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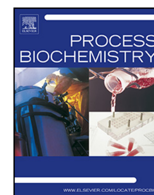

A new peptide from *Jatropha curcas* seeds: Unusual sequence and insights into its synthetic analogue that enhances proteolytic activity of papain

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ABSTRACT

A new peptide (1341 g/mol) from *Jatropha curcas* seeds was isolated. The linear sequence (APTLSG-GSVPRDAD) was deduced by de novo peptide sequencing, and further used as scaffold for synthesis of linear (1342 g/mol) and cyclic (1324 g/mol) synthetic analogues. The full peptide sequence was identified as inserted in a putative conserved domain of late-embryogenesis proteins which produced a significant alignment hit (100% of identity and *E*-value of 1e−05) with a hypothetical protein JCGZ_12502 of *J. curcas*. Whereas in the linear peptide predominated the double charged ion state (*m*/*z* 671.68), in the cyclic form was observed the mono charged ion state (*m*/*z* of 1325.19) and an unusual MS/MS fragmentation pattern. The differences between the forms were discrete in terms of ionic mobility, retention time (reverse phase) and net charge as function of pH. Circular dichroism spectra presented an intense negative peak at 198 nm which is assigned for its disordered contents. A negative peak at 222 nm in the spectrum of the circular form suggested its structure was not as disordered as the linear form. The peptides were neither haemolytic nor cytotoxic and did not inhibit phytopathogenic fungi. Surprisingly, the circular but not the linear peptide increased the proteolytic activity of papain.

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1. Introduction

Plant peptides demonstrate an astonishing functional variety and, therefore, have been frequently described in the scientific literature of the last years [1]. Cyclic peptides represent the most common kind isolated from plant species [2,3]. The genus *Jatropha*, for instance, is notorious for accumulating cyclopeptides in its latex where almost twenty molecules were reported [4]. From these, three (jatrophidin, curcacycline B and curcacycline A) were isolated from *Jatropha curcas*, a multipurpose small tree worldwide recognized for its oil production, chemical constituents and medicinal uses [5–7]. Nevertheless, in *J. curcas* seeds, there are only two reports describing the outstanding bioactive potential of peptides. In the first, the authors employed a novel method aiming to screen antimicrobial peptides, named cell membrane affinity chromatography, where it was possible to isolate and identify an antimicrobial peptide (KVFLGLK, JCpep7) [8]. More recently, it was published a study describing a rich source of bioactive peptides generated from protein hydrolysis that was able to inhibit the angiotensin, an enzyme involved in blood pressure control [9].

The field of finding polypeptides from plant biomass, even in rich sources such as species from the genus *Jatropha* is full of drawbacks to be explored, mainly due to sample preparation, appropriate fractionation and identification methods [10,11]. Based on this, the discovery of polypeptides sequences with unknown properties represents a challenge even as valuable tool to design scaffolds that can be used to explore its medicinal uses [12]. Therefore, the aim of this study was to investigate and explore the properties of two synthetic analogues peptides derived from a natural sequence of fourteen amino acids of *J. curcas* seeds that was used as draft.

Abbreviations: Lp, linear peptide; Cp, cyclic peptide; LEA, late embryogenesis proteins.

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2. Materials and methods

2.1. Chemicals

Organic solvents (dichloromethane, ethanol and acetic acid) were of analytical grade. Acetonitrile, polyvinylpolypyrrolidone (PVPP), ammonium bicarbonate, papain and trypsin were from Sigma Chemical Co. (São Paulo, Brazil). Solid phase extraction (SPE) C_{18}-E Phenomenex strata column (200 mg/3 mL) was purchased from Allcrom (São Paulo, Brazil). RP-HPLC-C_{18} column (PRP-3, 4.1 mm × 150 mm) was purchased from Hamilton. Mass spectrometry C18 BEH column (1.7 mm, 100 mm × 10 cm) and the purified peptide leucine enkephaline (555.277 Da) and [Glu1]-Fibrinopeptide (1568.65 Da) were purchased from Waters (Manchester, UK). Two synthetic analogue peptides, linear (1342.44 Da) and cyclic forms (1324.44 Da) with more than 90% of purity were purchased from GenOne Biotechnologies (Rio de Janeiro, Brazil).

2.2. Plant material

Seeds of *J. curcas* were collected in July 2012 in the experimental station at Fortaleza University (Fortaleza, CE). The seeds were dehulled and extracted for oil removal with 300 mL of hexane (five times) until clearness. The residual fine flour obtained was dried at 30 °C under shade.

