Identification of four novel members of Kunitz-like α-amylase inhibitors family from *Delonix regia* with activity toward Coleopteran insects

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**A B S T R A C T**

Crop improvement generally focuses on yield, seed quality and nutritional characteristics, rather than resistance to biotic and abiotic stresses. A clear consequence of this approach is the absence of natural anti-feedant toxins in some improved seed materials, allowing predation of commercial crops by insect herbivores. Cowpea (*Vigna unguiculata*), commonly cultivated by small farmers, is particularly affected by insect-pests that reproduce and develop inside stored seeds. One alternative to conventional pesticides for pest control is the use of biotechnological tools, such as the digestive enzyme inhibitors, that could be introduced in transgenic crops to enhance resistance. In this study, it was verified that the *in vivo* bioassays using artificial seeds containing 0.5%, 1.0% and 1.5% (w/w) of *Delonix regia* rich fraction, containing α-amylase inhibitors with effectiveness toward insect α-amylases and other sources, caused remarkable reduction in development and increased mortality of *Callosobruchus maculatus* cowpea weevil and to cotton boll weevil *Anthonomus grandis*. Therefore, attempts were made to isolate those inhibitors by SP-Sepharose ion exchange chromatography followed by high performance liquid chromatography on a Vydac C18-TP analytical column. Four inhibitor peaks were obtained with molecular masses of 6.0, 20 and 24 kDa. Their N-termini showed high sequence similarities with Kunitz-like inhibitor family members. These results provide evidence that *D. regia* synthesizes a multiple family of Kunitz-like α-amylase inhibitors, with different molecular masses and a wide biotechnological potential to control insect-pests.

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1. Introduction

*Vigna unguiculata*, commonly known as cowpea, is one of the most important legume crops in Africa, Asia and Latin America, where it has been consumed for centuries [22]. Cowpea seeds contain high concentrations of carbohydrates, proteins and lipids and are extremely valuable for low-income people who cannot afford to eat proteins derived from meat and fish that are usually expensive [26]. Moreover, cowpea has considerable ability to adapt to high temperatures, drought and salty soils that are present in most areas where cowpea is cultivated [27]. On the other hand, this crop is frequently attacked by the cowpea weevil *Callosobruchus maculatus* and other insect-pests. The female beetles lay eggs on the seed surface that hatch into larvae. The larvae burrow into the seeds and feed on them. As they cause serious damage the seeds become inadequate for home consumption or selling leading to serious economic losses to subsistence farmers [14]. Moreover, another insect-pest control here studied was the cotton boll weevil, *Anthonomus grandis* a major insect-pest of cultivated cotton, *Gossypium hirsutum* L., being responsible for significant cotton losses in South and North Americas. The adult female moves to the flower bud after alighting on the plant and then proceeds to construct an oviposition hole in which the egg is placed and which is then sealed. Floral buds and bolls are the main *A. grandis* food, in which female insects depend on nutrients from pollen grains and ovules for reproduction and eggs development [38]. In order to obtain metabolic energy for development, both pests rely on a multitude of hydrolytic enzyme. Among them, the α-amylases...
(α-1,4-glucan-4-glucanohydrolases) belong to a selective group of enzymes that occur in a wide variety of organisms and catalyze the hydrolysis of α-1,4-glycosidic bonds, transforming polysaccharides into mono- and disaccharides [15,24,38,43].

Aiming to control insect-pests, several strategies have been adopted, including the excessive use of chemical insecticides that are toxic to humans and animals, harmful to the environment and expensive to subsistence farmers [9]. Fortunately, seeds are able to produce several compounds such as alkaloids, phenols, chinatases, β-1,3-glucanases, lectins, arcelins and enzyme inhibitors to defend themselves against predators and phytopathogens [29,48]. The proteinaceous α-amylase inhibitors (α-AIs) inhibit digestive α-amylases [15,41], classified according to their tertiary structure in six different classes, namely, lectin-like, knotin-like, cereal-type, Kunitz-like, γ-purothionin-like and thauamatin-like [20], constitute an important biological tool that could be used to control insect development by reducing carbohydrate digestion [48].

