bean weevil *Zabrotes subfasciatus* α-amylase (ZSA). The other variant named α-AI2, in contrast to α-AI1, is not able to inhibit the first three α-amylases mentioned above, but can inhibit ZSA (Grossi-de-Sá and

Chrispells, 1997; Da Silva *et al.*, 2000; Yamada *et al.*, 2001). Therefore, it was showed that both inhibitors have an evolutionary relationship with phytohaemagglutinnins and arcelins and must be proteolytically processed and also pos-translation modified in order to be activated (Mirkov *et al.*, 1994).

Each family of α-amylase inhibitors shows particular specificity features. A small inhibitor from the knottin-like family found in *Amaranthus hypocondriacus* seeds inhibited insect α-amylases but was inactive against mammalian enzymes (Pereira *et al.*, 1999). Similar specificity has been shown in γ-purothionin-like proteins, which are able to inhibit insect digestive α-amylase (Bloch and Richardson, 1991). Nevertheless, a wide range of specificities was observed for cereal-like inhibitors isolated from cereal kernels. Members of this family showed inhibitory activity against α-amylases from birds, bacteria, insects and mammals (Franco *et al.*, 2002). Finally two more classes, the thaumatin-like and Kunitz-like inhibitors showed capability to reduce α-amylolytic activity from insects and endogenous α-amylases respectively (Malehorn *et al.*, 1994; Franco *et al.*, 2002; Nielsen *et al.*, 2004).

In order to identify novel α-amylase inhibitors, baru nuts (*Dipteryx alata*, Fabaceae Faboideae) were investigated as a possible source. Baru is an important commercial leguminous tree species from the Brazilian Cerrado. Here we report the isolation of four different classes of α-amylase inhibitors from baru seeds providing evidence, for the first time, of the existence of four different inhibitors classes in a single plant species. Furthermore, we also show the baru inhibitors effectiveness against insect-pest α-amylases, especially toward digestive enzymes of *C. maculatus* larvae. These α-amylase inhibitors from baru seeds are a new promise for crop protection through genetic engineering of *V. unguiculata* seeds with increased resistance to the cowpea weevil.

Materials and Methods

Extraction of digestive α-amylases. *Anthonomus grandis* larvae were obtained from Biological Control Department of EMBRAPA/ Cenargen (Brasília-DF, Brasil). *Acanthoscelides obtectus* and *Callosobruchus maculatus* were obtained from colonies of Centro de Análises Proteomicas e Bioquímica. Cotton boll weevil larvae were reared on an artificial diet (Monnerat *et al.*, 1999) at 25°C and 55% relative humidity. Furthermore, bruchids were reared at 28°C and 60% relative humidity in flasks containing dry seeds. By this way, it was utilized *Phaseolus vulgaris* seeds for *A. obtectus* and *Vigna unguiculata* seeds for *C. maculatus*. In all cases, the guts were surgically removed from larvae and placed into an iso-osmotic saline (0.15 M NaCl). Midguts were macerated and centrifuged at 3,000 g for 15 min at 4°C to remove gut walls and cellular debris. Porcine pancreatic α-amylase (PPA) was purchased from Sigma.

Purification of α-amylase inhibitors. Baru (*D. alata*) nuts were collected at Embrapa Cerrado field (Planaltina-GO, Brazil). Five hundred grams of shelled nuts were macerated and extracted with a solution of 0.6 M NaCl and 0.1% HCl, centrifuged at 4,500 g for 30 min at 4°C. Crude extract was precipitated with ammonium sulphate (100%), dialyzed against distilled water and lyophilized. This sample, named rich fraction, was applied onto a Red-Sepharose CL-6B affinity column in order to isolate cationic proteins. The resin was equilibrated with 0.5 M Tris-HCl buffer containing 5.0 mM $CaCl_2$, pH 7.0 and non-retained peaks were displaced with the same buffer. Retained proteins were eluted using a single step of 0.5 M Tris-HCl buffer pH 7.0 containing 3.0 M NaCl. Eluted fractions were monitored at 280 nm. The retained fraction was applied onto an analytical HPLC reversed-phase column (Vydac C-18TP 522) and proteins were eluted using an acetonitrile linear gradient (0-100%).