2.3. Organic extraction

The dried seed material (4.0 g) was extracted in a soxhlet apparatus with 100 mL of CH_2Cl_2 for 1 h. The procedure was repeated five times with fresh solvent. The CH_2Cl_2 soluble material was discarded and the residues were dried at room temperature. The dried material was extracted with 200 mL EtOH (50%) for 1 h and centrifuged at 10,000 × g for 20 min at 25 °C, and the supernatant was reserved. This procedure was repeated at least three times with fresh solvent, and at the end all supernatant were pooled and acidified by addition of HOAc to a final concentration of 2%.

The acidified supernatant was mixed for a period of 4 h with PVPP in a 1:100 (w/v) ratio at room temperature. After centrifugation at 10,000 × g for 20 min at 25 °C, the resulting clarified supernatant was evaporated under reduced pressure at 40 °C using an evaporator to a volume of ca. 50 mL and further lyophilized until achievement of dried power (0.4 g).

2.4. Solid-phase extraction

Samples of 100 mg of the dried powder cited above were dissolved in 100 mL of NH_4HCO_3 solution (50 mM) in a 1:1 (w/v) ratio of sample:buffer and applied to a C_{18}-E Phenomenex SPE strata column (200 mg/3 mL) previously conditioned in ACN (95%) and equilibrated with 60 mL of NH_4HCO_3 buffer. After applying the sample, the column was washed with 20 mL of NH_4HCO_3 buffer and the eluate discarded. The column was then eluted sequentially with 10 mL of 50% and 95% of ACN in NH_4HCO_3 buffer (50 mM). The eluates 0–50% ACN were lyophilized to yield the fraction (6 mg) used in this work. Typical yield of fraction 0–50% amounted to ca. 0.15% (w/w) of the dried plant material.

2.5. Chromatography

Gel filtration analysis: samples of 1 mg of the fraction 0–50% was dissolved in 1 mL of 40% CH_3CN containing 0.1% TFA. The resulted fraction (100 μL) was analyzed by gel filtration on a Superdex Peptide HR 10/30 column (GE Healthcare) using a mobile phase of 40% CH_3CN containing 0.1% TFA and flow of 1 mL/min. The mass spectrometry standard peptide leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) which gives a monoisotopic mass of 556.277 $[M+H]^+$ was used as reference peptide in gel filtration. The absorbance was monitored at 216 and 280 nm. This analysis was performed using an ÄKTA™ purifier 10 chromatography system, controlled by UNICORN software.

Reverse phase: the freeze-dried fraction 0–50% (1 mg) was dissolved in 0.1% (v/v) TFA/water (1000 μL) and subjected (100 μL) to RP-HPLC on a C_{18} column (Hamilton PRP-3, 4.1 mm × 150 mm) equilibrated with 0.1% (v/v) TFA/water (solvent A). After an initial 5-min wash with solvent A, elution was performed at a flow rate of 1 mL/min with a 0–95% linear gradient of ACN containing 0.1% (v/v) TFA (solvent B) during 20 min, then from 95 to 100% of solvent B in 5 min and a final 5 min wash with 100% of solvent B. The absorbance was monitored at 216 and 280 nm. All fractions were automatically collected and later lyophilized. The analysis was performed using the ÄKTA™ as before.

Multiple steps of HPLC fractionation: lyophilized PII fraction (0.5 mg) was dissolved in 0.1% (v/v) TFA/water (500 μL) and subjected (100 μL) to RP-HPLC on the same C18 column (Hamilton PRP-3, 4.1 mm × 150 mm) previously equilibrated as described. Elution was performed at a flow rate of 1 mL/min, with a 0–20% linear gradient of solvent B during 2 min, 20–40% of solvent B in 12 min, 40–100% of solvent B in 5 min, and a final 5 min wash with 100% of solvent B. The eluent was monitored by UV absorbance at 216 and 280 nm. All fractions were automatically collected and lyophilized as before. In the last step, 0.25 mg of lyophilized PII.C fraction was dissolved in 0.1% (v/v) TFA/water (250 μL) and subjected (100 μL) to RP-HPLC on the same C18 column (Hamilton PRP-3, 4.1 mm × 150 mm) and at the same conditions as described before. Finally, isolated fraction PII.C was collected and dried under vacuum for subsequent mass spectrometry analysis. The analysis was performed using the ÄKTA™ purifier 10 chromatography system.