Leguminous seeds are rich sources of proteinaceous α-amylase inhibitors, especially from lectin-like family [41]. Two different α-amylase inhibitor variants (α-AI1 and α-AI2) of the lectin-like type were purified from different varieties of Phaseolus vulgaris. They have high degree of amino acid sequence similarity, showed a 93% amino acid identity [32]. The three-dimensional structure of the AMY2/BASI complex obtained, macerated for 5 min on the ice and further centrifuged at 3000 g for 15 min at 4 °C to remove gut walls and cellular debris. The supernatants obtained, taken as the source of digestive α-amylases from C. maculatus and A. grandis, were kept at –20 °C for further use.

2.2. Purification of α-amylase inhibitors

Five hundred grams of mature and dry flamboyant seeds were macerated and extracted for 4 h at 4 °C with 0.1% of HCl containing 0.6 M of NaCl, and centrifuged at 4500 g for 30 min at 4 °C. The supernatant obtained (crude extract) was precipitated with ammonium sulfate (100%), dialyzed against distilled water (cut-off of 3.0 kDa) and further lyophilized. These proteins, named rich fraction, were dissolved in 0.5 M sodium phosphate buffer pH 6.2, and applied onto a cationic exchange SP-Sepharose column previously equilibrated with the same buffer. The non-retained protein fraction (NRF) was eluted with the equilibration buffer and the retained one desorbed by applying a linear gradient from 0 to 1.0 M of NaCl dissolved in the above buffer. All chromatography was conducted at flow rate of 0.1 mL min⁻¹. Eluted fractions (0.5 mL) were monitored at 280 nm. The retained protein fraction (RF) was dialyzed, lyophilized, dissolved in 0.1% TFA solution and further applied onto an analytical HPLC reversed-phase column (Vydac C18-TP 522) equilibrated in the same solution. Retained proteins were eluted with linear acetonitrile gradient (0–100%) at flow rate of 1 mL min⁻¹.

2.3. Extraction of digestive α-amylases from larvae of C. maculatus and A. grandis

Guts were surgically removed from larvae at 2nd instar and immersed in iso-osmotic saline (0.15 M NaCl). Midguts were separated, macerated for 5 min on the ice and further centrifuged at 3000 g for 15 min at 4 °C to remove gut walls and cellular debris. The supernatants obtained, taken as the source of digestive α-amylases from C. maculatus and A. grandis, were kept at –20 °C for further use.

2.4. α-Amylase activity and inhibition assay

The measurement of α-amylase activity was carried out using C. maculatus (CMA) and A. grandis (AOA) midgut extract and porcine pancreatic α-amylase (PPA) dissolved in 50 mM acetate buffer containing 5.0 mM CaCl₂, pH 6.0 as previously described by Franco et al. [15]. Starch 1% (w/v) was used as substrate in an incubation period of 25 min, at 37 °C. The enzymatic reaction was stopped by adding 3.5-dinitrosalicilic acid at 100 °C [2]. One α-amylase unit was defined as the amount of enzyme that increases the absorbance at 530 nm by 0.1 after 20 min. The inhibition assay of α-amylases was done by pre-incubation (20 min at 37 °C) of the seed protein fractions, SP-Sepharose fractions and HPLC derived peaks, at the standard concentrations of 50 μg mL⁻¹. Protein concentrations were determined according to Bradford [6]. Assays were done in triplicate.

2.5. Trypsin inhibition assays

Bovine pancreatic trypsin (BPT), were purchased from Sigma Co., St. Louis, USA and midguts from A. obectus, C. maculatus and A. grandis were used to enzymatic assays. Proteolytic inhibitory activities were tested against mammalian and insect enzymes using 10 μM fluorogenic peptides Z-CBZ-Phe-Arg-7-MCA and N-succinyl-Ala-Ala-Pro-Phe-MCA (Sigma), respectively. The assays were performed in 25 mM Tris–HCl, pH 7.5 and 20 mM DMSO according to Solomon et al. [50]. The reaction was stopped into a single endpoint with 1.9 mL of 0.2 M Na₂CO₃. The reaction was
then followed for up to 30 min in a DyNA Quant 500 fluorescence reader (Pharmacia-Biotech), with excitation at 365 nm and emission at 460 nm. The blank fluorescence readings (minus substrate) were subtracted. Assays were carried out in triplicate with endpoint fluorescence values differed by no more than 10%. Seed protein fractions and HPLC derived peaks were evaluated at the standard concentrations of 50 μg mL⁻¹. Protein concentrations were determined according to Bradford [6].