α-Amylase inhibitory assays. Enzymatic assays were carried out using insect and mammalian α-amylases, dissolved in 50 mM acetate buffer assay containing 5.0 mM $CaCl_2$, pH 6.5. For inhibitory assays, α-amylase inhibitors were pre-incubated with α-amylolytic enzymes in buffer assay for 20 min at 37°C. Assays with different HPLC peaks were carried out using protein standard concentrations of 41, 60, 623 and 69 $\mu g \cdot ml^{-1}$ from fractions I, II, III and IV, respectively. Other fractions were assayed at a standard concentration of 50 $\mu g \cdot ml^{-1}$. Starch 1% (w/v) was used as substrate in an incubation of 20 min at 37°C. The enzymatic reaction was stopped by adding 3,5-dinitrosalicilic acid at 100°C, according to Bernfeld (1955). One α-amylase unit was defined as the amount of enzyme that increased the absorbance at 530 nm by 0.1 after 25 min. Each assay was carried out in triplicate.

Molecular mass analysis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analyses (12.5%) were carried out as described by Laemmli (1970). Bromophenol blue was used as a tracking dye and proteins were visualized by silver staining. For mass spectrometry analyses, freeze-dried samples from HPLC proteins were prepared for MALDI-ToF analysis on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems) according to Franco *et al.* (2000).

***In vivo* bioassay.** In vivo assays were carried out using artificial seeds of 0.9 cm in diameter, 0.6 cm in height and a weight of 300 mg. Artificial seeds were constructed with *V. unguiculata* fine flour and *D. alata* rich fraction mixed in order to obtain uniform distribution. Standard concentrations of 0.5, 1.0 and 1.5% (w/w) from *D. alata* crude extract were used. Seeds with pure cowpea flour were used as negative control. Groups of five artificial seeds were added to plastic containers (10 ml) and 10 to 15 sexually mature females 48-72-h-old, previously coupled with males, were introduced for 24 h in the container for oviposition. After this period, all seeds were observed under a stereoscopic microscope in order to confirm oviposition, leaving just two eggs per seed. This experimental design evaluated the influence of baru crude extract on both immature stages (mortality after 15 days of development in the seed) and adults (longevity and fecundity of females). Mortality of immature stages was evaluated considering the initial number of eggs and adults obtained in each plastic container (n = 5 for each treatment and control). Effects on adults were analyzed using insects obtained in each treatment; males and females were jointed in couples and isolated in individual plastic containers with five

cowpea seeds (n = 10 for each treatment and control). Containers were observed every 24 h to evaluate longevity of insects and number of eggs deposited by females. For this, seeds of the containers were observed under stereoscopic microscope and total number of eggs counted, 10 replicates were used for each treatment. Data were analyzed using ANOVA and Dunnett test for median multiple comparisons ($p < 0.05$). Kruskal-Wallis test was used when the data were not normally distributed.

Results and Discussion

Purification and *in vitro* assays of *D. alata* α-amylase inhibitors. *D. alata* crude extract was precipitated with ammonium sulphate 100%, dialyzed and applied onto a Red-Sepharose CL-6B affinity column to isolate extremely basic proteins generating a single retained peak (DaRP) (Fig. 1A). Red-Sepharose has also been used for the isolation of α-amylase inhibitors from seeds of cowpea (*V. unguiculata*) (Melo *et al.*, 1999) and *Sorghum bicolor* (SIα1, SIα2 and SI1α3) (Bloch and Richardson, 1991). The inhibitory effects of DaRP against PPA and against insect α-amylases from *A. grandis* (AgA), *C. maculatus* α-amylases (CmA) and *A. obtectus* (AoA) were determined, as shown in Fig. 2. DaRP (Fig. 2A) strongly inhibited (80%) *C. maculatus* and *A. grandis* α-amylases (70%). This fraction did not inhibit α-amylases from *A. obtectus* or PPA. DaRP was applied onto an analytical reversed-phase HPLC column, yielding several peaks (Fig. 1B). Four of them demonstrated inhibitory activity against *C. maculatus* α-amylases. Proteins from fraction I (43.9%), II (45.21%), III (44.5%) and IV (49.3%) were capable of inhibiting CmA (Fig. 2B). These results indicate enhanced specificity toward insect enzymes. Several reports describe inhibitors with the ability to reduce the activity of insect and mammalian digestive enzymes (Grossi-de-Sá and Chrispells, 1997; Franco *et al.*, 2000; Gomes *et al.*, 2005). A microbial α-amylase inhibitor from *Streptomyces tendae*, the *Ragi* bifunctional α-amylase/trypsin inhibitor from finger millet and α-AI1 from common bean inhibit both mammalian and insect α-amylases (Pereira *et al.*, 1999). The inhibitor 0.19 from wheat showed inhibitory activity against PPA, AoA, ZSA and CmA. Nevertheless, some inhibitors are insect-specific such as 0.53, which inhibits α-amylases from bean and cowpea weevil, but has no activity toward PPA (Franco *et al.*, 2000). Other inhibitors isolated from wheat endosperm, with high sequence similarity to γ-thionins, were capable of inhibiting α-amylases from locust and cockroach guts (Bloch and Richardson, 1991). Finally, α-amylase inhibitors from the seeds of *A. hypochondriacus* (AAI) specifically inhibit insect α-amylases; however, it is inactive against the mammalian enzymes (Franco *et al.*, 2000). The inhibitors described in this report showed higher inhibitory activity toward insect enzymes, but was unable to inhibit mammalian α-amylases. This specificity may be advantageous if transgenic strategies are to be used in the control of insect-pests that attack seeds consumed by humans.

