Available online at www.sciencedirect.com
ScienceDirect
Pesticide Biochemistry and Physiology 87 (2007) 255–260
PESTICIDE
Biochemistry & Physiology
www.elsevier.com/locate/ypest

Isolation of a novel *Carica papaya* α-amylase inhibitor with deleterious activity toward *Callosobruchus maculatus*

L.R. Farias [a], F.T. Costa [a], L.A. Souza [a], P.B. Pelegrini [a], M.F. Grossi-de-Sá [b], S.M. Neto [a], C. Bloch Jr. [b], R.A. Laumann [b], E.F. Noronha [a], O.L. Franco [a,*]

[a] *Centro de Análises Proteômicas e Bioquímicas, Programa de Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília-DF, Brazil*
[b] *Cenargen/Embrapa, Brasília-DF, Brazil*

Received 28 April 2006; accepted 14 August 2006
Available online 22 August 2006

Abstract

Cowpea (*Vigna unguiculata*) is a subsistence crop for small and poor farmers from Latin America and Africa. This culture is commonly damaged by cowpea weevil (*Callosobruchus maculatus*), which burrow into stored seeds to fed on. Due to impact of larval predation, several plant defense studies have been developed, indicating that α-amylase inhibitors are able to impede and/or reduce bruchids digestive process. In this report, a novel α-amylase inhibitor from papaya seeds (*Carica papaya*) with activity against cowpea weevil enzymes was purified and biochemical characterized. Peeled seeds were macerated and extracted with a 0.6 M NaCl and 0.1% HCl solution. Crude extract was precipitated with ammonium sulphate (100%). After dialyses, this rich fraction was applied onto a CM-Cellulose column and retained peak was submitted to an analytic reversed-phase column HPLC (Vydac C-18TP) yielding several peaks. Only one fraction, with molecular mass of 4562 Da, showed significant inhibitory activity against *C. maculatus* α-amylases. Otherwise, no inhibitory activities against mammalian α-amylases were observed. Bioassays using artificial seeds containing *C. papaya* α-amylase inhibitor rich fraction (0.5% and 1.0%) were also conduced showing that α-amylase inhibitors were able to increase larval mortality (50%) and also decrease insect fecundity and adult longevity. These results showed the presence of an α-amylase inhibitor from *C. papaya* seeds with high specificity to insect enzymes, indicating that this inhibitor probably could be used, through genetic engineering, in the construction of transgenic plants with enhanced resistance toward cowpea weevil.

Keywords: *Callosobruchus maculatus*; α-Amylase inhibitors; *Carica papaya*; Plant defense

1. Introduction

Cowpea (*Vigna unguiculata*) crop is commonly cultivated by small and poor farmers from Latin America and Africa, being used as main sources of carbohydrate and proteins. This crop is severely attacked by several pests and pathogens as the cowpea weevil (*Callosobruchus maculatus*). This bruchid fed on starchy cowpea cotyledons during storage, causing dramatic degradation to dry seeds, obstructing the commercialization and human consume. Since metabolic energy, extremely necessary for larval development, could be originated from starch hydrolyzes, which produces mono- and disaccharides [1], several studies have been realized in order to understand α-amylase mechanisms. Actually an unique insect α-amylase, from digestive tract of *Tenebrio molitor* (TMA), has their three-dimensional structure elucidated. A single polypeptide chain of 471 amino acid residues length, one calcium ion, one chloride ion and 261 water molecules composed TMA structure [2]. Calcium ion seems important for enzyme stability, binding to one histidine residue, which interacts with fourth substrate sugar bound in the active site, forming a hinge between the catalytic-site and Ca^{2+}-binding site [2]. These interactions lead to a clear adaptation to acidic physiological larvae midgut environment [3].

* Corresponding author. Fax: +55 61 3347 4797.
E-mail address: ocfranco@pos.ucb.br (O.L. Franco).

