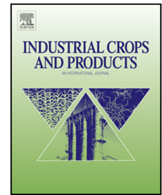
Industrial Crops and Products 70 (2015) 48–55

Contents lists available at ScienceDirect

Industrial Crops and Products

journal homepage: www.elsevier.com/locate/indcrop



Castor bean cake contains a trypsin inhibitor that displays antifungal activity against *Colletotrichum gloeosporioides* and inhibits the midgut proteases of the dengue mosquito larvae

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ARTICLE INFO

Article history:
Received 29 July 2014
Received in revised form 20 February 2015
Accepted 23 February 2015
Available online 19 March 2015

Keywords:
Ricinus communis
Trypsin inhibitor
Aedes aegypti
Colletotrichum gloeosporioides

ABSTRACT

A novel trypsin inhibitor, named RcTI, was purified from castor bean cake (*Ricinus communis* L.) by heat treatment followed by chromatography on anhydrotrypsin-Sepharose 4B and Resource Q. RcTI is a 14 kDa competitive inhibitor with pI 5.2 and a dissociation constant (Ki) of 1.9×10^{-5} mM. The amino-terminal sequence showed similarity with a 2S sulfur-rich seed storage protein (83%) and napin-like protein (48%). RcTI was stable over a broad pH range and is exceptionally resistant to heating as it retained high inhibitory activity toward trypsin after incubation at 100 °C for 2 h. RcTI (13 μg) inhibited the spore germination of the phytopatogenic fungus *Colletotrichum gloeosporioides* and promoted 91% inhibition of the proteases from the midgut of *Aedes aegypti* larvae. The results of the present study indicate that RcTI has biotechnological potential as an alternative agent to combat the important phytopathogen *C. gloeosporioides* and the larvae of *A. aegypti*.

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1. Introduction

Protease inhibitors are proteins or peptides that form complexes with proteases hindering catalysis (Fan and Wu, 2005). Protease inhibitors are ubiquitously distributed in animals, plants and microorganisms (Katunuma et al., 2003; Ishihara et al., 2006; Bijina et al., 2011) and are classified according to the type of proteases they inhibit; serine protease, cysteine protease, aspartic protease and metalloprotease inhibitors (Laskowski and Kato, 1980; Richardson, 1991). Plant serine proteases inhibitors are grouped in Kunitz, Bowman-Birk, potato I and II and squash families of inhibitors according to their primary and tridimensional structures, molecular masses and disulfide bond contents (Bhattacharyya and Babu, 2009; Rawlings et al., 2010). Additionally, there are 2S albumins (seed storage proteins) that behave as trypsin inhibitors (Mandal et al., 2002; Maleki et al., 2003).

In plant tissues, protease inhibitors, besides being considered storage proteins (Mandal et al., 2002) are associated with regulation and control of endogenous proteases during seed development and germination (Xavier-Filho and Campos, 1889; Richardson, 1991), regulation of programmed cell death (Solomon et al., 1999), response to abiotic stresses (Franco and Melo, 2000) and protection against pathogens and insects (Ryan, 2000; Carrillo et al., 2011).

Many previously studied trypsin inhibitors from plants belong to the Leguminosae, Solanaceae, Cucurbitaceae and Poaceae families (Garcia-Olmedo et al., 1987). However, few protease inhibitors from other families such Rutaceae (Shee and Sharma, 2007; Shee et al., 2007a,b) and Euphorbiaceae (Sritanyarat et al., 2006; Chaudhary et al., 2008) have been purified and characterized.

Within the Euphorbiaceae family, castor bean (*Ricinus communis* L.) is of great socioeconomic importance because its seeds are used mainly for biodiesel production. The oil extraction from castor bean seeds generates a protein-rich by-product known as the castor bean cake, but the presence of toxic and allergenic compounds hinders the use of this residue as a feeding source (Dubois et al., 2013). Nevertheless, a way to add value to the side product castor cake is to discover, purify and characterize bioactive molecules, such as protease inhibitors, that could have applications in agriculture and human health and use as biotechnological tools. To the best of our knowledge there are no reports to date on the biological properties of trypsin inhibitors purified from castor beans.

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http://dx.doi.org/10.1016/j.indcrop.2015.02.058
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In the present study a trypsin inhibitor was purified from castor bean cake and was biochemically characterized and tested against the phytopathogenic fungi *Fusarium oxysporum*, *Rhizoctonia solani* and *Colletotrichum gloeosporioides* and against the midgut proteases from *Aedes aegypti* larvae.

2. Materials and methods

2.1. Materials

Castor bean (*R. communis*) cake was obtained from OLVEQ – Indústria e Comércio de Óleos Vegetais Ltda., Ceara-Brazil. Sepharose 4B and Resource Q were purchased from GE Healthcare. Bovine pancreatic trypsin, Nα-benzoyl-L-arginine p-nitroanilide (BAPNA) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich Ltd. Reagents for SDS-PAGE were purchased from Sigma–Aldrich Ltd. and GE Healthcare. All other chemicals were of analytical grade for biochemical use. The filamentous fungi *F. oxysporum*, *R. solani*, and *C. gloeosporioides* were obtained from the Biochemistry and Molecular Biology Department, Federal University of Ceara, CE, Brazil. *A. aegypti* larvae (3rd stage of development) were obtained from the Department of Biology, Federal University of Ceara, CE, Brazil.

2.2. Purification of a castor bean cake protease inhibitor (RcTI)

Castor bean cake was defatted with 3 volumes (m/v) of petroleum ether. The defatted castor cake was air-dried at room temperature (23 ± 2 °C) until petroleum ether evaporates to obtain a fine dried powder.