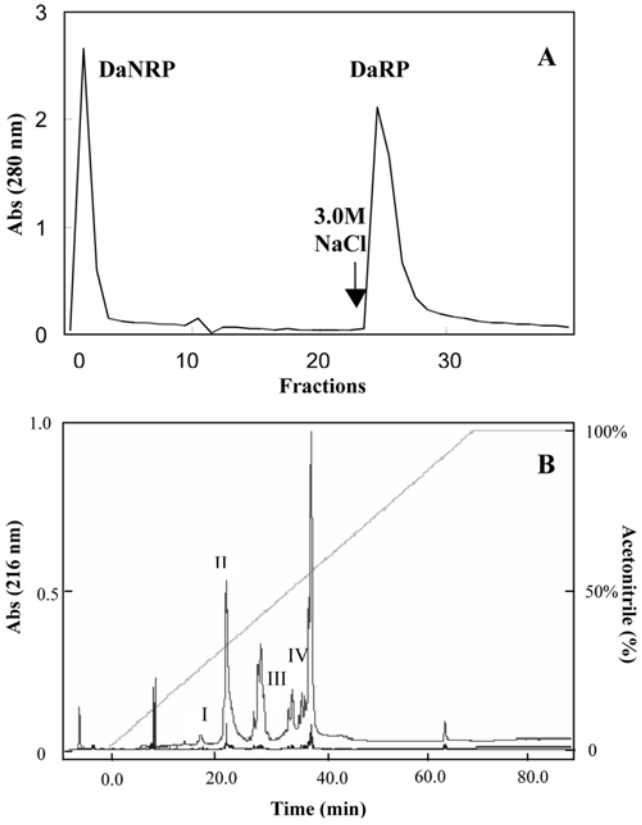

Fig. 1. (A) Chromatographic profile of *D. alata* crude extract using Red-Sepharose CL-6B affinity chromatography. Non-retained proteins (DaNRP) were washed with 0.5 M Tris-HCl buffer pH 7.0 containing 5 mM $CaCl_2$ and retained proteins (DaRP) were displaced with a single step of 0.5 M Tris-HCl buffer pH 7.0 containing 3.0 M NaCl (black arrow). Chromatography was monitored at 280 nm. (B) Reversed-phase HPLC (Vydac C18-TP) of retained protein (DaRP) using analytical column with a flow rate of 1 ml min^{-1}. TFA (0.1%) was used as an ion-pairing agent and the diagonal line indicates the linear gradient of acetonitrile (0-100%).

Molecular Mass Analyses. SDS-PAGE analysis of DaRP (Fig. 3A) showed the presence of proteins with a wide range of molecular masses, varying from 5 to 80 kDa. SDS-PAGE analyses of HPLC peaks showed the presence of 6.0 kDa proteins in fraction I and II, a major band of approximately 53.0 kDa in fraction III and IV and 6.0 to 115.5 kDa in fraction I (Fig. 3B). These data were corroborated by mass spectrometry analyses (MALDI-ToF). Fraction I (Fig. 4A) showed a major peptide with 11 kDa, which according to molecular mass could be classified into the cereal-like family (Fig. 4A). This α-amylase inhibitor family displays proteins with molecular masses of about 11-14 kDa, with some insect specific members such as the 0.53 wheat inhibitors (Franco *et al.*, 2000; Payan, 2004). Other inhibitors purified from wheat, named WRP25 and WRP26, are only able to inhibit insect α-amylases (Feng *et al.*, 1996). The prototype of the cereal inhibitor superfamily is a bifunctional α-amylase/trypsin inhibitor (RBI) from *Ragi*