0048-3575/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2006.08.004

In response to hydrolytic enzymes, several compounds found in nature are able to inhibit α-amylases, which include different molecules as carbohydrates [4] and proteins [5]. Among plant non-proteinaceous inhibitors, several examples are cited as acarbose, iso-acarbose, acarviosine-glucose, hibiscus acid and cyclodextrins [5–7]. Otherwise, proteinaceous α-amylase inhibitors are also found in plants as well in microorganisms and animals [8–11]. In plants, these inhibitors could occur as part of defense mechanisms, been particularly abundant in leguminous [12–15] and cereals [8,10,16–18]. Several reports described this inhibitory activity in several families [5,4], where mechanisms of action are deeply studied but not completely understood. Due to great diversity of α-amylase inhibitors and their expressive specificity variability, these inhibitors could be discerned according their effectiveness against target enzymes. α-AIs were reported to inhibit digestive enzymes from several sources, including mammalian, insects and fungi [19,20]. α-Amylase inhibitors found in wheat [10], barley [21] and Indian finger millet [22] are able to efficiently inhibit α-amylases from insect sources, showing inhibitory activity against α-amylases from *C. maculatus*, *Acanthoscelides obtectus*, *Zabrotes subfasciatus* [10], *T. molitor* [17] and *Bacillus subtilis* [23]. In spite of several α-amylase inhibitors have been described in plant seeds, just few studies indicated the presence of inhibitors in fruits. One single example was shown with a glycoprotein with inhibitory activity against pectin methylesterase isolated from kiwi fruit (*Actinidia chinensis*) [24]. Furthermore, a Kunitz-type trypsin inhibitor, a 24.0 kDa glycoprotein, was also purified from *Carica papaya* homogeneity. Nevertheless, this inhibitor was no challenged against α-amylases [42]. According our knowledge, no α-amylase inhibitor was isolated from papaya seeds before. Here, in this report, we described the isolation and molecular characterization of a novel α-amylase inhibitor from *C. papaya* seeds as an alternative control for the cowpea weevil *C. maculatus*.

2. Material and methods

2.1. α-Amylases

Commercially available α-amylase from porcine pancreas (PPA) and from human saliva were purchased from Sigma Co. Furthermore, *C. maculatus* larval guts of 17–20-day-old were dissected in iced-cold iso-osmotic solution containing 25 mM NaCl, homogenized, and centrifuged at 4000*g* for 20 min at 4 °C to remove gut walls and cellular debris. CMA supernatant was utilized for enzyme and inhibitory enzyme assays.

2.2. Isolation of C. papaya α-AI

Carica papaya seeds were extracted with a solution of 0.6 M NaCl and 0.1% HCl with continuous stirring for 5 h at 4 °C. Crude extract was centrifuged at 10,000*g* and 4 °C for 40 min. Supernatant was submitted to a fractionation with ammonium sulphate (100%). After dialysis against distilled water and liophilization, this fraction was applied onto an ionic exchange CM-Cellulose column equilibrated with 50 mM sodium acetate buffer, pH 6.5 containing 5.0 mM $CaCl_2$ and 10 mM NaCl. Non-retained proteins were displaced with same buffer and retained fractions were eluted with a linear gradient of 0.0–1.0 M of NaCl dissolved at same buffer. Major chromatography retained peak was applied onto a reversed-phase column HPLC (Vydac 218TP 1022 C-18) using a flow rate of 1.0 ml min^{-1}. Several peaks were collected separately, lyophilized and stored at −20 °C for further analyses.

2.3. Molecular mass analyses

The protein fractions eluted from the reversed-phase chromatography were analyzed by 15% SDS–PAGE according to Laemmli [47] with minor modifications using bromophenol blue as tracking dye. Samples were run for 45 min at 200 V and gels were silver stained. These fractions were also submitted to mass spectrometry analyses. Freeze-dried sample was prepared for matrix-assisted laser desorption time-of-flight analysis (MALDI-TOF) on a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA, USA). Samples were dissolved in 1.0% trifluoroacetic acid (TFA) and sinapinic acid (a saturated solution dissolved in acetonitrile/0.1% TFA 1:1, v/v) was added. 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 an 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 light. The signal was digitized at a rate of 500 MHz and averaged data was presented to a standard Voyager data system for manipulation.

