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Research article

New constitutive latex osmotin-like proteins lacking antifungal activity

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ABSTRACT

Proteins that share similar primary sequences to the protein originally described in salt-stressed tobacco cells have been named osmotins. So far, only two osmotin-like proteins were purified and characterized of latex fluids. Osmotin from *Carica papaya* latex is an inducible protein lacking antifungal activity, whereas the *Calotropis procera* latex osmotin is a constitutive antifungal protein. To get additional insights into this subject, we investigated osmotins in latex fluids of five species. Two potential osmotin-like proteins in *Cryptostegia grandiflora* and *Plumeria rubra* latex were detected by immunological cross-reactivity with polyclonal antibodies produced against the *C. procera* latex osmotin (CpOsm) by ELISA, Dot Blot and Western Blot assays. Osmotin-like proteins were not detected in the latex of *Thevetia peruviana*, *Himatanthus drasticus* and healthy *Carica papaya* fruits. Later, the two new osmotin-like proteins were purified through immunoaffinity chromatography with anti-CpOsm immobilized antibodies. Worth noting the chromatographic efficiency allowed for the purification of the osmotin-like protein belonging to *H. drasticus* latex, which was not detectable by immunoassays. The identification of the purified proteins was confirmed after MS/MS analyses of their tryptic digests. It is concluded that the constitutive osmotin-like proteins reported here share structural similarities to CpOsm. However, unlike CpOsm, they did not exhibit antifungal activity against *Fusarium solani* and *Colletotrichum gloeosporioides*. These results suggest that osmotins of different latex sources may be involved in distinct physiological or defensive events.

1. Introduction

Latex fluids have been recognized in over 20,000 plant species. The most accepted hypothesis suggests that the multiple compounds found in the fluid participate in plants' defenses (Hagel et al., 2008). Several classes of molecules have been identified in these fluids and are generally divided into secondary metabolites and proteins. Among the studied proteins are the chitinases, peptidase inhibitors, peptidases, lectins, and enzymes related to antioxidative metabolism (Konno, 2011). Peptidases have been recognized as the most common proteins found in laticifer fluids, and some studies have shown them to participate in plant protection against insects and fungi (Konno, 2011; Ramos et al., 2014). In contrast, only two osmotin-/thaumatin-like proteins were purified and characterized from laticifer fluids, and their functional status remains a point of discussion (Freitas et al., 2011a; Looze et al., 2009).

The genetic and biochemical characteristics of osmotin-/thaumatin-like proteins are considerably conserved in the plant kingdom (Liu et al., 2010). The reported proteins have amino acid sequences and three-dimensional structures that very closely resemble those of the originally described thaumatin and osmotin, which were isolated from *Thaumatococcus daniellii* fruit and cultured salt-adapted tobacco cells, respectively (Singh et al., 1987; Van der Wel and Loewe, 1972). They also share other molecular characteristics, including large numbers of cysteine residues

Abbreviations: CpLP, *Calotropis procera*; PrLP, *Plumeria rubra*; CgLP, *Cryptostegia grandiflora*; TpLP, *Thevetia peruviana*; HdLP, *Himatanthus drasticus*; CapLP, *Carica papaya* latex proteins.

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involving 5–8 disulfide bonds; pH and temperature stability; resistance to proteolysis; and molecular masses of approximately 20 kDa (Liu et al., 2010; Viktorova et al., 2012).

Osmotin-/thaumatin-like proteins can be induced by several hormonal signals, including abscisic acid or auxin, and environmental signals such as dehydration, salinity, and fungal infection (Ahmed et al., 2013). Therefore, their synthesis *de novo* can confer tolerance to several abiotic and biotic stresses, and their overexpression would have numerous applications in the field of agriculture (Das et al., 2011). Some osmotins have activity against fungal pathogens. However, these activities are specific to each protein and target fungi. The antifungal activity can involve cell membrane damage with cytoplasmic content leakage (Freitas et al., 2011b), cell wall degradation due to glucanase activity (Menu-Bouaouiche et al., 2003) or induction of reactive oxygen species via membrane receptors (Choi et al., 2013).

To date, two osmotin-/thaumatin-like proteins were purified from laticifer fluids. Osmotin was detected only in *Carica papaya* latex obtained from damaged plants, whereas healthy plant latices were osmotin free, suggesting that it is an inducible protein. However, this osmotin did not show antifungal activity (Looze et al., 2009). Conversely, the *Calotropis procera* latex osmotin showed constitutive activity against phytopathogenic fungi (Freitas et al., 2011a). Faced with these heterogeneous data, a search for new latex osmotins and their functional characterization is needed. Information about osmotin-like proteins in latex fluids is still restricted, and the role played by these proteins in the latex is poorly discussed. Here, new laticifer fluids were examined for osmotin and their activities against phytopathogens were assayed.