2.6. SDS–PAGE analyses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analyses (12.5%) were carried out as described by Laemmli [31]. Bromophenol blue was used as a tracking dye and protein fractions and HPLC derived peaks were evaluated at the final concentrations of 0.5%, 1.0% and 1.5% (w/v). Protein concentrations were determined according to Bradford [6]. Ten larvae standard concentrations: 0.5%, 1.0% and 1.5% (w/v). Protein concentrations were determined according to Bradford [6].

2.7. Callosobruchus maculatus feeding tests

Assays were carried out using artificial seeds of 0.9 cm in diameter, 0.6 cm in height and a weight of 300 mg, formulated to contain commercial V. unguiculata fine flour and D. regia SP-Sepharose retained protein fraction mixed in order to obtain uniform distribution. Flour was compacted by using a hand compressor. Rich fraction was utilized at the final concentrations of 0.5%, 1.0% and 1.5% (w/w) as previously described [15]. Protein concentrations were determined according to Bradford [6]. Artificial seeds with cowpea flour alone as the sole source of protein were used as negative control. Groups of five artificial seeds were placed in plastic containers (10 mL) and 10–15 sexually mature females (48–72-h-old), previously coupled with males, were introduced in the container for a 24-h oviposition period. After this period, all seeds were observed under stereoscopic microscope to confirm oviposition and remove eggs excess to leave two per seed. At the 20th day, the survivors’ larvae weights were measured and after 45 days the adult insects were counted. Insect mortality 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 placed in individual plastic containers containing five cowpea seeds (n = 10 for each treatment and control). Containers were observed every 24 h to evaluate longevity of insects and to count the number of eggs deposited by female with the aid of a stereoscopic microscope. Ten 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.

2.8. Anthonomus grandis feeding tests

Bioassays were carried out in six-well plates containing 5.0 mL of artificial diet sterilized in each well [12]. The SP-Sepharose retained protein fraction was incorporated into the diet at three standard concentrations: 0.5%, 1.0% and 1.5% (w/v). Protein concentrations were determined according to Bradford [6]. Ten larvae from first instar were fed on artificial diets per treatment. The negative controls were distilled water. After 7 days of incubation at 28 °C ± 2 and 55% relative humidity and photoperiod of 14 h, the dead larvae were counted. Each treatment involved three replicates. Mortality was calculated as the percentage of neonate larvae that completed their development through to emergence.

2.9. Amino acid sequence and in silico analyses

In order to carry out sequencing experiments, SDS–PAGE proteins were transferred to a PVDF membrane by using a semi-dry transfer blot. The N-terminal amino acid sequences of D. regia α-amylase inhibitors were determined on a Shimadzu PPSQ-23A Automated Protein Sequencer performing Edman degradation. PHT-amino acids were detected at 269 nm after separation on a reversed-phase C18 column (4.6 × 2.5 mm) under isocratic conditions, according to the manufacturer’s instructions. The amino acid sequences obtained were compared with those of proteins reported in the SWISSPROT Data Bank, using FASTA3 Program [42]. Alignment using the ClustalW Program [51] was performed to assess primary sequence similarities.