2.4. Enzyme and inhibitory enzyme assays

α-Amylases and α-amylase inhibitory activities were estimated according Bernfeld [25]. Enzyme and inhibitors, buffered with 50 mM acetate buffer, pH 6.5 containing 5.0 mM $CaCl_2$ and 10.0 mM NaCl, were pre-incubated for 20 min at 37 °C. α-AI from *C. papaya* (CpAI)[1] seeds was assayed at standard concentration of 50 μg ml^{-1} against PPA and CMA. Both enzymes were assayed at concentration of 25 μg ml^{-1}. 1.0% starch was utilized as substrate. After the

[1] *Abbreviations used:* CpAI, *C. papaya* α-amylase inhibitor; CMA, *C. maculatus* α-amylase; HSA, human salivary α-amylase; PPA, porcine pancreatic α-amylase; ZSA, *Z. subfasciatus* α-amylase; α-AIs, α-amylase inhibitors; TMA, *T. molitor* α-amylase; HSA, human salivary α-amylase; MALDI-TOF, matrix-assisted laser desorption time-of-flight analysis; NMR, nuclear magnetic resonance; SIα1, SIα2 and SIα3, *Sorghum* α-amylase inhibitors; WRP24 or 0.19, 0.53, WRP25, WRP26, WRP27, wheat α-amylase inhibitors.

addition of 3.5 dinitrosalicilic acid (DNS), reaction was stopped at 100 °C, being absorbance measured at 530 nm. One α-amylase unit (1UI) was defined as the amount of the enzyme that increased the absorbance at 530 nm by 0.1 OD during 20 min of assay. Each assay was carried out in triplicate. Distilled water was utilized as negative control and wheat α-amylase inhibitor at a standard concentration of 50 $\mu g\,ml^{-1}$ was used as positive control.

2.5. Bioassays

In vivo bioassays were performed with artificial seeds constructed with cowpea flour. For this, after retired the epicarp of seeds cotyledons were grounded in a flourmill. Flour was compacted using hand compressor, in artificial seed (about 0.9 cm diameter × 0.6 cm high, weight 300 mg). For feeding tests, papaya rich fraction was aggregated to the flour to obtain desired concentrations. Bioassays were done using seed containing three different concentrations of papaya seeds crude extracts (1.5%, 1.0% and 0.5% w:w). 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 stereoscopic microscope in order to confirm oviposition, leaving just two eggs per seed. This experimental design evaluated the influence of papaya crude extract on both immature stages (mortality after 15 days of developed in seed) and adults (longevity and fecundity of females). Mortality of immature stages was evaluated considering 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 joint 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 evaluated 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 were used when the data was not normally distributed.

3. Results and discussion

3.1. Purification of CpAI and inhibitory enzyme assays

In order to isolate a novel defense factor toward *C. maculatus* larvae, a crude extract from *C. papaya* seeds was precipitated with ammonium sulphate (100%). Other fractions (0–50% and 60–80%) were unable to inhibit α-amylolytic activity (data not shown). Rich-fraction (100%) was assayed against CMA and PPA causing 61.5% and 12% of enzymatic activity inhibition, respectively (Fig. 1). None inhibitory activity was observed against HSA (data not shown). This fraction was applied, after dialysis, onto an ionic-change chromatography CM-Cellulose column generating several fractions (Fig. 2A). CM-Cellulose non-retained fraction showed no inhibitory activity against α-amylases tested (data not shown). Otherwise, retained peak III, displaced with 0.4 M NaCl, was able to reduce 30% of CMA activity, but was unable to inhibit PPA (Fig. 1). This peak was applied onto a reversed-phase column HPLC (Vydac C-18TP), yielding 18 peaks (Fig. 2B). Inhibition assays were also carried out and only peak VIII, after re-chromatography in order to reduce contaminants (Fig. 2C) inhibited specifically CMA (57.3%). Furthermore, PPA was not inhibited by this fraction (Fig. 1). Several compounds have been shown inhibitory activity against insect enzymes as proteins, carbohydrates and metals [43–45], with different specificities. *C. papaya* α-amylase inhibitor (CpAI) probable shows selectivity toward insect enzymes, a desirable characteristic for bioinsecticides. Other inhibitors showed previously reported activity against digestive enzymes from *C. maculatus* as the wheat inhibitors named 0.19, 0.53 and WRP25 [10]. Additionally, wheat α-amylase inhibitors inhibit digestive α-amylases from rice weevil, red flour beetle and yellow mealworm, but were unable to inhibit human salivary enzyme (HSA) [17]. *S. bicolor* α-AIs (SIα1, SIα2 and SIα3) were assayed toward α-amylases from several sources, being able to efficiently inhibit locust and cockroach enzymes. Otherwise, sorghum inhibitors were unable to inhibit porcine, barley and *Bacillus* spp. α-amylases [26]. Furthermore, lectin-like α-amylase inhibitors showed similar specificity pattern, while α-AI1 variant showed specificity toward PPA, CMA and *Callosobruchus chinensis* α-amylases [19]; α-AI2 variant showed activity against ZSA but no inhibition toward α-amylase affected by α-AI1 and vice versa [15]. α-Amylase inhibitors isolated from *Amaranthus hipocondriacus* seeds revealed a specific inhibitory activity against insect