Reverse phase of synthetic peptides: an aliquot of 100 μL containing 50 μg of a mixture of Lp and Cp (1:1) dissolved in 0.1% (v/v) TFA/water was subjected to RP-HPLC on the same C18 column (Hamilton PRP-3, 4.1 mm × 150 mm) and same conditions (see topic 'Reverse phase') as previously described.

Liquid chromatography: the *J. curcas* seed peptides were separated using a nano-ACQUITY UPLC Chromatography™ system (Waters, Manchester, UK) employing a C18 BEH column (1.7 mm, 100 mm × 10 cm). The sample was diluted in water containing 0.1% formic acid and the separation was carried out using a gradient from 5% to 80% (v/v) of acetonitrile in 0.1% formic acid (v:v) at flow rate of 600 nL/min.

2.6. Mass spectrometry

Mass spectrometry analysis was performed using a Q-TOF type Synapt HDMS™ operated in the positive ion mode (+). For the external calibration, [Glu^1]-fibrinopeptide (100 fmol/μL) was applied as the lock mass compound. Accurate LC–MS data were collected employing data-dependent analysis (DDA) mode for the most intensive precursor ions. The MS/MS peptide fragmentation (collision induced dissociation, CID) spectra from DDA acquisition were converted into raw files and after in pkl-files using Mass Lynx and the Protein Lynx global server (PLGS) softwares respectively.

Ion mobility spectrometry: the following adopted conditions were capillary 4.0 kV, gas pressure 0.3 psi, cone 50 V, extractor 4 V. The IMS gas was nitrogen at a flow rate of 24 mL/min, the velocity and the wave height were operated at 300 m/s and 8 V, respectively. The instrument control and data analysis of experiments were performed using the MassLynx and V4.1 software respectively.