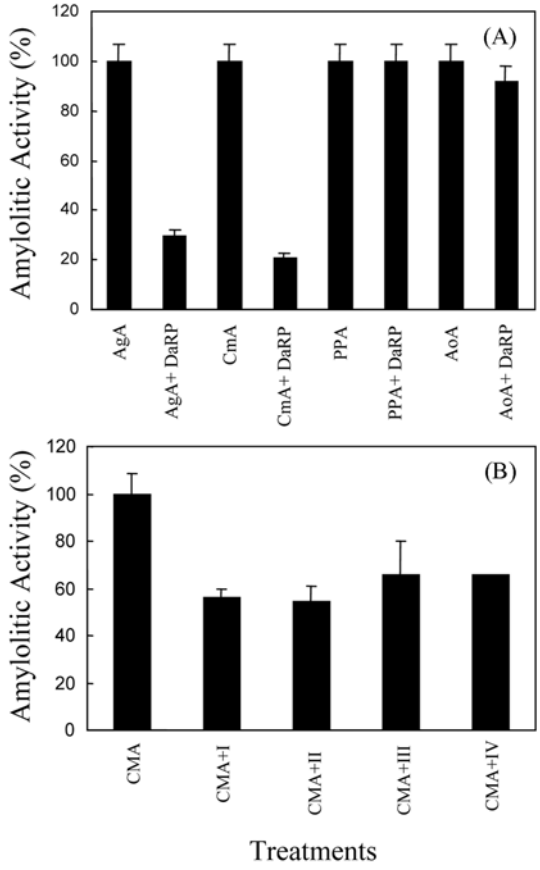

Fig. 2. Inhibitory activity of the *D. alata* retained peak (DaRP) toward *A. grandis* (AgA), *C. maculatus* (CmA), PPA (porcine pancreatic α-amylase) and *A. obtectus* (AoA) α-amylases (A); and inhibitory activity of HPLC fractions toward CmA (B). Each assay was carried out in triplicate. Vertical bars correspond to the standard deviation.

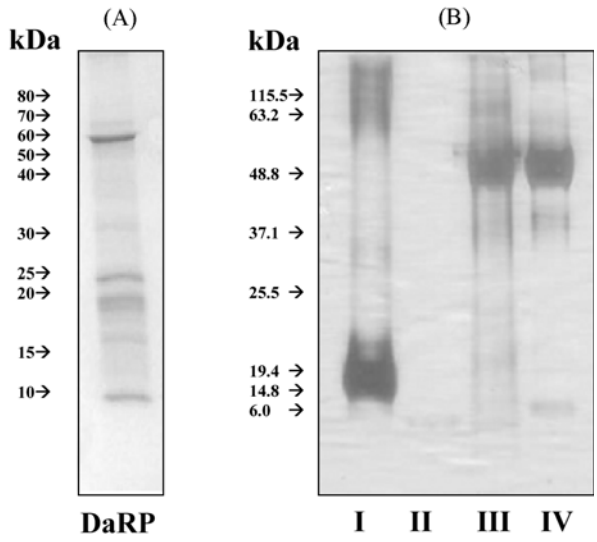

Fig. 3. SDS-PAGE analysis of *D. alata* Red-Sepharose retained peak (DaRP) (A) and HPLC fractions (B) with capability of CMA inhibition: 1-Peak I, 2-peak II, 3-peak III and 4-peak IV. Proteins were visualized by silver staining.

(Indian finger millet), which inhibits α-amylases from various sources (Strobl *et al.*, 1998). Fraction II analysis (Fig. 4B) demonstrated peptides with 5 kDa and 6 kDa, typical of γ-thionins-like, small and stable proteins, some of them positively charged, which are an important component of the plant defense response (Colilla *et al.*, 1990; Thomma *et al.*, 2002; Pelegrini *et al.*, 2005). γ-Thionins purified from cowpea seeds (*V. unguiculata*) inhibit α-amylases from *Bacillus* sp., *A. oryzae*, *V. unguiculata* seeds and *C. maculatus* larvae (Melo *et al.*, 1999). Fraction IV (Fig. 4C) presented peptides with 20 kDa, similar to proteins from Kunitz-like, also identified in barley as a α-amylase/subtilisin inhibitor (BASI). This inhibitor plays a role in plant defense by inhibiting subtilisin-like serine proteinases of pathogens and pests (Mundy *et al.*, 1984; Vallee *et al.*, 1998; Nielsen *et al.*, 2004). Proteins from fraction IV can also be classified into the thaumatin-like family, with a molecular mass of ≈22 kDa, being capable of inhibiting insect α-amylases and also showing antifungal activity (Malehorn *et al.*, 1994; Batalia *et al.*, 1996; Franco *et al.*, 2002) (Fig. 4C). Finally, according to SDS-PAGE analysis (Fig. 3B) lectin-like proteins (55 kDa) were found in the same fraction that will be further purified and studied. Lectin inhibitors from common bean (*P. vulgaris*) (α-AI1 and α-AI2) are evolutionarily related to plant defense proteins such as phytohemaglutinins and arcelins and share a common tridimensional structure (Chrispeels *et al.*, 1991; Suzuki *et al.*, 1994). Despite having similar structures, the mode of action of these proteins in protecting seeds is totally different (Mirkov *et al.*, 1994; Kluh *et al.*, 2005). Phytohemaglutinins and arcelins are toxic to insects due to their binding to midgut epithelial cells (Paes *et al.*, 2000) and α-AIs due to their antinutritional effects (Kasahara *et al.*, 1996). The *D. alata* inhibitors identified probably do not have a similar tertiary structure as they belong to different protein hyperfamilies of α-amylase inhibitors, however, they evolved a similar biological function. In summary, this report clearly provides evidence that a single plant could be able to synthesize different classes of α-amylase inhibitors, and not just one or two, as previously observed (Franco *et al.*, 2002). These data improve the knowledge about plant defense mechanisms, indicating that they are probably much more complex than we could imagine, utilizing different strategies to control a single pest and/or pathogen.