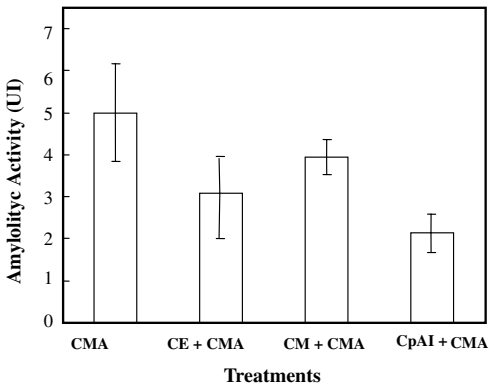

Fig. 1. Inhibitory α-amylolitic (CMA) assays against *C. papaya* crude extract (CE), retained fraction three from CM-Cellulose (CM) and purified CpAI. Vertical bars correspond to standard deviation. Each assay was carried out in triplicate.

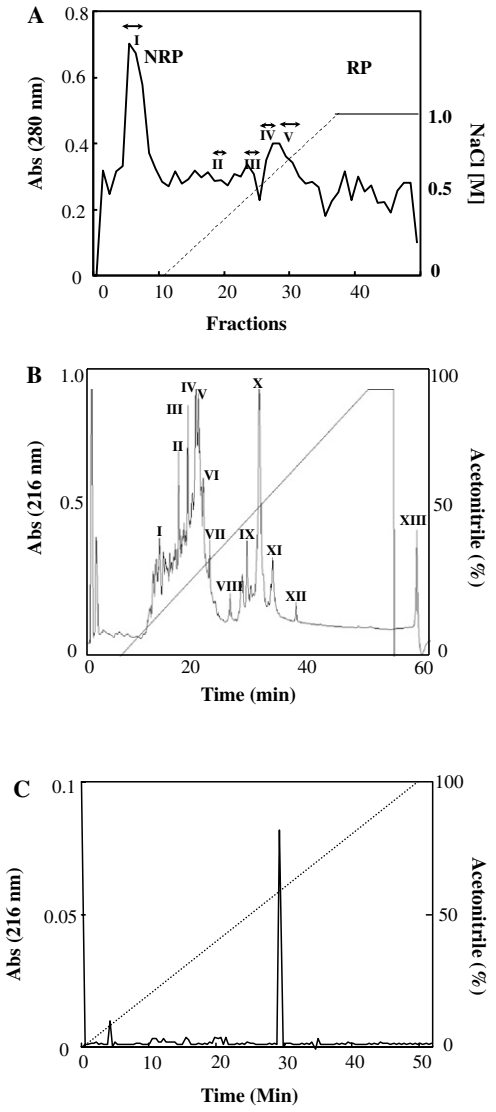

Fig. 2. (A) CM-Cellulose chromatography profile of *C. Papaya* seeds crude extract. NRP corresponds to non-retained peak and RP to retained peak. Dotted line represents the 0–1 M NaCl linear gradient. (B) CM-Cellulose retained peak was applied onto a reversed-phase HPLC (Vydac C-18TP) generating XIII peaks. (C) Re-chromatography from HPLC peak XII at same reversed-phase column. Diagonal lines represents linear acetonitrile gradient (0–100%).

α-amylases, being unable to inhibit mammalian α-amylases [27].