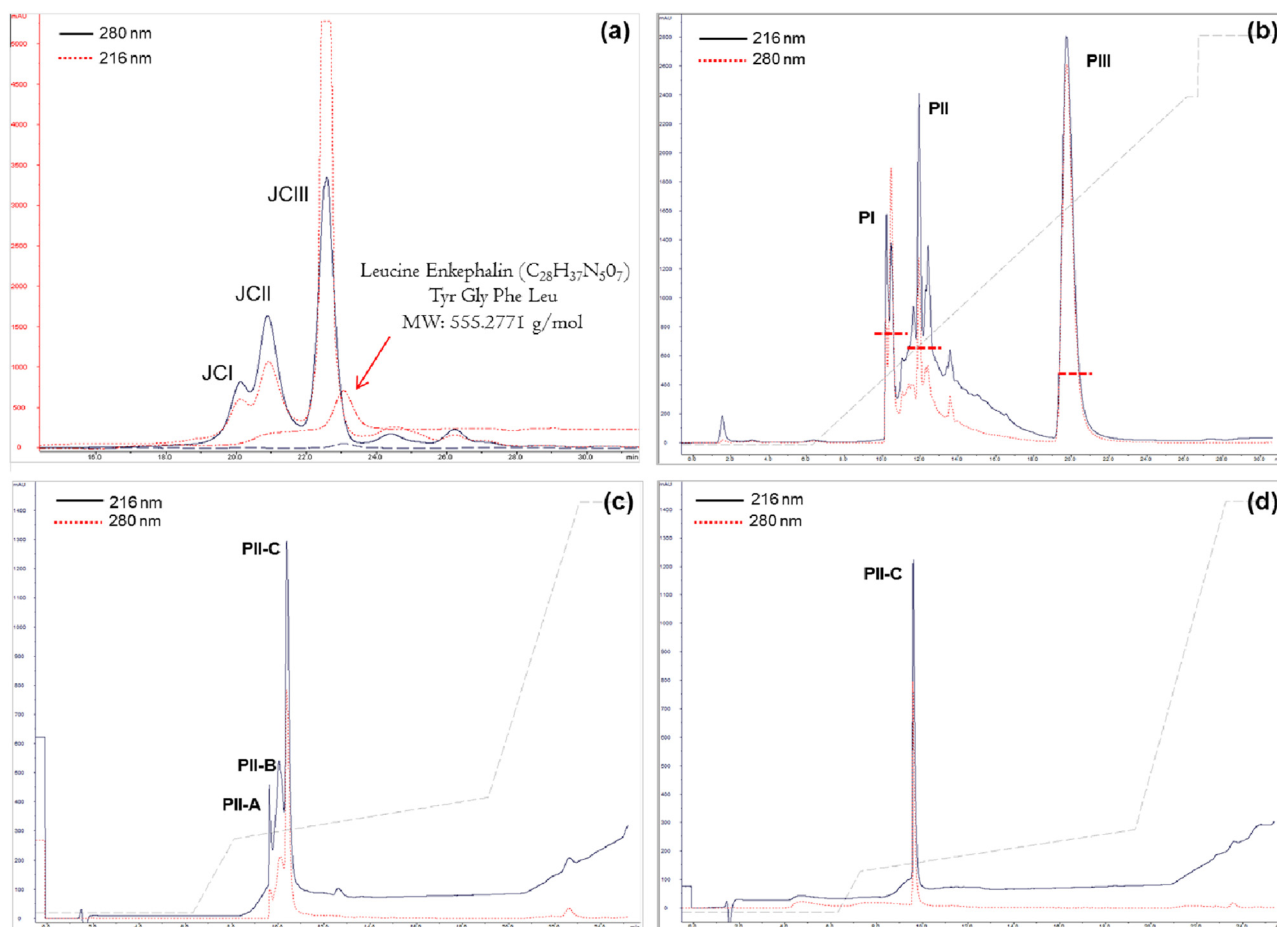

Fig. 1. Chromatographic profile and separation steps of isolation of a new peptide from *Jatropha curcas* seeds. (a) Gel filtration analyses on a Superdex Peptide HR 10/30 column (GE healthcare) showing its three sub-fractions JCI, JCII and JCIII; (b) linear gradient (0–95%) of RP-C18 analyses, using a Hamilton PRP-3 (4.1 mm × 150 mm) column, showing its three sub-fractions (PI, PII and PIII); (c) stepwise separation (0–20%, 20–40% and 40–95% in ACN) by RP C18 of sub-fraction PII (showing its three new sub-fractions PII-A, PII-B and PII-C) and PII-C (d), respectively.

2.7. Sequencing analyses

De novo sequencing by tandem mass spectrometry: for sequence reconstruction of unknown peptides based on the acquired mass data, de novo sequencing approach was employed to interpretation of MS/MS peptide fragmentation spectra from DDA acquisition.

Jatropha Genome Database search: a searching in Jatropha Genome Database (http://www.kazusa.or.jp/jatropha/) version 4.5, was employed to confirm the presence of deduced primary amino acid sequences described in this study. It was confirmed and considered only those sequences that appeared one time and exhibited exactly the same amino acids residues in coding sequences.

Identification and sequence similarities: the full length coding sequence (contig) where deduced primary amino acids sequences were inserted, were used as input (query) in protein BLAST tool for identification. The most significant hit in each search were considered. For guide tree construction, was chosen the full length coding sequence (contig) of the ion 671.79 *m*/*z*. ClustalW (1.1.0) tool was employed using the non-redundant protein sequence (nr) database from GenBank (Viridiplantae taxa was used as search set). The most eight significant hits with *E*-value greater than 10^{17} were considered.

2.8. Synchrotron radiation circular dichroism (SRCD) spectroscopy

The SRCD spectra of the linear and cyclic synthetic analogues peptide (7 mM), in aqueous solution, were obtained at the CD1 beamline at the Institute for Storage Ring facilities (ISA synchrotron) at the University of Aarhus, Denmark. Measurements were collected over the wavelength range from 280 to 175 nm with 1 nm interval and 2 s dwell time, at 25 °C, using a 0.0016 cm path length demountable Suprasil quartz cell (Hellma Ltd, UK).

The spectra were processed using CDTools software [13], performing the average of the three scans collected, the baseline subtraction (baseline consisted of pure water), smoothing with a Savitzky–Golay filter, and calibration against a spectrum of a camphorsulfonic acid standard taken at the beginning of the data collection. Final processed SRCD spectra were expressed in delta epsilon units, using a mean residue weight of 112.

2.9. Theoretical determination of net charge of Lp and Cp in pH range

Liquid charge of each peptide (Lp and Cp) was conducted through a calculation procedure that took into account pK_a ionizations of terminal (NH_2 and COOH) and lateral groups (ionizable groups) of the 14 amino acids residues (Lp and CP) of APTLSGGSVPRDAD sequence in a range pH of 1–10 [14].

2.10. Biological prospecting

Hemolytic activity was investigated by exposing Lp and Cp (200–0.7 μM) to 2% human erythrocytes for 24 at 25 °C in PBS buffered medium. Blood samples were obtained from healthy

Table 1
The amino acid sequence alignment of *J. curcas* new peptide with closely related sequences in the non-redundant protein sequence (nr) database from NCBI.