***In vivo* bioassays.** Bioassays revealed an enhanced mortality rate and reduced insect longevity when *C. maculatus* were fed on a diet containing three different baru crude extract concentrations (0.5, 1.0 and 1.5%). Using the baru rich fraction concentration of 1.0% a mortality rate of 42% was observed (Fig. 5A). Furthermore, a longevity reduction of 2.5% was observed with 1.5% baru crude extract (Fig. 5B). No difference was observed in larval weight, fecundity and growth time bioassays (data not shown). Research on starch

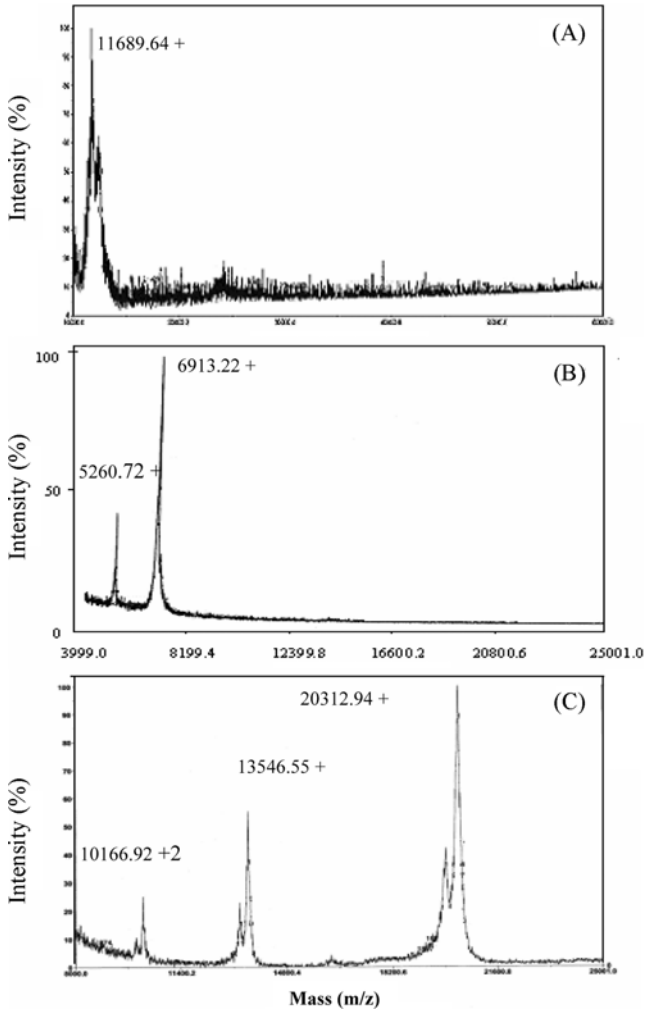

Fig. 4. Mass spectrum (MALDI-ToF) of α-amylase inhibitors found in *D. alata* of fraction I (A), II (B) and IV (C) from HPLC.