3.2. Molecular mass analyses

CpAI SDS–PAGE and MALDI-TOF analyses showed a majority protein of 4286 Da (Fig. 3) and minor contaminants. These contaminants could be generated by glycosilated isoforms, as observed in α-AIs from common bean seeds and rye kernels [11,46]. This molecular mass probably indicates that CpAI could be included into γ-purothionins-like α-amylase inhibitors family. γ-Thionins are involved in plant defense processes through a wide variety of mechanisms: modifications of membrane permeability [28,29],

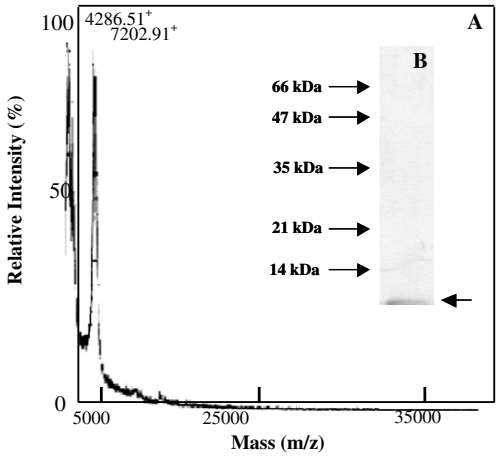

Fig. 3. MALDI-TOF spectrum (A) and SDS–PAGE (B) analyses of purified CpAI. Gel was silver stained and bromophenol blue was utilized as tracking dye.

blockage of protein synthesis [41] and digestive enzyme inhibition [30]. They are small and cationic proteins with 45–54 amino acid residues length, with high disulfide bonds content that could act against insect, bacteria and fungi [31]. Many γ-thionins are studied as monomers, but they can also occur as oligomers [32,33]. Analyses by X-ray crystallography and NMR showed that γ-thionins structures are typical two-layers αβ sandwich that create an amphipatic molecule [34–36]. Despite of these results suggested that CpAI pertains to γ-purothionin family, further studies must be carry in order to classify this proteinaceous compound.

3.3. Feeding tests

In order to evaluate *in vivo* effects of CpAI toward *C. maculatus* larvae, CpAI rich fraction was mixture to cowpea flour and three parameters were analyzed: adult longevity (Fig. 4A), adult fecundity (Fig. 4B) and larvae mortality (Fig. 4C). Results from bioassays showed that inhibitor concentrations of 0.5% and 1.0% (w:w) smoothly reduced adult longevity (Fig. 4A). Furthermore, seeds with an enhanced inhibitor concentration (1.5%) caused remarkable larval mortality (50%) and a clear decrease in adult fecundity (50%). These deleterious effects probably are caused by the presence of CpAI. However, we could not discard the possibility that other minor contaminants could reduce *C. maculatus* development. Franco et al. [37] reported the *in vivo* efficiency of wheat α-AIs (0.19 and 0.53) toward bean weevil *A. obtectus*. Artificial seeds containing several concentrations of wheat α-AIs (0.25%, 0.5% and 1.0% w/w) caused a remarkable decrease on larval growth and an enhanced in insect mortality [37]. According to Feng et al. [17] WRP24 α-amylase inhibitor caused growth inhibition of *T. castaneum* larvae and a substantial loss of weight in adult insects. Although wheat α-amylase inhibitors have showed efficiency on insect α-amylase dependent control, these inhibitors also are associated to baker's