Identification	Organism	Sequence alignment	ID (NCBI)	%Identity	*E*-value
Hypothetical protein JCGZ_12502	*Jatropha curcas*	APTLSGGSVPRDAD	KDP32041.1	14/14 (100%)	3e−04

donors certified by the Centre of Hematology and Hemotherapy of State of Ceará (Ceará, Brazil) in terms of infectious agents.

Cytotoxicity was performed against the tumorigenic cell lines HCT-116 (human colon), Ovcar-8 (human ovarian) and HL-60 (human leukaemia) according to the method reported previously [15].

Inhibition of spore germination and vegetative growth of phytopathogenic fungi (*Fusarium solani* and *Rhizoctonia solani*) were performed according to the method reported previously [16].

Proteolytic assays and inhibition of the proteolytic activity of papain and trypsin were according to the method reported previously [17].

3. Results and discussion

3.1. Peptide prospecting in J. curcas seeds

The goal of this study was to prospect polypeptides in seeds of *J. curcas*. The complexity of the plants tissues which contains a large number of secondary metabolites and other substances makes peptides findings a challenger research field [12,18]. After three initial strategies of peptide achievement: contaminants removal (dichloromethane), extraction (ethanol 50%), and analyte enrichment (C-18 solid phase extraction); two chromatographic profiling analyses (fraction 0–50% ACN) were performed on a gel filtration Superdex Peptide HR 10/30 and in a high resolution reverse-phase separation (HPLC) column, respectively. The first chromatogram (Fig. 1a) showed three fractions wherein two of them (JCI and JCII) presented relative molecular mass greater than of leucine enkephaline (555 g/mol), chosen as molecular weight reference, and one (JCIII), the most intense, in the neighbourhood of the reference. In the second chromatogram (Fig. 1b) the resolved peaks were grouped in the fractions PI, PII and PIII.

Previous mass spectrometry analyses of PI, PII and PIII indicated the presence of ions with isotopic distribution and mass/charge ratio compatible with peptides in fraction PII (Fig. 2a). Thus further studies of PII fraction were performed by means of multi-steps of high resolution reverse-phase (C-18) separation (Fig. 1c, d) which enabled the isolation of a new peptide (PII-C) of monoisotopic molecular mass of 1341.58 Da $[M+H]^+$ from *J. curcas* seeds (Fig. 2b). Its fourteen amino acids residues (APTLSGGSVPRDAD)

