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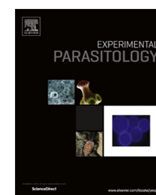
Experimental Parasitology 135 (2013) 116–125

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

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



Activity of recombinant and natural defensins from *Vigna unguiculata* seeds against *Leishmania amazonensis*

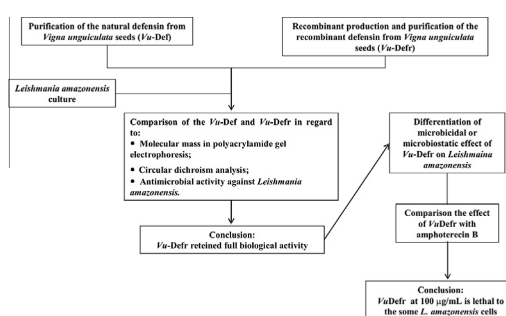
Géssika Silva Souza [a,1], Viviane Veiga do Nascimento [a,1], Laís Pessanha de Carvalho [b], Edésio José Tenório de Melo [b], Keysson Vieira Fernandes [c], Olga Lima Tavares Machado [c], Claudio Andres Retamal [b], Valdirene Moreira Gomes [a], André de Oliveira Carvalho [a,*]

[a] Laboratório de Fisiologia e Bioquímica de Microrganismos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes-RJ 28013-600, Brazil
[b] Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes-RJ 28013-600, Brazil
[c] Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes-RJ 28013-600, Brazil

HIGHLIGHTS

- The best conditions to improve the overexpression of *Vu*-Defr as Terrific Broth medium.
- The CD spectra of *Vu*-Defr indicating that the recombinant peptide was correctly folded.
- *Vu*-Defr eliminated 54.3% and 46.9% of the *Leishmania amazonensis* growth at 24 and 48 h, respectively.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 30 November 2012
Received in revised form 27 May 2013
Accepted 12 June 2013
Available online 28 June 2013

Keywords:
Antimicrobial peptides
Neglected disease
Human pathogen
Recombinant expression
New antiparasitic substances

ABSTRACT

Antimicrobial peptides (AMPs), which are differentiated from other antibiotic peptides, such as gramicidins and polymyxins, because they are synthesized by large enzymatic complex and bear modified amino acids including D-amino acids, are short polymers of L-amino acids synthesized by ribosomes upon which all living organisms rely to defend themselves from invaders or competitor microorganisms. AMPs have received a great deal of attention from the scientific community as potential new drugs for neglected diseases such as Leishmaniasis. In plants, they include several families of compounds, including the plant defensins. The aim of the present study was to improve the expression of recombinant defensin from *Vigna unguiculata* seeds (*Vu*-Defr) and to test its activity against *Leishmania amazonensis* promatigotes. Recombinant expression was performed in LB and TB media and under different conditions. The purification of *Vu*-Defr was achieved by immobilized metal ion affinity and reversed-phase chromatography. The purified *Vu*-Defr was analyzed by circular dichroism (CD), and its biological activity was tested against *L. amazonenis* promastigotes. To demonstrate that the recombinant production of *Vu*-Defr did not interfere with its fold and biological activity, the results of all experiments were compared with the results from the natural defensin (*Vu*-Def). The CD spectra of both peptides presented good superimposition indicating that both peptides present very similar secondary structure and that the *Vu*-Defr was correctly folded. *L. amazonensis* treated with *Vu*-Defr led to the elimination of 54.3% and 46.9% of the

Abbreviations: AMPs, antimicrobial peptides; *Vu*-Defr, recombinant *Vigna unguiculata* defensin; *Vu*-Def, natural *Vigna unguiculata* defensin; LIC, ligation-independent cloning; EK, enterokinase; Trx, thioredoxin tag; His, histidine tag; CD, circular dichroism; TB, Terrific Broth; LB, Luria–Bertani.

* Corresponding author. Address: Av. Alberto Lamego, 2000 Laboratório de Fisiologia e Bioquímica de Microrganismos/Centro de Biociências e Biotecnologia/Universidade Estadual do Norte Fluminense-Darcy Ribeiro, Parque Califórnia, Campos dos Goytacazes-RJ CEP 28013 600, Brazil. Fax: +55 22 2726 1520.
E-mail address: andre@uenf.br (A.O. Carvalho).

[1] These authors contributed equally to this work.

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http://dx.doi.org/10.1016/j.exppara.2013.06.005

parasites at 24 and 48 h of incubation time, respectively. *Vu*-Def eliminated 50% and 54.8% of the parasites at 24 and 48 h, respectively. Both were used at a concentration of 100 μg/mL. These results suggested the potential for plant defensins to be used as new antiparasitic substances.