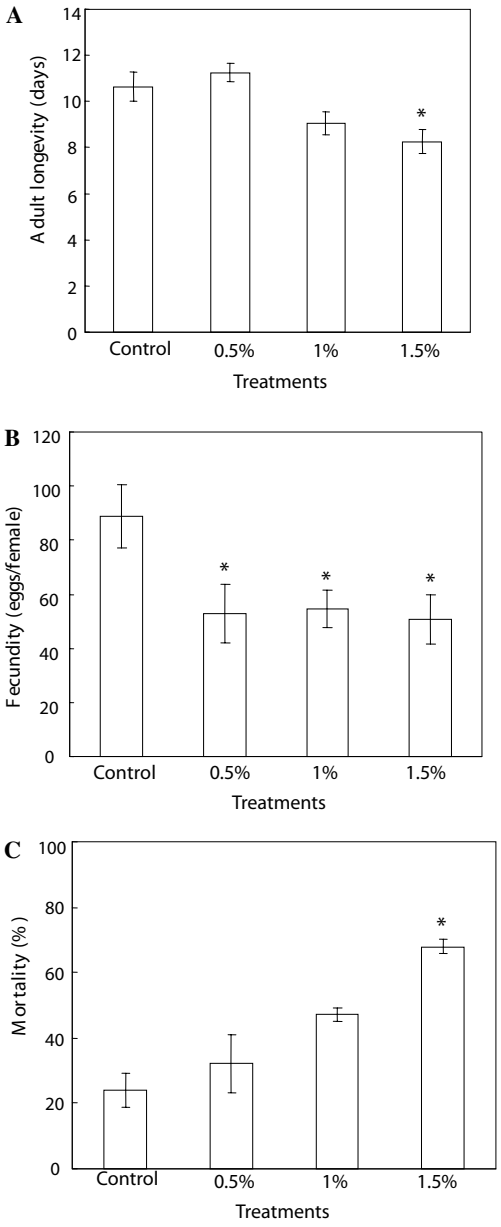

Fig. 4. Feeding tests using *C. papaya* rich fraction at standard concentrations of 0.5%, 1.0% and 1.5%. Bioassays evaluate (A) adult longevity (B) female fecundity and (C) larval mortality. Each experiment was carried out in triplicate and vertical bars represents standard deviation. Asterisk indicates treatments statistically different than control (Dunnet test $p < 0.05$).

Asthma, due to glycosylated subunits of α-amylase inhibitors that increased IgE-binding capacity to allergens [38]. These data suggest that different inhibitors from alternative sources must be isolate, specially inhibitors with a double activity. One example is observed in common bean seeds, which are able to synthesize inhibitors with the ability of inhibit α-amylases and degrade chitin [44]. A great number of genes conferring pest resistance have been incorporated into different crops [1] including digestive enzyme inhibitors, which are thought to inhibit larvae growth by slowing down the digestion process, reducing carbohydrate assimilation. Transgenic peas and adzuki beans expressing *Phaseolus vulgaris* α-AI1 have enhanced resistance to certain species of Bruchidae, whose digestive α-amylases are affected by α-amylase inhibitors [39,40]. When α-AI2 was expressed in peas, a partial protection against bruchids was obtained. Therefore, the α-AI2 gene could also be used to extend the time before the weevil damage, reaching the break-even cost of spraying, and thus removing the necessity of chemical pesticides during storage seeds [40]. None study was done in order to produce transgenic plants expressing fruit α-amylase inhibitors until now. In summary, here was reported for the first time the isolation of an α-amylase inhibitor from fruit seeds. This inhibitor, purified from papaya seeds showed high specificity toward insect enzymes. These surprisingly findings suggests the implication of CpAI in the plant defense mechanism indicating that this inhibitor probably could be utilized, by using genetic engineering techniques, as an alternative strategy to enhance cowpea crop resistance.

Acknowledgment

This work was supported by CAPES, CNPq, EMBRAPA and UCB.

References

[1] C.R. Carlini, M.F. Grossi-de-Sá, Plant toxic proteins with insecticidal properties. A review on their potentialities as bioinsecticides, Toxicon 40 (2002) 1515–1539.
[2] S. Strobl, K. Maskos, G. Wiegand, R. Hurber, F.X. Gomis-Ruth, R. Glockshuber, Crystal structure of yellow mealworm α-amylase at 1.64 resolution, J. Mol. Biol. 278 (1998) 617–628.
[3] V. Buonocore, E. Poerio, V. Silano, M. Tomasi, Physical and catalytic properties of α-amylase from *Tenebrio molitor* L. larvae, Biochem. J. 153 (1976) 621–625.
[4] F. Payan, Structural basis of the inhibition of mammalian and insect α-amylase by plant protein inhibitors, Biochim. Biophys. Acta 1696 (2005) 171–180.
[5] O.L. Franco, D.L. Rigden, F.R. Melo, M.F. Grossi-de Sá, α-Amylase inhibitors and their interaction with insect α-amylases. Structure, function and potential crop protection, Eur. J. Biochem. 269 (2002) 397–412.
[6] M.J. Kim, S.B. Lee, H.B. Lee, S.Y. Lee, J.S. Haek, D. Kim, T.W. Moon, J.F. Robyt, K.H. Park, Comparative study of the inhibition of α-glucanosidase, α-amylase, and cyclomaltodextrin glunosyltransferase by acarbose, isoacarbose, and acarviosine-glucose, Arch. Biochem. Biophys. 371 (1999) 277–283.
[7] C. Harsawasdi, J. Kawabata, T. Kasai, α-Amylase inhibitors from Roselle (*Hibiscus sabdariffa* Linn.) tea, Biosci. Biotechnol. Biochem. 64 (2000) 1041–1043.
[8] C.A. Ryan, Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens, Ann. Rev. Phytopathol. 28 (1990) 425–449.
[9] V. Silano, α-Amylase inhibitors, in: J. Kruger, D. Lineback (Eds.), Enzymes and Their Role in Cereal Technology, American Association of Cereal Chemists, St. Paul, MD, USA, 1987, pp. 141–199.
[10] O.L. Franco, D.J. Ridgen, F.R. Melo Jr., C. Bloch, C.P. Silva, M.F. Grossi-de-Sá, Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities, Eur. J. Biochem. 267 (2000) 2166–2173.
[11] J. Iulek, O.L. Franco, M. Silva, C.T. Slivinski Jr., C. Bloch, D.J. Rigden, M.F. Grossi-de-Sá, Purification, biochemical characterization and partial primary structure of a new α-amylase inhibitor from