3. Results and discussion

3.1. Purification and biochemical characterization of D. regia α-amylase inhibitors

To find novel Kunitz-like α-amylase inhibitors in D. regia seeds, several steps of purification were applied to 3.0 mg of proteins and resulted in a purification fold of 40 times to the most active α-amylase inhibitor isolated with a yield of 66.7% (Table 1). Moreover, other inhibitors here purified showed lower purification folds. In order, proteins of D. regia seeds were extracted and precipitated with ammonium sulfate (100%). After dialyses and lyophilization, the protein-rich fraction obtained was applied onto a SP-Sepharose ion exchange column from which one retained protein fraction (RF) containing cationic proteins (Fig. 1A) emerged. Cation exchanger resins have been employed for purification of several plant defense proteins including α-amylase [5,48] and proteinase inhibitors [32]; defensins [19] and 2S albumins [45]. Enzymatic assays revealed that the RF fraction inhibits human salivary and porcine pancreas amylases in 53.2% and 22.1%, respectively. Additionally RF inhibits α-amylases from the bruchids C. maculatus (97.7%), A. obtectus (48.5%), from cotton boll weevil A. grandis (84.7%), from the fungus A. oryzae (27.9%) and the plant H. vulgarum (60.8%) (Fig. 2A). RF was then applied onto an analytical reversed-phase HPLC column (Vydac C18-TP 522), yielding three major peaks (Fig. 1B). Fractions I, II and III demonstrated a clear inhibitory activity of approximately 52%, 35% and 34% against C. maculatus α-amylases (CMA), respectively (Fig. 2B). Moreover, much lower activity against porcine pancreas amylase (PPA) was observed (Fig. 2B). None inhibitory activity was obtained against serine proteinases from bovine pancreas, A. obtectus, C. maculatus and A. grandis (data not shown). This is not the first inhibitor reported to inhibit CMA and other insect amylases. Earlier studies have shown that α-amylase inhibitors isolated from cereal kernels, legume and papaya seeds have inhibitory activity against bruchid enzymes [10,12,13,20,24,28]. In summary, our data indicated that D. regia inhibitors seem to be more specific to insect α-amylases. This specificity is a desirable characteristic, which may increase

| Purification step | Protein (μg) | Inhibitory activity (U) | Yield (%) | Specific activity (U mg⁻¹) | Purification fold |
|-------------------|-------------|------------------------|-----------|---------------------------|------------------|
| Crude extract     | 3000        | 1200                   | 100       | 0.4                       | 1.0              |
| Ammonium sulfate  | 2300        | 980                    | 81.6      | 0.4                       | 1.0              |
| Sp-Sepharose      | 1150        | 930                    | 77.5      | 0.8                       | 2.0              |
| HPLC Dr1          | 50          | 800                    | 66.7      | 16.0                      | 40               |
| HPLC Dr2          | 340         | 280                    | 23.3      | 0.8                       | 2.0              |
| HPLC Dr3          | 340         | 280                    | 23.3      | 0.8                       | 2.0              |
| HPLC Dr4          | 110         | 410                    | 34.1      | 3.8                       | 9.3              |

Table 1
Overview of amylase inhibitors purification. The inhibitory activity was determined by the Bernfeld method (19) toward C. maculatus enzymes as described in the text, using starch as a substrate. Accordingly, one enzyme unit is defined as the amount of enzyme necessary to increase 0.1 in optical density at 530 nm.


3.2. Molecular mass analyses

The \( \alpha \)-amylase inhibitors isolated from \textit{D. regia}, were designated Dr1 (from fraction I), Dr2 and Dr3 (fraction II) and Dr4 (fraction III), were analyzed by SDS–PAGE (Fig. 3). The \( \alpha \)-amylase inhibitor Dr1, that first emerged from the reversed-phase column Vydac C18-TP 522 (fraction I), showed a single protein band of approximately 24 kDa, similar to Kunitz-like inhibitors isolated from soybean [16], black-eyed pea [17] and the native plant \textit{Prosopis juliflora} [18]. \( \alpha \)-Amylase inhibitor Dr3 (fraction III) shows a molecular mass of about 20 kDa, with similarity to those reported for cereal kernels [20]. It is noteworthy that so far only one trypsin inhibitor of 22 kDa, with two disulfide bridges, had been described for flamboyant seeds [25,30,40]. Thus, this report clearly shows and reinforces that a single plant could synthesize different \( \alpha \)-amylase inhibitors, as previously observed in \textit{D. alata} seeds [5]. Moreover, these data suggest that the plant defense systems are extremely complexes and far more studies are required for full comprehension of the underlying mechanisms.