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1. Introduction

Antimicrobial peptides (AMPs), are differentiated from other antibiotic peptides, such as gramicidins and polymyxins, which are synthesized by large enzymatic complex and bear modified amino acids including D-amino acids (Sieber and Marahiel, 2005; Choi et al., 2009), are short polymers of L-amino acids synthesized by ribosomes upon which all living organisms rely to defend themselves from invaders or competitor microorganisms (Broekaert et al., 1997; Bulet et al., 1999; Gao et al., 2009; Zasloff, 2002). In complex organisms, these components are part of the innate immune system, which presents broad spectra of inhibitory activity against Gram-negative and -positive bacteria, fungi, other parasites, and even enveloped viruses such as human immunodeficiency virus and herpes simplex virus (Hancock and Diamond, 2000).

Of all the plant AMPs, the family of plant defensins, which belongs to a large superfamily of AMPs named defensins, stands out. These defensins are found in fungi (Mygind et al., 2005), mollusks (Charlet et al., 1996), insects (Bulet et al., 1999), birds and mammals (Ganz, 2004; van Dijk et al., 2008; Tomasinsig and Zanetti, 2005). In plants they are composed of 45–54 amino acid residues including eight strictly conserved Cys residues, are approximately 6 kDa and have a basic pI (Carvalho and Gomes, 2011). The three-dimensional structure consists of three anti-parallel β-strands and one α-helix and is stabilized by four intramolecular disulfide bonds formed between the four pairs of Cys residues (Almeida et al., 2002; Fant et al., 1998, 1999; Janssen et al., 2003; Lay et al., 2003; Liu et al., 2006). These peptides also present two structural motifs called the Cys-stabilized α-helix β-strand motif (Cornet et al., 1995) and the γ-core motif (Yount and Yeaman, 2004). The latter is shared with many other AMPs. Plant defensins are endowed with multiple biological activities, such as the ability to halt protein translation in a cell-free system (Mendez et al., 1990, 1996), inhibit α-amylase activity (Bloch and Richardson, 1991; Mendez et al., 1996; Santos et al., 2010; Pelegrini et al., 2008), Ca^{+2} and Na^{+} channels (Kushmerick et al., 1998; Spelbrink et al., 2004) and trypsin activity (Huang et al., 2008; Wijaya et al., 2000), regenerate dehydroascorbate to ascorbic acid in the presence of glutathione (Huang et al., 2008) and maintain plant self-incompatibility (Doughty et al., 1998; Nasrallah et al., 1991). They also play roles in the plant response to abitoic stresses (Koike et al., 2002; Mirouze et al., 2006). The most prominent activity of those cited above are the activities related to plant defense, with includes antifungal activity (Almeida et al., 2002; Games et al., 2008; Mello et al., 2011; Osborn et al., 1995; Portieles et al., 2010; Rogozhin et al., 2011; Solis et al., 2007; Tavares et al., 2008; Vijayan et al., 2008;), antibacterial activity (Chen et al., 2005; Osborn et al., 1995; Terras et al., 1992, 1993; Wong et al., 2006; van der Weerden et al., 2008), and activity against dicotyledonous obligate root plant parasites (Letousey et al., 2007). In regard to the antimicrobial activity, it is interesting that plant defensins are active against human pathogens such as *Candida* (Games et al., 2008; Tavares et al., 2008) and have more recently demonstrated activity against the *Leishmania donovani* protozoan (Berrocal-Lobo et al., 2009).

More than 350 million individuals in 88 countries are at risk of infection from parasitic diseases including Leishmaniasis. It is endemic in American, Asian and African countries and in southern European countries. There is increasing concern that currently used drugs will soon become ineffective due to parasite resistance becoming more prevalent and the fact that there is no effective vaccination against Leishmaniasis (Cobb and Denny, 2010; Renslo and McKerrow, 2006). Consequently, there is a need for new drugs to treat this neglected disease. AMPs have received a great deal of attention from the scientific community as possible new therapeutic substances to offset the rise of these pathogens (Giuliani et al., 2007; Yeung et al., 2011).

We have reported the cloning (Carvalho et al., 2006) and overexpression of the recombinant *Vigna unguiculata* defensin (*Vu*-Defr) in *Escherichia coli* (Santos et al., 2010), which was originally obtained from the seeds of this plant. In this work, we introduced some modifications to improve the expression of *Vu*-Defr and altered the purification system to improve the purification process. Additionally, as previously mentioned, despite the broad inhibitory activity of plant defensins, almost nothing is known about their activity against protozoa. For this reason, we explored *Vu*-Defr activity against the protozoan *Leishmania amazonensis*. We also compared the activity of *Vu*-Defr to the natural *V. unguiculata* defensin (*Vu*-Def) in order to demonstrate the correct expression and folding of *Vu*-Defr.