Secale cereale (rye), Int. J. Biochem. Cell Physiol. 32 (2000) 1195–1204.

[12] J.J. Marshall, C.M. Lauda, Purification and properties of phaseolamin an inhibitor of α-amylase from the kidney bean, *Phaseolus vulgaris*, J. Biol. Chem. 250 (1975) 8030–8037.

[13] A.P. Giri, M.V. Kachole, Amylase inhibitors of pigeonpea (*Cajanus cajan*) seeds, Phytochemistry 47 (1998) 197–202.

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

[15] M.F. Grossi-de-Sá, T.E. Mirkov, M. Ishimoto, G. Colucci, K.S. Bateman, M.J. Chrispeels, Molecular characterization of a bean α-amylase inhibitor that inhibits the α-amylase of the Mexican bean weevil *Zabrotes subfasciatus.*, Planta 203 (1997) 295–303.

[16] J.I. Abe, U. Sidenius, B. Svensson, Arginine is essential for the α-amylase inhibitory activity of the α-amylase/subtilisin inhibitor (BASI) from barley seeds, Biochem. J. 293 (1993) 151–155.

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

[18] H. Yamagata, K. Kunimatsu, H. Kamasaka, T. Kuramoto, T. Iwasaki, Rice bifunctional α-amylase/subtilisin inhibitor: characterization, localization, and changes in developing and germinating seeds, Biosci. Biotechnol. Biochem. 62 (1998) 978–985.

[19] J. Moreno, M.J. Chrispeels, A lectin gene encodes the α-amylase inhibitors the α-amylase inhibitors of the common bean, Proc. Natl. Acad. Sci. USA 86 (1989) 7885–7889.

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

[21] M. Richardson, Seed storage proteins: the enzyme inhibitors, in: L. Rogers (Ed.), Methods in Plant Biochemistry, vol. 5, Academic Press, London, UK, 1990, pp. 261–307.

[22] F.A.P. Campos, M. Richardson, The complete amino acid sequence of bifunctional α-amylase/trypsin inhibitor from seeds of ragi (Indian finger millet, *Eleusine coracana* Gaertneri), FEBS Lett. 152 (1983) 300–304.

[23] K. Takase, Site-directed mutagenesis reveals critical importance of the catalytic site in the binding of α-amylase by wheat proteinaceous inhibitor, Biochemistry 33 (1994) 7925–7930.

[24] C. Balastrieri, D. Castaldo, A. Giovane, L. Quaglioulo, L. Servillo, A glycoprotein inhibitor of pectyl methylesterase in kiwi fruit (*Actinidia chinensis*), Eur. J. Biochem. 193 (1990) 183–187.

[25] P. Bernfeld, Amylase α and beta, Methods Enzymol. 1 (1955) 149–154.

[26] C. Bloch Jr., M. Richardson, A new family of small (5 kDa) protein inhibitors of insect α-amylases from seeds or sorghum (*Sorghum bicolor* (L) Moench) have sequence homologies with wheat γ-purothionins, FEBS Lett. 279 (1991) 104–107.

[27] A. Chagolla-Lopez, A. Blanco-Labra, A. Patthy, R. Sánchez, S. Pongor, A novel α-amylase inhibitor from amaranth (*Amaranthus hypocondriacus*) seeds, J. Biol. Chem. 269 (1994) 23675–23680.

[28] M.D. Castro, W. Fontes, L. Morhy, C. Bloch Jr., Complete amino acid sequence from γ-thionins from maize (*Zea mays* L) seeds, Protein Peptide Lett. 3 (1996) 267–274.

