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Structural insights regarding an insecticidal *Talisia esculenta* protein and its biotechnological potential for *Diatraea saccharalis* larval control

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ABSTRACT

Talisin is a seed-storage protein from *Talisia esculenta* that presents lectin-like activities, as well as proteinase-inhibitor properties. The present study aims to provide new *in vitro* and *in silico* biochemical information about this protein, shedding some light on its mechanistic inhibitory strategies. A theoretical three-dimensional structure of Talisin bound to trypsin was constructed in order to determine the relative interaction mode. Since the structure of non-competitive inhibition has not been elucidated, Talisin-trypsin docking was carried out using Hex v5.1, since the structure of non-competitive inhibition has not been elucidated. The predicted non-coincidence of the trypsin binding site is completely different from that previously proposed for Kunitz-type inhibitors, which demonstrate a substitution of an Arg^{64} for the Glu^{64} residue. Data, therefore, provide more information regarding the mechanisms of non-competitive plant proteinase inhibitors. Bioassays with Talisin also presented a strong insecticide effect on the larval development of *Diatraea saccharalis*, demonstrating LD50 and ED50 of ca. 2.0% and 1.5%, respectively.

1. Introduction

Seed proteins play an important role in human and animal nutrition, providing a major contribution to dietary protein. These proteins may be classified as storage, structural and biologically active proteins (Fukusima, 1991). The major function of the storage proteins in plant tissues appear to be a nutritional resource for seed germination or tuber development (Yeh et al., 1997). Furthermore, storage proteins play a dual role in repository and defense mechanisms (Van Damme et al., 2002; Gaidamashvili et al., 2004). Vicilins, legume seed-storage proteins of globulin nature, from cowpeas (Sales et al., 1996) and other legumes, bind strongly to chitin and have a highly detrimental effect on the larval development of *Callosobruchus maculatus* (Yunes et al., 1998). Moreover, Kunitz-type proteinaceous inhibitors reversibly interact with enzyme targets, forming stable complexes influencing their catalytic activities in competitive and non-competitive ways (Prabhu and Pattabiraman, 1980; Bhattacharyya et al., 2006; Oliveira et al., 2007). These inhibitors have been widely isolated and characterized from plants (Macedo et al., 2000a, 2000b; Mello et al., 2001; Oliveira et al., 2002; Macedo et al., 2004a, 2004b; Ramos et al., 2008) normally occurring as single polypeptide chains (Negreiros et al., 1991; Wu and Lin, 1993; Sattar et al., 2005). These proteinaceous compounds have been implicated in various physiological functions, such as the regulation of proteolytic cascades and safe storage of proteins, as well as defense molecules against plant pests and pathogens (Xavier-Filho, 1992). Kunitz-type inhibitors are characterized by molecular masses around 20 kDa, a low cysteine content forming two disulphide bonds and a common structural fold composed of a β-trefoil formed by 12 antiparallel β-strands with long interconnecting loops presenting one or two reactive sites for serine proteinases (Song and Suh, 1998; Krauchenco et al., 2003, 2004; Khamrui et al., 2005).

Previous study (Freire et al., 2009) showed the biochemical characterization and cloning of the major protein from *Talisia esculenta* seeds (Talisin), a member of the Sapindaceae family. The deduced peptide presented high similarity with several storage proteins and all demonstrating amino acid sequences that were clearly related to the Kunitz family of the proteinase inhibitor (Freire et al., 2009). It has also been shown that Talisin could be a saccharide-binding protein characterized by a proven interaction with carbohydrates on neutrophil or mononuclear cells (Freire et al., 2003) and to with the chitin component of the peritrophic membrane (or equivalent structures) in the *C. maculatus* (Macedo et al., 2002). Dixon plot and negative staining against bovine trypsin indicate that Talisin presents a non-competitive inhibition of trypsin (Freire et al., 2009). Talisin

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also showed insecticidal activity against several insects (Macedo et al., 2004a, 2004b, 2011).

Talisin's structural data are not available; in this report, we obtained the theoretical three-dimensional structure of Talisin by homology modeling. We used a molecular docking strategy in order to investigate the effects of this protein on the growth and development of *Diatraea saccharalis*, the major insect pest of sugarcane in Brazil and other South American countries, which is responsible for significant economic damage.

2. Materials and methods

2.1. Plant material and chemicals

T. esculenta (Sapindaceae) seeds were collected in the State of Ceará (Brazil). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Amersham Biosciences (Uppsala, Sweden).

2.2. Insects

D.saccharalis (Lepidoptera: Crambidae) larvae were from a laboratory colony and provided by Dr. J.R.P. Parra (Departamento de Entomologia, Fitopatologia e Zoologia Agrícola, Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo, Piracicaba, SP, Brazil). The colony was housed in standard conditions (25 ± 1 °C, 60 ± 10% relative humidity and L14:D10 photoperiod).