2. Materials and methods

2.1. Biological material

Cowpea (*V. unguiculata* L. Walp.) seeds were supplied by Universidade Federal do Ceará, Fortaleza, Brazil.

E. coli strain Rosetta-gami 2 (DE_3) pLysS [genotype: Δ(*ara–leu*)*7697* Δ*lacX74* Δ*phoA PvuII phoR araD139 ahpC galE galK rpsL* (DE_3) F'[*lac*$^{+}$ *lacI*^{q} *pro*] *gor522*::Tn*10 trxB* pLysSRARE23 (Cam^{R}, Str^{R}, Tet^{R})^{4})] was acquired from Novagen. Luria–Bertani (LB, 0.5% yeast extract, 1% tryptone, 1% NaCl) and Terrific Broth (TB, 2.4% yeast extract, 1.2% tryptone, 0.45% glycerol, 1.3% K_2HPO_4, 0.38% KH_2PO_4) were used as expression media, both supplemented with ampicillin (50 μg/mL) and chloramphenicol (34 μg/mL).

L. amazonensis, Josefa strain, at the promastigote stage, was supplied by Laboratório de Biologia Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, Brazil. The protozoan was cultivated in 5 mL of Warren's medium (90% Brain heart broth, Fluka, containing 10% heat-inactivated fetal bovine serum), enriched with 0.01% folic acid and 0.4% hemin at 28 °C with transfer to new medium every three days.

2.2. Purification of the natural defensin

The natural defensin from seeds of *V. unguiculata* (*Vu*-Def) was purified as previously described (Carvalho et al., 2001) with modifications (Santos et al., 2010) and is described in details as follows. Fine flour (100 g) was prepared from the seeds of *V. unguiculata* in a mill. Proteins and peptides were extracted from this flour with 500 mL extraction buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 1.5% EDTA) for 2 h at 4 °C with constant agitation. The homogenate was centrifuged at 15,000*g* and the supernatant was fractionated between 0% and 30% relative ammonium sulphate saturation by 18 h at 4 °C. After centrifugation at the same

conditions, the supernatant was fractionated between 30% and 70% relative ammonium sulphate saturation by 18 h at 4 °C. Again centrifuged at the same conditions and the precipitate was redissolved in distilled water and heated at 80 °C for 15 min. The resulting suspension was centrifuged at 8000*g* and the supernatant extensively dialyzed against distilled water for three days. The dialyzed solution was recovered by freeze drying, and named F/30–70, and then submitted to further purification by chromatographic methods. Initially a DEAE-Sepharose column (10 × 1.5 cm), equilibrated with 20 mM Tris–HCl (pH 8.0) at flow rate of 1 mL/min, was used to obtain a basic fraction, called F1, non-retained in the column and eluted in the equilibrium buffer. Bound proteins (F2) were eluted with 1 M NaCl in the same buffer. F1 fraction was pooled, concentrated by freeze drying and submitted to one cycle of gel filtration chromatography in Sephadex G-50 (column of 50 × 1.5 cm) equilibrated and developed in 20 mM Tris–HCl buffer (pH 8.0) at 0.3 mL/min. The column was calibrated with several proteins of known molecular masses (bovine serum albumin, ovalbumin, soybean trypsin inhibitor and ribonuclease). The peak containing peptides with molecular mass varying from 6 to 10 kDa was collected, concentrated by freeze drying and resuspended in 0.1% (v/v) trifluoroacetic acid (TFA) and injected onto an HPLC C2C18 reversed-phase column (μRPC C2/C18 ST 4.6/100, GE Healthcare) attached to a C8 guard column (Pelliguard, Sigma). The chromatography was developed at a flow rate of 0.5 mL/min with 100% solvent A (0.1% TFA and 2% acetonitrile) for 10 min, 0–100% solvent B (80% acetonitrile containing 0.1% TFA) over 80 min, 100% solvent B over 85 min, 100–0% solvent B over 90 min, and 100% solvent A for 5 min.