3.3. Protein sequence comparisons

N-terminal sequences of \textit{D. regia} inhibitors here reported (Table 2) showed clear identity to trypsin inhibitors that belong to the Kunitz-like family. The highest similarity was found with EcTI, a bifunctional inhibitor from \textit{Enterolobium contortisiliquum} [1]. For instance, highly conserved glycine 2 and 3 were found in these bifunctional inhibitors [1,11,38,47], probably due to their structural role. Interestingly low degree of similarities (20–40%) were found between the N-terminal sequences of the novel inhibitors of \textit{D. regia} and those previously described for this species [40]. Thus, the novel inhibitors described here are different from those isolated from \textit{D. regia} by Pando et al. [40] and constitute new members of the plant Kunitz-like inhibitor family with the ability of inhibit \( \alpha \)-amylases. These data suggest that \textit{D. regia} synthesizes diverse inhibitors that are probably involved with the defense mechanisms against insect-pests. However, an important question that needs to be addressed is the influence of the geographic location where seeds of \textit{D. regia} were collected. For instance, the inhibitors described by Pando et al. [40] were isolated from \textit{D. regia} seeds collected in Mata Atlantica whose environmental conditions and biotic stresses are different from those present in Cerrado.

Thus it is possible that differences in abiotic and biotic factors among these two ecosystems could trigger the synthesis of different inhibitors within a same plant species.

3.4. In vivo bioassays

The \textit{in vivo} effects of \textit{D. regia} inhibitors toward \textit{C. maculatus} were evaluated by incorporating \textit{D. regia} SP-Sepharose retained protein fraction into artificial cowpea seeds. The larval weight (Fig. 4A) and adult insect mortality (Fig. 4B) were analyzed. The inhibitor concentrations of 0.5% and 1.0% (w/w) caused low larval weight reduction (\(-25\%\)) (Fig. 4A) and adult insect mortality of approximately 80% (Fig. 4B). At 1.5% (w/w) concentration, larval weight was reduced to almost 50% and adult insect mortality increased to 85% as compared with controls. Furthermore, a clear deleterious \textit{in vivo} activity of \textit{D. regia} inhibitors was also obtained against cotton boll weevil \textit{A. grandis}, where 0.5% and 1.0% were able to cause 33% of larval mortality. Moreover highest concentration was able to improve the larval mortality at 43%, suggesting that these inhibitors could be used to improve cotton resistance (Table 3). Similar results were observed in bioassays using \textit{P. pubescens} \( \alpha \)-amylase inhibitors [48]. Other inhibitors were also detrimental for various insect-pests. The purified wheat inhibitors 0.19 and 0.53 were both able to inhibit \textit{A. obtectus} development and decrease survival [15]. The \( \alpha \)-amylase inhibitor isolated from rye kernels (0.5% and 1.0%, w/w) [12] enhanced mortality to 83% and significantly diminished the cotton boll weevil adult longevity. Furthermore, artificial seeds containing 1.5% (w/w) of \( \alpha \)-amylase...
inhibitor from papaya seeds also caused a remarkable larval mortality (50%) [13].

A vast number of genes conferring pest resistance have been expressed in heterologous systems [7,45] or incorporated into different crops [35,49], including digestive enzyme inhibitors that inhibit larval growth by slowing down the digestion process, reducing carbohydrate assimilation. Transgenic peas expressing \( P. vulgaris \) \( \alpha \)-AI1 have enhanced resistance to certain species of Bruchidae, whose digestive \( \alpha \)-amylases are affected by \( \alpha \)-amylase inhibitors [36]. When \( \alpha \)-AI2 was expressed in peas, a partial protection against bruchids was obtained. Therefore, expression of \( \alpha \)-AI2 gene could protect peas against the damage promoted by pea weevils, removing the necessity of using chemical pesticides during seed storage [35]. Moreover transgenic cowpeas expressing \( \alpha \)AI-1 have these defenses increased, strongly inhibiting the development of \( C. maculatus \) and \( C. chinensis \) in insect bioassays, indicating the efficiency of genetic modified plants expressing amylase inhibitors [49]. Just as important as the proof of protection of transgenic crops against insect-pests is the demonstration that the new crops present no health risk to consumers, as reviewed by Payan [44]. For example, transgenic plants expressing \( \alpha \)-AI1 demonstrated minimal detrimental effects on the nutritional value.