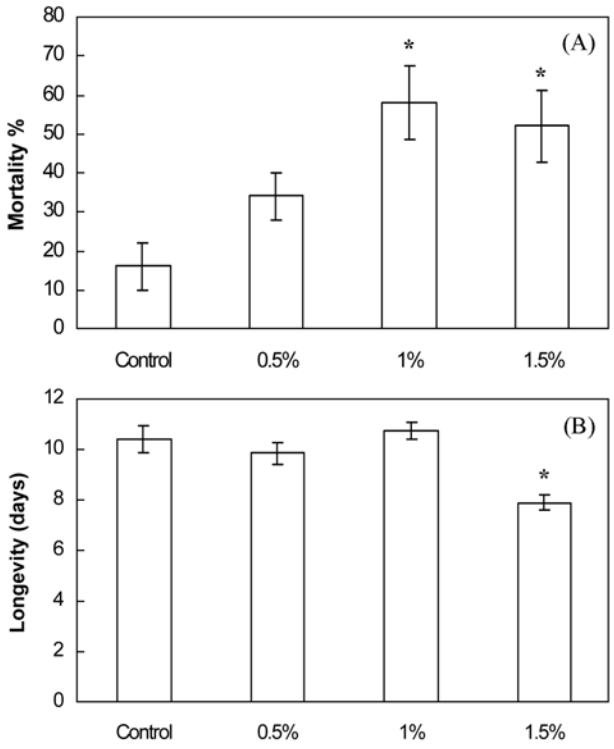

Fig. 5. *In vivo* effects of *D. alata* crude extracts in three concentrations of 0.5, 1.0 and 1.5% (w/w) in *C. maculatus* (A) survival and (B) longevity. Each experiment was carried out in triplicate and vertical bars represents standard deviation. Asterisk indicates treatments statistically different from control (Dunnet test $p < 0.05$).

digestion as a target for control of starch-dependent insects was stimulated in recent years after results showed that α-amylase inhibitors from *P. vulgaris* seeds are detrimental to the development of cowpea weevil *C. maculatus* and Azuki bean weevil *Callosobruchus chinensis* (Ishimoto and Kitamura, 1989; Ishimoto *et al*., 1999). Moreover, the inhibitors 0.19 and 0.53 purified from wheat showed similar results to those obtained in this report, strongly inhibiting *A. obtectus* larval growth and clearly showing a delay in larval development caused by its antinutritional properties (Franco *et al.*, 2005). A similar result was described with a Kunitz-type inhibitor isolated from *Adenanthera pavonina*, which presented a mortality rate of 50% with 0.5% of the inhibitor concentration (Macedo *et al.*, 2004). Furthermore, an inhibitor named pBIII was cloned, expressed and purified from rye kernels and evaluated *in vivo* toward the cotton boll weevil *A. grandis*. Results showed an enhanced mortality rate of weevils (83%) with a low inhibitor concentration (0.89% (w/w)) indicating its effectiveness in Coleopteran control (Dias *et al.*, 2005). Therefore, when α-amylase inhibitors effectiveness toward insect pests were compared to deleterious effects caused by proteinase inhibitors, the latter have been shown to be more lethal (Gomes *et al.*, 2005). Similar effects were caused by vicilins from resistant cowpea seeds, which showed low mortality but were strictly involved in a drastic reduction of insect emergence of *C. maculatus* (Sales *et al.*, 2005).

The growing number of studies that describe specificity profiles and attempt to understand which factors are involved in the interaction between insect α-amylases and their cognate inhibitors is notable. These studies can be used, in a near future, for the design of specific bioinsecticides, as well as the utilization of bioinsecticide proteins from fungi and plant sources sources (Murad *et al.*, 2006). The discovery of baru inhibitors adds a novel piece to the plant-insect interaction puzzle, suggesting that these compounds could be useful to cowpea pest management programs as an alternative strategy to *C. maculatus* control. Moreover, the tridimensional structure and the evolutionary relationships among the α-amylase inhibitors described here will only be known with certainty after complete purification and primary structure determination. These new informations will allow the understanding of the mechanisms of action of these proteins and will offer new insights for rational design of specific bioinsecticides.

Acknowlegments Universidade Católica de Brasilia, CNPq, FAPEMIG and CAPES supported this work.

References

Batalia, M. A., Monzingo, A. F., Ernst, S., Roberts, W. and Robertus, J. D. (1996) The crystal structure of the antifungal protein zeamatin, a member of the thaumatin-like, Pr-5 protein family. *Nat. Struct. Biol.* **3**, 19-23.

Bernfeld, P. (1955) Amylases a and b. *Meth. Enzymol.* **1**, 149-154.

Bloch Jr., C. and Richardson, M. (1991) A new family of small (5kDa) protein inhibitors of insect α-amylases from seeds pf sorghum (*Sorghum bicolor* (L) Moench) have sequence homologies with wheat g-purothionins. *FEBS Lett.* **279**, 101-104.

Chrispeels, M. J. and Raikhel, N. V. (1991) Lectins, lectin genes, and their role in plant defense. *Plant Cell* **3**, 1-9.

Colilla, F. J., Rocher, A. and Mendez, E. (1990) g-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. *FEBS Lett.* **270**, 191-194.