2.3. Expression of recombinant Vu-Def in E. coli

Initially the induction was performed in LB or TB media. Cells containing the *Vu*-Defr clone were grown at 37 °C in both media supplemented with antibiotics until the optical density at 600 nm reached an absorbance value between 0.5 and 1.0, at which point the cells were induced with 1 mM IPTG. After 3 h of induction at 30 °C/27 °C, cells were harvested by centrifugation (15,400*g* for 10 min at 4 °C), resuspended in resuspension buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) and immediately lysed by sonication with two bursts of 30 s in the presence of a inhibitor cocktail (Protease inhibitor cocktail for general use, Sigma) and 0.1% Triton X-100. This crude lysate was centrifuged at 15,400*g* for 5 min at 4 °C, and the supernatant protein content was quantified by the bicinchoninic acid method (Sigma) with albumin from chicken egg whites (Sigma) used as the protein standard.

Improved induction conditions were used to produce *Vu*-Defr in large cultures as follows. Singles colonies were used to inoculate a starter culture of TB medium, which was incubated at 37 °C for 16 h at 250 rpm. This culture was diluted 50-fold into fresh TB medium and incubated in the same conditions until the optical density at 600 nm reached a value between 0.5 and 1. At a density between these values, IPTG was added to the culture to a final concentration of 1 mM. The culture was maintained under induction at 27 °C for 3 h at 250 rpm. After this period, the cells were sedimented, resuspended and lysed as described above.

2.4. Gel electrophoresis

Protein analyses were performed on a 12% SDS–PAGE as described by Laemmli (1970) or tricine–SDS–PAGE as described by Schägger and von Jagow (1987). Prior to electrophoresis, samples were reduced by boiling them in sample buffer containing 125 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% saccharose, 0.25% bromophenol blue containing 5% β-mercaptoethanol for 12% SDS–PAGE and 125 mM Tris–HCl pH 8.0, 2.5% SDS, 15% saccharose and 0.25% bromophenol blue containing 5% β-mercaptoethanol for the tricine gel. Gels were stained with Coomassie Brilliant blue R-250 or silver precipitation and protein markers from 66,000 to 20,000 Da were used as standards (Sigma) for SDS–PAGE and from 19,950 to 2510 Da for tricine–SDS–PAGE (MW-SDS-17S, Sigma).

The best expression condition was evaluated after induction in the conditions described above. Aliquots of 10 μL of the supernatants of the inductions in the different conditions were loaded on gels as described above. The gels were stained with freshly prepared stain solution. The bands that corresponded to the induced recombinant defensin were submitted to densitometric analysis by with the program Gel-Perfect (Bozzo and Retamal, 1991; Retamal et al., 1999). The estimated induction of *Vu*-Defr was calculated for each condition by comparing the area of the smallest band that was considered 100%.

2.5. Obtention of the Vu-Defr

A volume of the supernatant of the crude cell lysate corresponding to 30 mg of protein was applied to a Ni^+-NTA agarose column (Qiagen) previously equilibrated with resuspension buffer without inhibitor cocktail. The resin was washed with seven volumes of the same buffer containing 55 mM imidazole and followed by seven volumes of the same buffer containing 500 mM imidazole. The non-retained and retained samples were dialyzed against distilled water and recovered by lyophilization. The retained sample and that eluted with 500 mM imidazole, which corresponded to *Vu*-Defr fused to the Trx and His tags, were treated with the endoproteinase enterokinase (EK).

The treatment with the recombinant EK (Sigma) was performed according to the instruction manual. After cleavage, the sample was reloaded on the Ni^+-NTA agarose column in the same conditions as described above to eliminate the Txr and His tags from the mature sequence of the peptide. The obtained non-retained fraction were dialyzed and lyophilized. The quality of cleavage conditions was monitored by electrophoresis.

The non-retained fraction of the second Ni^+-NTA agarose column was diluted in 0.1% (v/v) trifluoroacetic acid (TFA, Fluka) and injected onto an HPLC (Prominence, Shimadzu) C18 reversed-phase column (250 × 4.6 mm, Shimadzu) (attached to a C8 pre-column (20 × 4.6 mm, Pelliguard, Sigma–Aldrich). The solvent flow rate was 0.6 mL/min and the solvent progressed from 100% solvent A (0.1% TFA in water) for 5 min, 0–10% solvent B (100% 2-propanol (Merck) containing 0.1% TFA) for 10 min, 28% solvent B for 125 min, 65% solvent B for 1 min, returned to 0% solvent B for 1 s and then remained at 0% solvent B for 6 min. The elution of the fractions was monitored by on-line measurement of the absorbance at 220 and 280 nm.

2.6. Circular dichroism spectroscopy

Vu-Defr and *Vu*-Def, both at 15 μg/mL, were dissolved in ultrapure water (TEDIA) and transferred to a 5 mm path length quartz cuvette. Circular dichroism (CD) spectra for both peptides were separately recorded in a J815 CD spectrometer (Jasco) from the far-ultraviolet range (185–275 nm) at 20 °C. Five scans for each peptide were performed per spectrum at a speed of 10 nm/min. The plot was drawn based on an average of five scans.

