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Research article

First insights into the diversity and functional properties of chitinases of the latex of *Calotropis procera*

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ABSTRACT

Chitinases (EC 3.2.1.14) found in the latex of *Calotropis procera* (Ait) R. Br. were studied. The proteins were homogeneously obtained after two ion exchange chromatography steps. Most proteins were identified individually in 15 spots on 2-D gel electrophoresis with isoelectric points ranging from 4.6 to 6.0 and molecular masses extending from 27 to 30 kDa. Additionally, 66 kDa proteins were identified as chitinases in SDS-PAGE. Their identities were further confirmed by mass spectrometry (MS) analysis of the tryptic digests of each spot and MS analysis of the non-digested proteins. Positive reaction for Schiff's reagent suggested the proteins are glycosylated. The chitinases exhibited high catalytic activity toward to colloidal chitin at pH 5.0, and this activity underwent decay in the presence of increasing amounts of reducing agent dithiothreitol. Spore germination and hyphae growth of two phytopathogenic fungi were inhibited only marginally by the chitinases but were affected differently. This suggested a complex relationship might exist between the specificity of the proteins toward the fungal species. The chitinases showed potent insecticidal activity against the Bruchidae *Callosobruchus maculatus*, drastically reducing survival, larval weight and adult emergence. It is concluded that closely related chitinases are present in the latex of *C. procera*, and the first experimental evidence suggests these proteins are involved more efficiently in defence strategies against insects rather than fungi.

1. Introduction

Latex is the protoplasm of specialised cells termed laticifers (Hagel et al., 2008). This fluid has been described in almost 20,000 plant species, including monocots and dicots (Lewinsohn, 1991). In general, latex contains a complex mixture of several secondary metabolites and proteins, which have shown toxic effects to animals, insects and fungi (Agrawal and Konno, 2009; Freitas et al., 2011a; Ramos et al., 2014). The peptidases are among the major proteins identified in latex fluids, and they can be found in several isoforms (Domsalla and Melzig, 2008). The major hypothesis is that these enzymes participate as defensive molecules against insects and fungi (Konno, 2011). In contrast, latex chitinases have not been extensively studied. Chitinases (EC 3.2.1.14) are hydrolytic enzymes with potential to degrade chitin, a polysaccharide present in fungal cell walls and peritrophic membranes of insects (Wang and Granados, 2001; Latgé, 2007). Therefore, these enzymes have gained importance for their biotechnological applications in the agricultural field to control pathogens and insects, as an environmentally safe agent (Hamid et al., 2013).

Chitinases have been described in some latex fluids, including those of *Hevea brasiliensis* (Martin, 1991), *Carica papaya* (Azarkan et al., 1997) and *Euphorbia characias* (Spanò et al., 2015). One of the most interesting aspects of the latex chitinases for studies in plant biology is that they are synthesised constitutively at high concentrations (Sytwala et al., 2015) compared to chitinases from other plant tissues, which are produced mainly in response to biological or hormonal stimuli (van Loon et al., 2006). Another motivating fact is that the chitinolytic enzymes are present in latex

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fluids as several isoforms (Azarkan et al., 1997). Three chitinase isoforms with different molecular masses were purified and characterized from *Ficus microcarpa* latex (Taira et al., 2005). Similarly, six basic chitinases were identified in *H. brasiliensis* latex (Martin, 1991), and two chitinase-like proteins are accumulated abundantly in the latex of *Morus alba* (Kitajima et al., 2010). The physiological function of each isoform is still unknown, since the different chitinase isoforms can exhibit differential biological activities, as was described by three chitinases from *F. microcarpa* (Taira et al., 2005).

The present work describes the isolation and biochemical characterisation of chitinases from the *Calotropis procera* (Ait) R. Br. latex and provides the insights into physiological functions of these constitutive proteins in plant defence against fungi and insects.

2. Materials and methods

2.1. Reagents

Dialysis membrane with a cut-off value of 8 kDa, glucuronidase (EC 3.2.1.31), propidium iodide, 3,3'-diaminobenzidine (DAB), colloidal chitin and Schiff's reagent were obtained from Sigma-Aldrich (São Paulo, Brazil). The ion exchange columns (Mono-Q and CM-Sepharose Fast Flow), reversed-phase column (type C_{18}, Hichrom), molecular mass markers, dithiothreitol (DTT), iodoacetamide (IAA), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), IPG buffer 3–10 and PhastGel Blue R were obtained from GE Healthcare (São Paulo, Brazil). Sequencing-grade trypsin (EC 3.4.21.4) was obtained from Promega (São Paulo, Brazil). The culture media Sabouraud Dextrose Agar (SDA) and Yeast Peptone Dextrose (YPD) were obtained from Difco and HiMedia (São Paulo, Brazil), respectively. Unless otherwise mentioned, the other reagents were of analytical grade.