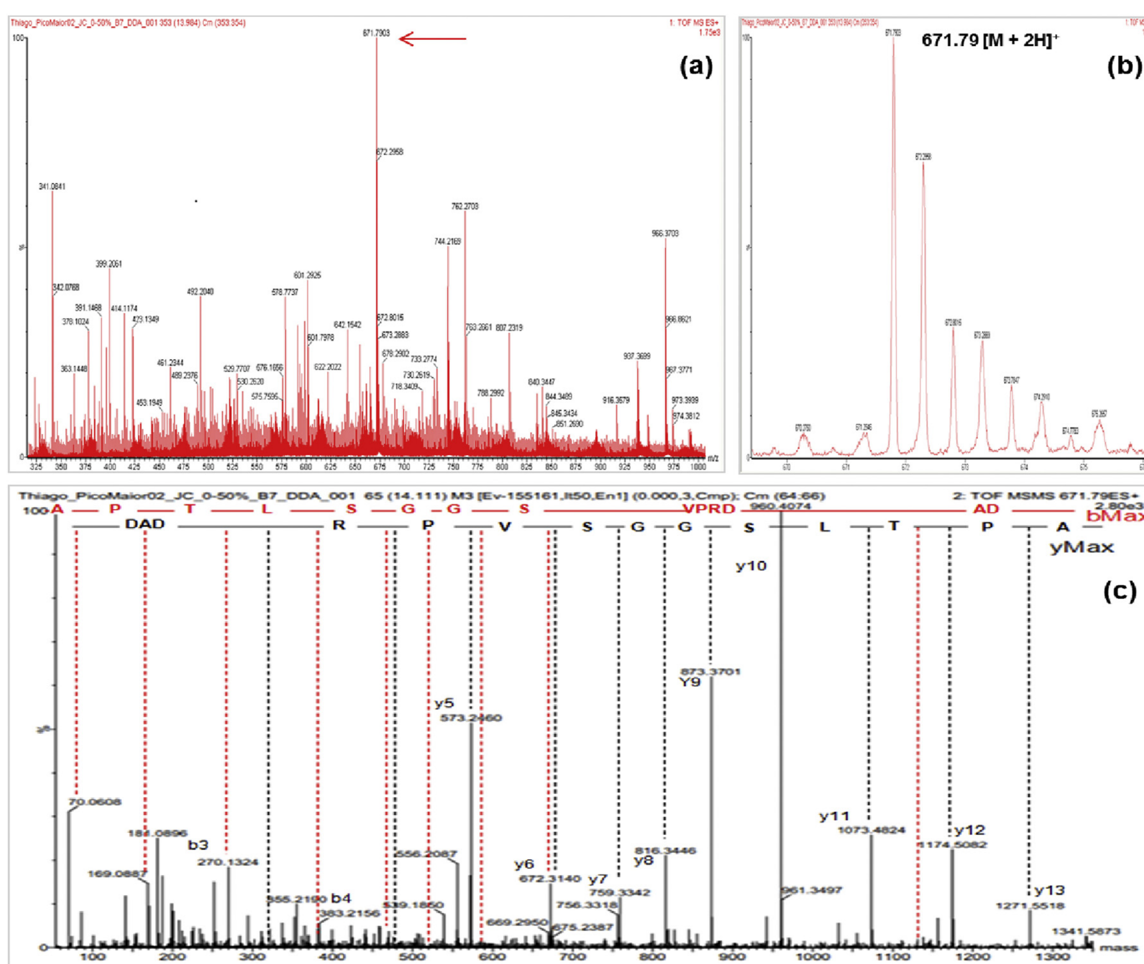

Fig. 2. Mass spectrometry analyses of the ion 671.79 $(M+2H)^+$. (a) Exploratory profile by liquid chromatographic (nanoACQUITY UPLC C-18 BEH) coupled to mass spectrometry (SYNAPT HDMS, Waters) of ions present in *Jatropha curcas* seed fraction PII; (b) isotopic distribution of the double charged ion of *m*/*z* 671.79 present in fraction PII-C; and (c) primary amino acid sequence (APTLSGGSVPRDAD) deduced by de novo sequencing of ion of 671.79 $(M+2H)^+$.