![Fig. 2.](image)

(A) Inhibitory activities of \( D. regia \) rich fraction (RF) against digestive \( \alpha \)-amylases from \( C. maculatus \) (CMA), \( A. obtectus \) (AOA), \( A. grandis \) (AGA), \( A. oryzae \) (AZA), \( H. vulgareum \) (HVA), human saliva (HSA) and porcine pancreas (PPA). (B) Moreover HPLC fractions I, II and III were here evaluated toward CMA (black bars) and PPA (white bars). Assays were carried out in triplicate. Vertical bars correspond to the standard deviation.

![Fig. 3.](image)

SDS–PAGE analysis of fractions I, II and III obtained by HPLC. Proteins were visualized by silver staining. Black arrows indicate major sequenced proteins.
of pea fed to rats until 30% of the diet [46]. Another approach utilized to improve resistance in plants consists in the use of biotechnology and a toolbox of promoters with defined specificities, which could be a valuable resource in controlling the expression of α-AI transgenes in desired plant tissues [21]. This strategy, allied to discovery of novel α-amylase inhibitors could bring clear benefits to agribusiness. So far, until now, no study was done to produce insect-resistant transgenic crops expressing Kunitz-like α-amylase inhibitors; despite of great efforts should be made to discover novel Kunitz-like α-amylase inhibitors from alternative plant sources. Then a panel of such inhibitors and promoters could be challenged against plant predators and the gene/promoter combo may be introduced in transgenic crops, enhancing protection against insect-pests.

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Table 2

Alignment of D. regia α-amylase inhibitors (DR1–4) and others well described plant Kunitz-like inhibitors. Asterisks showed conserved glycine residues.

| Name     | SWISSPROT number | Organism        | Sequence                             | References |
|----------|------------------|-----------------|--------------------------------------|------------|
| DR1      | –                | Delonix regia   | SG--LLVWQGIQH- - - - - - - - - - -   | This report|
| DR2      | –                | Delonix regia   | SG--KNMNSMWH- - - - - - - - - - -    | This report|
| DR3      | –                | Delonix regia   | SGOG-- - - V1MNH- - - -QMMHSHADK     | This report|
| DR4      | –                | Delonix regia   | SGOGKAAKMTHRYHRKFRPDA- - - - - - -   | This report|
| EcTi     | –                | Enterolobium    | KGODLELA- - - KGSQETCPHVRVQA         | [1]        |
| BvcTi-3  | P83036           | Leucaena        | KGODLELA- - - KGSQECSPRYVQT          | [37]       |
| RASI     | P29421           | Bauhinia variegata | NKGQKTSMS- - - - - - - - - - - - - - | [11]       |
| BASI     | P07596           | Oryza sativa    | KGODLMATAPRV- - - -FCPLVQAE          | [36]       |
| DRTI     | P83667           | Hordeum         | KGODMTAPOHQR- - - -CPLQYVQD          | [45]       |
|          |                  | vulgareum      | GGQVTR-- - - - - - - - - - - - - - - | [39]       |

Table 3

Evaluation of larval mortality of boll weevils developed in artificial diet in the presence of different concentrations (w/v) of rich fraction of D. regia. Each assay was performed in triplicate, and the results did not differ more than 12%. Statistical differences (Dunnett test, p < 0.05) between control and test groups are marked with an asterisk.

| Treatment | Mortality (%) |
|-----------|---------------|
| Control   | 18.8          |
| 0.5%      | 33.3*         |
| 1.0%      | 33.3*         |
| 1.5%      | 43.0*         |

Fig. 4. In vivo effects of D. regia SP-Sepharose retained protein fraction at 0.5%, 1.0% and 1.5% (w/w) concentrations on C. maculatus larval weight (after 10 days) (A) and adult insects survival (B). Assays were carried out in triplicate. Vertical bars represent standard deviation. Significant statistical differences (Dunnett test, p < 0.05) between control and test groups are marked with an asterisk.
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