A crude extract was obtained by extraction of the defatted cake with 50 mM Tris–HCl, pH 7.5, (1:10, m/v), for 30 min, at 4 °C, followed by centrifugation at 12,000 × g, 30 min, at 4 °C. The supernatant (crude extract) obtained was heated at 100 °C for 30 min and cooled under ice water. To remove coagulated debris, the heat-treated extract was centrifuged at 12,000 × g for 15 min at 4 °C.

The heat-treated extract was subjected to affinity chromatography on anhydrotrypsin-Sepharose 4B column (6.5 × 2.1 cm) pre-equilibrated with 50 mM Tris–HCl, pH 7.5, buffer containing 50 mM galactose (to avoid ricin interaction) and 500 mM NaCl. The retained proteins were eluted with 50 mM glycine–HCl, pH 2.2, containing 500 mM NaCl at 45 mL h^{-1} flow rate. Fractions of 2 mL were collected and read at 280 nm. The fractions containing antitrypsin activity were pooled, dialyzed against distilled water at 4 °C, freeze-dried, and dissolved in 50 mM Tris–HCl, pH 8.5. This sample was applied to a Resource Q column pre-equilibrated with 50 mM Tris–HCl, pH 8.5. The column was washed extensively with the equilibration buffer to remove unbound proteins and those retained were desorbed by stepwise elution with increasing concentrations of NaCl (25, 50, 100, 200, 400, 500 and 1000 mM), prepared in 50 mM Tris–HCl, pH 8.5. Fractions of 2 mL were collected at 60 mL h^{-1} flow rate, read at 280 nm, analyzed for antitrypsin activity, and the purified inhibitor, named RcTI, subjected to further analysis.

2.3. Protein quantification

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

2.4. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to the methodology described by Laemmli (1970) adapted for use in plates (10.5 × 10 cm). The samples were prepared by dilution (1:3, v/v) in sample buffer (62.5 mM Tris–HCl, pH 6.8, containing 1% [m/v] SDS and 0.1% [m/v] bromphenol blue) in the presence or absence of 0.5% 2-β-mercaptoethanol and boiled for 3 min. The samples (20 μg) were loaded on the 3.5% stacking gel and fractionated in the 12.5% separating gel at a constant current of 20 mA per gel using a Hoefer miniVE vertical electrophoresis system (Amersham Pharmacia Biotech, San Francisco CA, USA). Proteins were visualized by incubation of the gel after electrophoresis with Coomassie Brilliant Blue G-250 (Candiano et al., 2004). Phosphorylase B (97.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa) were used as molecular mass markers.

2.5. Assessment of the glycoprotein nature of RcTI

The presence of covalently bound carbohydrate to the RcTI structure was assessed after SDS–PAGE and specific staining by the periodic acid-Schiff (PAS) method (Kapitany and Zebrowski, 1973). SDS–PAGE (Laemmli, 1970) of RcTI (10 μg) was carried out using the Hoefer SE 600 Series electrophoresis unit (GE Healthcare). Fetuin (10 μg), a glycoprotein, and soybean trypsin inhibitor (SBTI) (10 μg) were used as positive and negative control, respectively. The proteins were loaded (10 μg) concomitantly on the gel and run as above. After electrophoresis, the gel was immersed in a fixing solution of 12% (v/v) trichloroacetic acid (TCA) for 30 min, transferred and kept incubated for 60 min in 1% (v/v) periodic acid and stained with the Schiff's reagent for 50 min in the dark. The gel was washed repeatedly with a solution of 0.5% (m/v) sodium metabisulphite and documented using a digital camera.

2.6. Amino-terminal sequencing

This was done in a Shimadzu Co® PPSQ-10 Automated Protein Sequencer performing Edman degradation. Phenylthiohydantoin amino acid derivatives were detected at 269 nm after separation on a RP-HPLC C18 column (4.6 × 2.5 mm) eluted at isocratic conditions according to the supplier's instructions. The amino-terminal sequence of RcTI was subjected to search for similar sequences in the database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) using the BLASTP search tool (Altschul et al., 1990). Those with the highest percentage of identity were selected and aligned using the ClustalW program.

2.7. Two-dimensional gel electrophoresis

To determine the isoelectric point and confirm the molecular mass of RcTI, 45 μg were solubilized in 250 μl of rehydration buffer (8 × 103 mM urea, 1.0 × 103 mM thiourea, 10% [v/v] glycerol, 2% [m/v] CHAPS, IPG buffer pH 3–10 and 0.001% [m/v] bromophenol blue) and placed into contact with immobilized pH gradient polyacrylamide gel strip (IPG), 13 cm, pH 3–10 (GE Healthcare) for 12 h. Isoelectric focusing (IEF) was performed in Ettan IPGphor-II system (Amersham Bioscience®) using the schedule: 200 V for 1 h; 500 V for 1 h, 1000 V for 1 h and a gradient from 4000 V up to 18,000 V/h total, exposed to 50 μA continuous electric current, at 20 °C. After IEF, the strip was incubated in the equilibrium and reducing solution (50 mM Tris–HCl, pH 8.8, containing 30% [v/v] glycerol, 6 × 103 mM urea, 2% [m/v] SDS, 2% [m/v] DTT, 0.1% [m/v] bromophenol blue) for 15 min under gentle stirring. Next, the strip was washed with an alkylation solution (50 mM Tris–HCl, pH 8.8, containing 30% [v/v] glycerol, 6 × 103 mM urea, 2% [m/v] SDS, 2.5% [m/v] iodoacetamide, 0.1% [m/v] bromophenol blue) for 15 min under gentle orbital shaking. For electrophoresis in the second dimension, the strip was placed on the top of a 12.5% polyacrylamide gel that was submitted to 30 mA constant current and 250 V as maximum voltage. This procedure was performed on a

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