Da Silva, M. C. M., Grossi-de-Sá, M. F., Chrispeels, M. J., Togawa, R. C. and Neshich, G. (2000) Analysis of structural and physico-chemical parameters involved in the specificity of binding between α-amylases and their inhibitors. *Protein Eng.* **13**, 167-177.

Dias, S. C., Franco, O. L., Magalhães, C. P, de Oliveira-Neto, O. B., Laumann, R. A., Figueira, E. L. Z., Melo, F. R. and Grossi-de-Sá, M. F. (2005) Molecular cloning and expression of an α-amylase inhibitor from rye with potential for controlling insect pests. *Protein J.* **24**, 113-123.

Feng, G. H., Richardson, M., Chen, M. S., Kramer, K. J., Morgan, T. D. and Reeck, G. R. (1996) α-Amylase inhibitors from wheat: amino acid sequences and patterns of inhibition of insect and human α-amylases. *Insect Biochem. Mol. Biol.* **26**, 419-426.

Franco, O. L., Rigden, D. J., Melo, F. R., Bloch Jr., C., Silva, C. P. and Grossi-de-Sá, M. F. (2000) Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. *Eur. J. Biochem.* **267**, 2166-2173.

Franco, O. L., Rigden, D. J., Melo, F. R. and Grossi-de-Sá, M. F. (2002) Plant α-amylase inhibitors and their interaction with insect α-amylases. Structure, function and potential crop protection. *Eur. J. Biochem.* **269**, 397-412.

Franco, O. L., Melo, F. R., Mendes, P. A., Paes, N. S., Yokoyama, M., Coutinho, M. V., Bloch Jr, C. and Grossi-de-Sá, M. F. (2005) Characterization of two *Acanthoscelides obtectus* α-amylases and their inactivation by wheat inhibitors. *J. Agric. Food Chem.* **53**, 1585-1590.

Gomes, A. P. G., Dias, S. C., Bloch, C. Jr., Melo, F. R., Furtado, J. R. Jr., Monnerat, R. G., Grossi-de-Sa, M. F. and Franco, O. L. (2005) Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* **140**, 313-319.

Grossi-de-Sá, M. F. and Chrispeels, M. J. (1997) Molecular cloning of bruchid (*Zabrotes subfasciatus*) α-amylase cDNA and interaction of the expressed enzyme with bean α-amylase inhibitors. *Insect Biochem. Mol. Biol.* **27**, 271-281.

Ishimoto, M. and 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. Ent. Zool.* **24**, 281-286.

Ishimoto, M., Yamada, T. and Kaga, A. (1999) Insecticidal activity of an α-amylase inhibitor-like protein resembling a putative precursor of α-amylase inhibitor in the common bean, *Phaseolus vulgaris* L. *Biochim. Biophys. Acta* **1432**, 104-112.

Kasahara, K., Hayashi, K., Arakawa, T., Philo, J. S., Wen, J., Hara, S. and Yamaguchi, H. (1996) Complete sequence, subunit structure, and complexes with pancreatic α-amylase of an α-amylase inhibitor from *Phaseolus vulgaris* white kidney beans. *J. Biochem.* **120**, 177-183.

Kluh, I., Horn, M., Hýblová, J., Hubert, J., Dolecková-Maresová, L., Voburka, Z., Kudlíková, I., Kocourek, F. and Mares, M. (2005) Inhibitory specificity and insecticidal selectivity of α-amylase inhibitor from *Phaseolus vulgaris*. *Phytochem.* **66**, 31-39.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriphage T4. *Nature* **227**, 680-685.

Macedo, M. L. R., de Sá, C. M., Freire, M.das G.M. and Parra, J. R. P. (2004) A Kunitz-type inhibitor of Coleopteran Proteases, isolated from *Adenanthera pavonina* L. Seeds and its effect on *Callosobruchus maculatus*. *J. Agric. Food Chem.* **52**, 2533-2540.

Malehorn, D. E., Borgmeyer, J. R., Smith, C. E. and Shah, D. M. (1994) Characterization and expression of an antifungal zeamatin-like protein (Zlp) gene from *Zea mays*. *Plant Physiol.* **106**, 1471-1481.

Melo, F. R., Sales, M. P., Pereira, L. S., Bloch Jr., C., Franco, O. L. and Ary, M. B. (1999) α-Amylase inhibitors from cowpea seeds. *Protein Pept. Lett.* **6**, 385-390.

Mirkov, T. E., Whalstrom, J. M., Hagiwara, K., Finardi-Filho, F., Kjemtrup, S. and Chrispeels, M. J. (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.