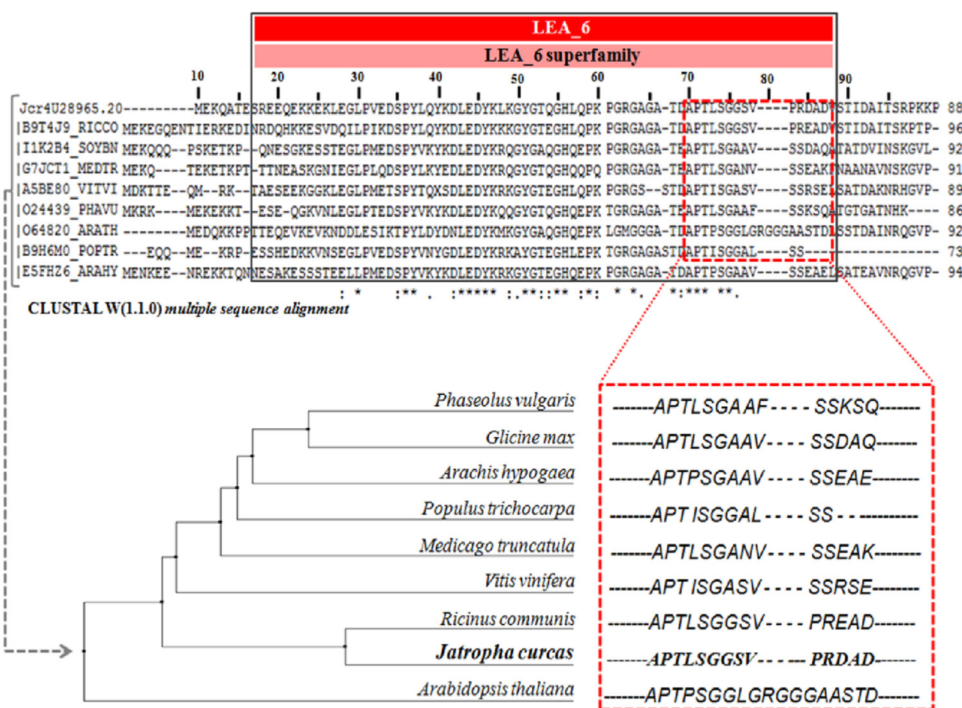

Fig. 3. Phylogenetic tree to further illustrate amino acid sequence homologies of conserved LEA_6 superfamily domain. The red dashed line highlights and compares the peptide sequence (APTLSGGSVPRDAD) of *Jatropha curcas* with the other plant sequences. *J. curcas* sequence is represented by the contig Jcr4U28965.20 and its alignment has been done with ClustalW. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were unambiguously deduced by MS de novo peptide sequencing and confirmed by manual searching in *Jatropha* Genome Database (Fig. 2c). Many of the ions detected in PII fraction were subsequently sequenced using the same strategies described for the isolated peptide (Supplementary Table S1).

According to Claeson [11], the main problem faced to study highly purified polypeptides from plant biomass is due to contaminants such as pigments, salts, carbohydrates and polyphenols, which represent one of the drawbacks encountered when dealing with complex sample such as vegetal tissue. Interference removal, analyte enrichment and high resolution separations are priorities and essentials steps preceding posterior mass spectrometry analyses and peptide characterization to access the primary amino acids sequences of peptides with unknown properties and functions [19,20].

3.2. Peptide identity and origin

The APTLSGGSVPRDAD sequence of *J. curcas* peptide, used as input query to search for similar sequences in the non-redundant protein sequence (nr) database from NCBI, produced a significant alignment (100% of identity and *E*-value of 1e−05) with a hypothetical protein JCGZ_12502 of *J. curcas*, confirming thus its primary sequence (Table 1). A dendrogram based on the alignment of this hypothetical protein with the eigth most significant hits of BlastP evidenced peptide localization (APTLSGGSVPRDAD) in the putative conserved domain of LEA_6 superfamily, a family of late embryogenesis abundant protein whose levels increases highly during the last stage of seed maturation (Fig. 3). The identity of this peptide and its related LEA superfamily function suggests a post-translation processing as origin mechanisms.

3.3. Structural analyses of synthetic analogues

Many reports in literature have showed that linear and cyclic peptides exhibit functional differences which are attributed to its individual structural arrangement [21]. For this reason, both synthetic peptides, linear (1341.36 g/mol) and cyclic (1323.19 g/mol), designed on the APTLSGGSVPRDAD sequence, were acquired and studied.

According to mass spectrometry analyses, both peptide forms produced differences regarding its charge state and MS/MS fragmentation pattern (Supplementary Fig. S1). Meanwhile, in the MS spectrum of the linear form the double charged ion state (671.68 *m*/*z*) predominated. In the cyclic form it was observed the mono charged ion state (1325.19 *m*/*z*). In respect to MS/MS fragmentation pattern, the differences were much remarked. The intensity of cyclic precursor ion was greater than in its analogue linear precursor ion. On the other hand, its daughter ions exhibited a complex and indistinguishable pattern of fragmentation when compared with ions of linear precursor fragments, which were easily attributed to its correspondent amino acids (APTLSGGSVPRDAD). These features are mainly due to particular behaviour of cyclic peptides during collision-induced dissociation [22]. Initially, fragmentation of cyclic precursor ion generates a linear precursor and just after this occurs fragmentation as predicted by conventional fragmentation rules such as in linear peptide [23]. Thus, while the sequence of amino acids of a linear peptide can be deduced directly subtracting the mass of two consecutive peaks, in cyclic peptides what is observed is the superposition of ions series of each linear peptide after opening of cyclic structure.

The SRCD spectra of the linear peptide in aqueous solutions presented an intense negative peak at 198 nm, which is assigned to its disordered content [24] (Supplementary Fig. S2). Additionally, it was possible to observe a small positive peak at 184 nm, which was observed in the SRCD spectra of proteins that belong to the group of intrinsically disordered proteins (IDPs), such as the MEG-14 and β-synuclein [25,26]. The SRCD spectra of the cyclic peptide also presented the 198 and 184 nm peak, suggesting its low content of ordering in aqueous solution, but a negative peak at 222 nm was also observed, suggesting the cyclic peptide is not as disordered as the linear peptide. The Lp and Cp were eluted with retention