2.2. Biological materials

2.2.1. Plants

Plants of the species *Calotropis procera* (Ait) R. Br. (Apocynaceae), grown in the city of Fortaleza, Ceará, Brazil, were identified by a local taxonomist at the Herbarium Prisco Bezerra of the Federal University of Ceará, and a voucher was deposited under number 32663.

2.2.2. Fungi

The phytopathogenic fungi *Fusarium oxysporum* and *Colletotrichum gloeosporioides* were obtained from a collection of the Federal University of Ceará and maintained on SDA at 27 °C, a photoperiod of 12 h and a relative humidity of 65%, as described by Souza et al. (2011).

2.2.3. Insects

Callosobruchus maculatus (Coleoptera) insects were isolated from infested beans (*Vigna unguiculata*) obtained at a local market and kept at 27 ± 2 °C, a relative humidity of 65% and a photoperiod of 12 h on *V. unguiculata* seeds, as described by Ramos et al. (2010).

2.3. Latex fractionation and collection

Latex was collected and fractionated as described by Freitas et al. (2007). Briefly, after breaking of the shoot apex, latex was collected in distilled water to a final ratio of 1:1 (v:v) and centrifuged at 4 °C for 10 min at 5000 × g. The supernatant was dialysed against distilled water using membranes with a cut-off of 8000 Da at 8 °C for 3 days. The resulting material was again centrifuged and lyophilised, yielding a clear material rich in proteins and devoid of rubber and secondary metabolites. The soluble proteins were estimated as described by Bradford (1976), using bovine serum albumin (BSA) as a standard.

2.4. Ion exchange chromatography on CM-Sepharose column

Latex proteins were submitted to ion exchange chromatography using a CM-Sepharose Fast Flow column, as described by Ramos et al. (2006). Briefly, non-retained proteins on a column were eluted with 50 mM sodium acetate buffer (pH 5.0). This fraction, termed PI-CM, was dialysed against distilled water, lyophilised and used in all further assays. The retained proteins were eluted first with 50 mM sodium acetate buffer (pH 5.0) containing 0.2 M and then the still bound proteins eluted with 0.3 M NaCl.

2.5. Ion exchange chromatography on Mono-Q column

The PI-CM fraction (2 mg mL^{-1}), dissolved in 20 mM Tris-HCl buffer (pH 8.0), centrifuged previously at 20,000 × g at 4 °C for 20 min and filtered using a 0.22 μm filter (Millipore®), was subjected to ion exchange chromatography using a Mono-Q column coupled to an FPLC system, equilibrated previously with 20 mM Tris-HCl buffer (pH 8.0). Retained proteins were eluted using a linear gradient from 0 to 400 mM NaCl for 40 min at a 1 mL min^{-1} flow rate. The peaks obtained were dialysed against distilled water and lyophilised. All fractions were dissolved in 25 mM ammonium bicarbonate, denatured with 0.2% RapiGest™ SF (Waters, Milford, USA), reduced with 10 mM dithiothreitol, alkylated with 10 mM iodoacetamide and further digested by trypsin during 12 h at 37 °C. The identification of proteins was performed by ESI-QUAD-TOF analyses after passing the mixture of peptides on a nanoACQUITY UPLC core system as carefully described in Cavalcante et al. (2016).

2.6. One-dimensional gel electrophoresis (1-D SDS PAGE)

Electrophoresis was performed in 12.5% polyacrylamide gels containing 0.1% SDS, as described by Laemmli (1970), with slight modifications. The samples, dissolved in 62.5 mM Tris-HCl buffer (pH 6.8) containing 1% SDS, in the presence or absence of 5% β-mercaptoethanol, were heated for 5 min at 100 °C and centrifuged for 5 min. Protein bands were visualized after staining with 0.1% Coomassie Brilliant Blue R-250. Phosphorylase B (97.0 kDa), bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as molecular mass markers.

2.7. Two-dimensional gel electrophoresis (2-D SDS PAGE)

The purity, isoelectric point and possible presence of isoforms in the isolated fraction were first approached by 2-D gel electrophoresis, as described by Freitas et al. (2007). The isolated fraction (125 μg) was dissolved in 200 μL of 7 M urea, 2 M thiourea, 1% DTT, 1% CHAPS, 1% IPG buffer 3–10 and bromophenol blue and then added to a strip (11 cm) containing immobilised pH (3–10). The isoelectric focussing step was performed using Ettan IPGphor II equipment (GE Healthcare), as described by the manufacturer. After isoelectric focussing, the pH strips were incubated in equilibrium solution (50 mM Tris-HCl, pH 7.0) containing 1% DTT, 30% glycerol, 6 M urea, 2% SDS and bromophenol blue) for 20 min and again afterward in the same solution containing 2.5% IAA instead of 1% DTT. The second dimension was performed in a vertical system in 12.5% polyacrylamide gels (14 × 14 cm). The gels were scanned using an Imagescanner machine (Amersham Biosciences, Uppsala, Sweden) and analysed by ImageMaster 2D Platinum 6.0 software (Amersham Biosciences).