2. Material and methods

2.1. Chemicals

Acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), iodoacetamide (IAA), phenylmethylsulphonyl fluoride (PMSF), RESOURCE-S, CM-Sepharose Fast Flow, CNBr-activated Sepharose™ 4B, and molecular mass markers were from GE Healthcare Life Science (São Paulo, SP, Brazil). Freund's complete and incomplete adjuvant, papain, goat anti-rabbit IgG conjugated with alkaline phosphatase, p-nitrophenyl phosphate disodium, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and nitro blue tetrazolium (NBT) were from Sigma–Aldrich (São Paulo, SP, Brazil). Sequencing-grade modified trypsin was from Promega (Madison, WI, USA). Others chemicals were of analytical grade.

2.2. Plant material

Healthy plants of *Carica papaya* L. (Caricaceae) and the Apocynaceae members *Calotropis procera* (Ait.) R. Br., *Plumeria rubra* L., *Cryptostegia grandiflora* R.Br., *Thevetia peruviana* (Pers.) Schum., and *Himatanthus drasticus* (Mart.) Plumel, all grown in city of Fortaleza-CE, Brazil, or its vicinities, were used as source of fresh latex. All plants were identified and the vouchers were deposited at the Herbarium of the Universidade Federal do Ceará, Brazil.

2.3. Latex processing

The latices were released by cutting off the ends of the branches and were then collected into distilled water at a 1:2 (v/v) dilution. The protein fractions were isolated as described by Freitas et al. (2007) and termed as latex proteins (LP) according to their source: CpLP, PrLP, CgLP, TpLP and HdLP, corresponding to *C. procera*, *P. rubra*, *C. grandiflora*, *T. peruviana* and *H. drasticus* latex proteins, respectively. *Carica papaya* (CapLP) latex proteins were obtained from the green fruits of a healthy specimen and processed as reported above.

2.4. Rabbit polyclonal antibodies

CpOsm was purified as previously described by Freitas et al. (2011a), and its purity was checked by polyacrylamide gel electrophoresis (Laemmli, 1970). CpOsm (0.5 mg) was dissolved in 0.5 mL saline and mixed with 0.5 mL of Freund's complete adjuvant. The suspension (1 mL) was injected intramuscularly into an adult New Zealand white male rabbit followed by booster injections (21, 35, and 42 days) of the same CpOsm dose with Freund's incomplete adjuvant, as described by Ramos et al. (2006). Serum samples were collected weekly from day 21 and maintained at −20 °C until use.

2.5. Purification of anti-CpOsm antibodies

Specific anti-CpOsm antibodies were purified by immunoaffinity chromatography using CpOsm immobilized on a Sepharose 4B column. CpOsm was immobilized on a CNBr-activated Sepharose™ 4B matrix, as described by the supplier (GE Healthcare). CpOsm (3 mg mL^{-1}) was dissolved in coupling buffer (0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl), centrifuged for 10 min at 10.000 × *g* at 4 °C, and incubated for 2 h at 25 °C with matrix. Excess uncoupled CpOsm was removed by washing with coupling buffer. The remaining active groups of the matrix were blocked using 0.1 M Tris–HCl buffer (pH 8.0) for 4 h at 25 °C. This column was named as CpOsm-Sepharose 4B column and was used to purify anti-CpOsm antibodies.

After loading the immunized rabbit whole serum (1 mL) onto the CpOsm-Sepharose 4B column, the non-retained proteins were eluted with 50 mM sodium phosphate buffer (pH 6.0), and the retained proteins (anti-CpOsm antibodies) were eluted with 50 mM glycine-HCl buffer (pH 3.0). Protein fractions (0.5 mL) were collected at a 1 mL min^{-1} flow rate, and the absorbance was monitored at 280 nm. The specific anti-CpOsm antibodies were dialyzed against distilled water and lyophilized. Further, the purified anti-CpOsm antibodies were chemically coupled to a Sepharose 4B column and used as an immunoaffinity matrix to identify osmotins in the studied laticifer fluids. These specific antibodies were also used in all immunological assays.

2.6. Enzyme linked immunosorbent assay (ELISA)

ELISA assays were performed as previously reported by Ramos et al. (2007). The presence of osmotins in different latices was estimated by using purified anti-CpOsm IgG as the primary antibody (1:2000 dilution) and goat anti-rabbit IgG conjugated to alkaline phosphatase (1:5000 dilution) as the secondary antibody. Experiments were developed in 96-well microplates, and latex fractions were prepared at a concentration of 1 mg mL^{-1} in triplicate. The reactions were detected using the substrate p-nitrophenyl phosphate disodium (5 mg mL^{-1}) and quantified by reading the absorbance at 405 nm using an automated microplate reader (ELx800 Absorbance Microplate Reader, BioTek Customer Care).

2.7. Dot blot and Western blot

Dot blot assays were performed by adding 10 μL (1 mg mL^{-1}) of each latex fraction to a nitrocellulose membrane (0.45 mm, Hybond-P, Amersham-GE Healthcare Life Sciences), drying for 5 min, and then incubating in PBS buffer (pH 7.4) containing 5% (w/v) non-fat milk for 2 h at 25 °C. The membranes were probed with specific anti-CpOsm primary antibody (1:2000 dilution) and goat anti-rabbit IgG conjugated to alkaline phosphatase as the