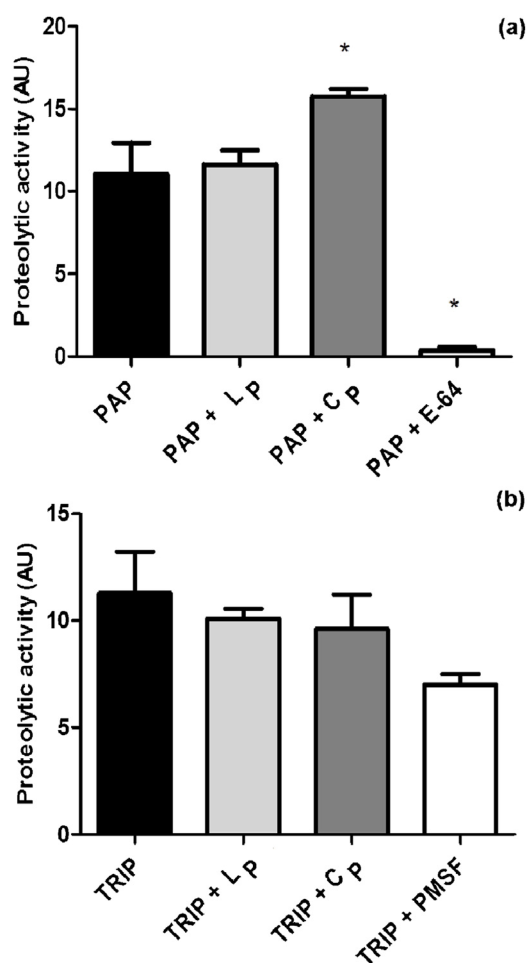

Fig. 4. Evaluation of the effect of linear (Lp) and cyclic (Cp) synthetic peptides against proteolytic enzymes papain (a) and trypsin (b). An amount of 100 μg of each peptide was utilized in these assays. Azocasein 1% was used as enzyme substrate. The absorbance of the reactions was monitored in a spectrophotometer at 440 nm. Assays were performed in triplicate and the specific cysteine protease inhibitor E-61 (0.18 mM) and the serine protease inhibitor PMSF (5 mM) were used as positive controls for papain (PAP) and trypsin (TRIP), respectively. $^{*}p < 0.05$ indicates statistical difference compared with the control group PAP ($n = 3$, ANOVA Dunnett's test).

times of 11.68 and 11.89 min respectively, which corresponded an acetonitrile percentage of 27.1 and 28.2%. This result suggests that reverse phase chromatographic analyses of analogues synthetics revealed a small difference regarding its hydrophobic interaction with C-18 columns. The cyclic peptide has a limited number of conformations and major structural restrictions, thus exposes much less its hydrophilic residues beyond absence of free amino and carboxyl groups in its ends, which in turn enhances the effect of its hydrophobic residues (Supplementary Fig. S2).

Theoretical calculation of net charge of Lp and Cp in a pH range of 1–10 revealed the same charge state (−1) amongst pH 4–9, being that cyclic peptide stands with this value in pH 10. The charge sums of aspartic, arginine and proline residues are the major responsible by this charge state. In cyclic molecule, the amino and carboxyl groups at the ends not contribute to this charge state because cyclization forms an amide bond (Supplementary Fig. S2).

3.4. Biological functions and applications

Both synthetic peptides were examined for biological properties and possible activities. Lp and Cp did not cause haemolysis of human erythrocytes even at the concentration of 200 μM. Absence of cytotoxic effects was further confirmed by assays with cancer cell lines. Therefore it was concluded that the peptides were not toxic to cell at the higher concentrations assayed (25 μg/mL). Surprisingly, Cp but not Lp significantly enhanced the proteolytic activity of papain, a cysteine peptidase (Fig. 4). Recent studies addressed to the expression and localization of cysteine peptidases in developing seeds of *J. curcas* showed a nice correlation between the expression of peptidases with the middle and later embryogenesis stages [27]. The higher levels of expression were associated to the anatomic changes in integument and cellular endosperm corroborating the hypothesis that the produced peptidases should participate of programmed cell death as part of the remodelling tissue at the final stage of embryogenesis. Some of the cysteine peptidases of *J. curcas* are papain-like enzymes and therefore could have their activity similarly modulated by the peptide we report here and thus, increased the programmed cell death at the final stage of maturation of the seeds. The enhancer activity however, reserved to the cyclic form (Cp), should be supported, as least in part, by the higher resistance to enzymatic hydrolysis in a medium highly hydrolytic. Even, in linear sequences ending points are suitable sites of recognition for degradation that does not occur in closed sequences [21,28]. Therefore our proposal remains to be probed. This research is especially relevant due to its application in detecting and discovery of polypeptides with unknown functions and properties such as revealed by the mentioned cyclic peptide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2015.05.002

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