Monnerat, R., Dias, S. C., Oliveira-Neto, B., Nobre, S. D. and Grossi-de-Sá, M. F. (1999) Criação de bicudo do algodoeiro *Anthonomus grandis* em dieta artificial e estabelecimento para bioensaios com *Bacillus thuringiensis*. *IV Congresso Brasileiro de Algodão*, p. 214-216, Ribeirão Preto, Brazil.

Mundy, J., Hejgaard, J. and Svendsen, I. (1984) Characterization of a bifunctional wheat inhibitor of endogenous α-amylase and subtilisin. *FEBS Lett.* **67**, 210-214.

Murad, A. M., Laumann, R. A., Lima, T. A., Sarmento, R. B., Noronha, E. F., Rocha, T. L., Valadares-Inglis, M. C. and Franco, O. L. (2006) Screening of entomopathogenic *Metarhizium anisopliae* isolates and proteomic analysis of secretion synthesized in response to cowpea weevil (*Callosobruchus maculatus*) exoskeleton. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.* **142**, 365-370.

Nielsen, P. K., Bonsager, B. C., Fukuda, K. and Svensson, B. (2004) Barley alpha-amylase/subtilisin inhibitor: structure, biophysics and protein engineering. *Biochim. Biophys. Acta* **1696**, 157-164.

Paes, N. S., Gerhardt, I. R., Coutinho, M. V., Yokoyama, M., Santana, E., Harris, N., Chrispeels, M. J. and Grossi-de-Sá, M. F. (2000) The effect of arcelin-1 on the structure of the midgut of bruchid larvae and immunolocalization of the arcelin protein. *J. Insect Physiol.* **46**, 393-402.

Payan, F. (2004) Structural basis for the inhibition of mammalian

and insect α-amylases by plant protein inhibitors. *Biochim. Biophys. Acta* **1696**, 171-180.

Pelegrini, P. B. and Franco, O. L. (2005) Plant g-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins. *Int. J. Biochem. Cell Biol.* **37**, 2239-2253.

Pelegrini, P. B., Murad, A. M., Grossi-de-Sa, M. F., Mello, L. V., Romeiro, L. A., Noronha, E. F., Caldas, R. A. and Franco, O. L. (2006) Structure and enzyme properties of *Zabrotes subfasciatus* α-amylase. *Arch. Insect Biochem. Physiol.* **61**, 77-86.

Pereira, P. J. B., Lozanov, V., Patthy, A., Huber, R., Bode, W., Pongor, S. and Strobl, S. (1999) Specific inhibition of insect α-amylase in complex with the Amaranth α-amylase inhibitor at 2.0 resolution. *Structure* **7**, 1079-1088.

Richardson, M. (1990) Seeds storage proteins: the enzyme inhibitors, In: *Methods in Plant Biochemistry* **5**, 261-307.

Sales, M. P., Andrade, L. B., Ary, M. B., Miranda, M. R., Teixeira, F. M., Oliveira, A. S., Fernandes, K. V. and Xavier-Filho, J. (2005) Performance of bean bruchids *Callosobruchus maculatus* and *Zabrotes subfasciatus* (Coleoptera: Bruchidae) reared on resistant (IT81D-1045) and susceptible (Epace 10) *Vigna unguiculata* seeds: relationship with trypsin inhibitor and vicilin excretion. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **142**, 422-426.

Strobl, S., Maskos, K., Wiegand, G., Huber, R., Gomis-Rüth, F. X. and Glockshuber, R. (1998) A novel strategy for inhibition of α-amylase in complex with the *Ragi* bifunctional inhibitor at 2.5 resolution. *Structure* **6**, 911-921.

Suzuki, K., Ishimoto, M. and Kitamura, K. (1994) cDNA sequence and deduced primary structure of an alpha-amylase inhibitor from a bruchid-resistant wild common bean. *Biochem. Biophys. Acta* **1206**, 289-291.

Thomma, B. P., Cammue, B. P. and Thevissen, K. (2002) Plant defensins. *Planta* **216**, 193-202.

Vallee, F., Kadziola, A., Bourne, Y., Juy, M., Rodenburg, K. W., Svensson, B. and Haser, R. (1998) Barley alpha-amylase bound to its endogenous protein inhibitor BASI: crystal structure of the complex at 1.9 Å resolution. *Structure* **6**, 649-659.

Yamada, T., Hattori, K. and Ishimoto, M. (2001) Purification and characterization of two α-amylase inhibitors from seeds of tepary bean (*Phaseolus acutifolius* A. Gray). *Phytochem.* **58**, 59-66.