2.3. Extraction and purification of T. esculenta protein

Defatted *T. esculenta* seeds were finely ground and extracted (meal to buffer ratio of 1:5) with 150 mM NaCl for 24 h at 4 °C and then centrifuged at 10,000 g for 30 min at the same temperature. The clear supernatant (crude extract or CE) was used to determine the protein content. The CE was diluted in 150 mM NaCl and applied to a Sephadex G-100 column (2.5 cm × 80 cm) equilibrated with the same solution. The protein-rich fraction was recovered and applied to a chitin column (1.5 cm × 10 cm) equilibrated with 50 mM phosphate buffer, pH 7.6, and eluted with 100 mM HCl. The purified protein was dialyzed and lyophilized.

2.4. Protein quantification

Protein concentrations were determined by the dye-binding method of Bradford (1976), with bovine serum albumin as the standard.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of protein was carried out according to Laemmli (1970), using 5% (w/v) stacking and 17% separating gels under reducing and non-reducing conditions. Proteins were stained with Coomassie Brilliant Blue R-250, and molecular mass was determined using molecular-mass references of phosphorylase B (94 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin (20.1 kDa), and R-lactalbumin (14.4 kDa).

2.6. Insect bioassays

Effects of Talisin on *D. saccharalis* development were evaluated using the artificial medium system previously developed by Green et al. (1976). For the bioassays, neonate *D. saccharalis* larvae were selected and fed an artificial diet containing 1%, 1.5% and 2% of Talisin (w/w). Control larvae were fed with untreated diet. Each treatment was set up in glass containers (8.5 cm long × 2.5 cm diameter) and five larvae were transferred to each glass container (n = 75). After the larvae reached the fourth instar at standard conditions, the relationship between protein content and the weight and number of larvae were determined. Linear regression analysis was used to describe the response of *D. saccharalis* to various doses of Talisin. The effective dose for a 50% response (ED_{50}) was defined as the concentration of Talisin that decreased the mass of the insect to 50% of that of control larvae. The lethal dose (LD_{50}) corresponded to the concentration of Talisin that reduced the number of insects to 50% of the number found in control diet.

2.7. Midgut preparation

Proteinases were obtained from the midguts of fourth-instar larvae, according to Macedo et al. (2007a). Fourth-instar larvae were cold-immobilized and the midgut, along with its contents, was removed in cold 150 mM NaCl and stored frozen (−20 °C). Guts from larvae of *D. saccharalis* were subsequently homogenized in 150 mM NaCl, centrifuged for 5 min at 6000 g at 4 °C, and the supernatants pooled and kept on ice for enzymatic assays.

2.8. Enzyme assays

Bovine pancreatic trypsin and midgut extracts were used for the enzymatic assays. Trypsin-like activities were assayed using N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate (Erlanger et al., 1961). The substrate was used at a final concentration of 1 mM in 1% (v/v) dimethyl sulfoxide (DMSO) and pH 8.0 (0.1 M Tris-HCl buffer, pH 8.0).

The ability of Talisin to inhibit the trypsin-like activities from insect midgut larvae, was determined by incubating a mixture of midgut extracts with different concentrations of Talisin. Incubation was performed in five repetitions. The inhibitory activity was measured by the difference in enzyme activity with and without Talisin.

The effect of Talisin on the proteolytic activity of midgut extracts was measured using 1 mM BAPNA at pH 8.0 after incubation with Talisin at 37 °C for 20 min. The residual enzymatic activity was assayed as described above. The assays were run in triplicate with appropriate blanks.

2.9. Digestion of Talisin

The digestion of Talisin by midgut extract was carried out as described by Macedo et al. (2002). The midguts of fourth-instar larvae were dissected, extracted in 1 mL of 0.1 M Tris buffer, pH 8.0, and processed as described above. Talisin was incubated with this homogenate in Tris buffer (final concentration, 2 mg/mL). The Talisin:midgut protein ratio was 1:1. Digestion was done for 1, 2, 4, 6, 12 and 24 h at 30 °C and was stopped by immersing the tubes in boiling water for 2 min. The degradation of bovine serum albumin (BSA) (2 mg/mL) was used as a positive control for serine proteinase activity. The digestion was stopped as described above. The relative molecular masses of the digestion products were estimated by 12.5% SDS-PAGE using protein markers of known molecular mass.

2.10. In silico analysis and molecular modeling

An alignment of the Talisin sequence, using Bioinfo Meta-Server (http://meta.bioinfo.pl/submit_wizard.pl) and ClustalW (Thompson et al., 1994; Kumar et al., 2008) was performed for the proteins, in order to find a template with high identity and analyze primary sequence similarities, observing the characters with reactive sites and disulfide bonds. Homology modeling was performed using Modeller 9v6 (Berman et al., 2000), the template found with the highest identity was the protein under the Protein Data Bank (PDB) accession code 1R8N with 1.75 Å of resolution, a Kunitz-type inhibitor resolved by X-ray diffraction. Fifty models were built and the best one was chosen based on the DOPE scores (Laskowski et al., 1993) and overall