[29] K. Thevissen, A. Ghazi, G. De Samblanx, C. Brownlee, R.W. Osborn, W.F. Broekaert, Fungal membrane responses induced by plant defensins and thionins, J. Biol. Chem. 271 (1996) 15018–15025.

[30] R. Wijaya, G.M. Neumann, R. Condron, A.B. Hughes, G.M. Polya, Defense proteins from seed of *Cassia fistula* include a lipid transfer protein homologue and protease inhibitory plant defensin, Plant Sci. 159 (2000) 243–255.

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

[32] F.R. Melo, D.J. Rigden, O.L. Franco, M.B. Ary, M.F. Grossi-de-Sá, C. Bloch Jr., Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking, Proteins 48 (2002) 311–319.

[33] X. Song, J. Wang, F. Wu, X. Li, M. Teng, W. Gong, cDNA cloning functional expression and antifungal activities of a dimeric plant defensin SPE10 from *Pachyrrhizus erosus* seeds, Plant Mol. Biol. 57 (2005) 13–20.

[34] C. Bloch Jr., S.U. Patel, F. Baud, M.J.I.M. Zvelebil, M.D.H. Carr, NMR structure of an anti-fungal γ-thionin protein SIα1: similarity to scorpion toxin, Proteins 32 (1998) 334–349.

[35] S. Romagnoli, F. Fogolari, E. Catalano, L. Zetta, G. Schaller, K. Urech, M. Giannattasio, L. Ragona, H. Molinari, NMR solution structure of viscotoxin C1 from *Viscum album* species *Coloratum ohwi*: toward a structure-function analysis of viscotoxins, Biochemistry 42 (2003) 12503–12510.

[36] M. Villa-Parelló, A. Sánchez-Vallet, F. García-Olmedo, A. Molina, D. Andreu, Synthetic and structural studies on *Pyrularia pureba* thionin: a single-residue mutation enhances activity against gram-positive bacteria, FEBS Lett. 536 (2003) 215–219.

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

[38] R. Sanchez-Monge, L. Gomez, D. Barber, C. Lopez-Otin, A. Armentia, G. Salcedo, Wheat and barley allergens associated with baker's asthma, Biochem. J. 281 (1992) 401–405.

[39] R.E. Shade, H.E. Schroeder, J.J. Pueyo, L.L. Tabe, T.J.V. Murdock, M.J. Higgins, M.J. Chrispells, Transgenic pea seeds expressing the α-amylase inhibitor of the common bean are resistant to bruchid beetles, Biotechnology 12 (1994) 793–796.

[40] R.L. Morton, H.E. Schroeder, K.S. Bateman, M.J. Chrispeels, E. Armstrong, T.J.V. Higgins, Bean α-amylase inhibitor 1 in transgenic peas (*Pisum sativum*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions, Proc. Natl. Acad. Sci. 97 (2000) 3820–3825.

[41] E. Mendez, A. Moreno, F. Colilla, F. Pelaez, G.G. Limas, R. Mendez, F. Soriano, M. Salinas, C. de Haro, Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin, γ-hordothionin, from barley endosperm, Eur. J. Biochem. 194 (1990) 533–539.

[42] M. Azarkan, R. Dibiani, E. Goormaghtigh, V. Raussens, D. Baeyens-Volant, The papaya Kunitz-type trypsin inhibitor is a highly stable 3 β-sheet glycoprotein, Biochim. Biophys. Acta 1746 (2006) 1063–1072.

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

[44] C.S. Dayler, P.A. Mendes, M.V. Prates, C. Bloch Jr., O.L. Franco, M.F. Grossi-de-Sá, Identification of a novel bean alpha-amylase inhibitor with chitinolytic activity, FEBS Lett. 579 (2005) 5616–5620.

[45] S. Sivakumar, M. Mohan, O.L. Franco, B. Thayumanavan, Inhibition of insect pest α-amylases by little and finger millet inhibitors, Pestic. Biochem. Physiol. 85 (2006) 155–160.

[46] N.M. Young, P. Thibault, D.C. Watson, M.J. Chrispeels, Post-translational processing of two alpha-amylase inhibitors and an arcelin from the common bean, *Phaseolus vulgaris*, FEBS Lett. 446 (1999) 203–206.

[47] U.K. Laemmli, Cleavage of structural proteins using assembly of the head of the bacteriophage T4, Nature 227 (1970) 680–685.