2.7. Growth inhibition assay on L. amazonensis promastigotes treated with Vu-Def and Vu-Defr

An aliquot of the cell culture of *L. amazonensis* promastigotes was counted in a Neubauer chamber, and the protozoa cell number

was adjusted to 1.7×10^6/mL. *Vu*-Def and *Vu*-Defr were separately diluted in dimethyl sulfoxide (DMSO), Warren's medium and sterilized by filtration (Millex-GV 0.22 μm, Millipore). Twenty micrograms of *Vu*-Def or *Vu*-Defr, 3 μL of 100% DMSO (1.5% final concentration), 100 μL of Warren's medium containing the protozoa were added to wells of a culture plate, and the final volume was brought to 200 μL with Warren's medium. The final concentration of *Vu*-Def or *Vu*-Defr was 100 μg/mL. The culture was incubated at 28 °C for until 48 h. The protozoan cell growth was determined at 24 and 48 h by cell counting in a Neubauer chamber. Controls were performed as described by excluding *Vu*-Def or *Vu*-Defr. The assay was done in triplicate, and the results shown are the average of the cell growth with regard to time for each treatment. Calculations of the standard deviation and Tukey's test were done with Prism software (version 5.0).

2.8. Time course of growth inhibition assay of L. amazonensis promastigotes treated with Vu-Defr

Culture of *L. amazonensis* promastigotes was prepared as described in item 2.7 with cell number adjusted to 1.43×10^6 parasites/mL. *Vu*-Defr was prepared as described in item 2.7, also at the concentration of 100 μg/mL. The growth of *L. amazonensis* promastigotes were monitored in the times of 0, 8, 16, 24, 32 and 48 h by cell counting as described in item 2.7. A positive control with amphotericin B (Cristália) at 100 μg/mL, prepared as *Vu*-Defr, was included. For the 0 h counting, immediately after the parasite has been added to the medium containing the *Vu*-Defr and amphotericin B, the culture was homogeneous mixed and counted.

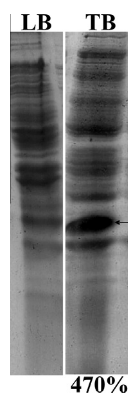

Fig. 1. Electrophoretic visualization and densiometric analysis in SDS–PAGE of the best expression conditions of *Vu*-Defr. Samples from the induced medium in LB and TB; the arrow indicates the recombinant defensin (*Vu*-Defr). Below this gel is indicated the increased percent expression of *Vu*-Defr induced in TB medium in comparison to the LB medium. Gel stained with Coomassie.

3. Results

3.1. Expression of Vu-Defr in E. coli

Previously we had expressed the *Vu*-Defr in *E. coli* at 37 °C for 3 h by induction with 1 mM IPTG. After induction the *Vu*-Defr was extracted from the bacteria by sonication pulses and the extract, after a centrifugation step, was heated to 90 °C for 30 min (Santos et al., 2010). To improve the overexpression of *Vu*-Defr, were introduced new modifications to the previously used method. The pre-inoculum was growth at 37 °C for 16 h at 250 rpm, changed the induced medium from LB medium to TB medium, decreased the temperature of the induction phase from 37 to 27 °C and after the bacterial extraction we abolished the warm step. Densitometric analysis in SDS–PAGE demonstrated that the protein band corresponding to *Vu*-Defr increased 470% in this new condition in comparison to that one used previously (Fig. 1).

The crude bacterial lysate obtained contain the *Vu*-Defr fused with thioredoxin (Trx) and histidine (His) tags and analysis in SDS–PAGE demonstrated that the recombinant *Vu*-Defr presented a molecular mass of approximately 27 kDa (Fig. 2A, white arrow). The His tag allow the interaction of the recombinant protein with metals and for this reason we submitted the crude bacterial extract to a metal affinity chromatography in a Ni^+-NTA agarose. The column was developed with three buffers, the equilibrium buffer in which the crude bacterial extract was load into the column, the equilibrium buffer containing 55 mM imidazole in which the non-retained fraction was eluted from the column and the equilibrium buffer containing 500 mM imidazole in which the retained fraction was eluted from the column. This last one corresponds to the fraction that has affinity to the Ni coupled to the column. Analysis in SDS–PAGE demonstrated that the retained fraction presented only one protein band of molecular mass of approximately 27 kDa (Fig. 2A, arrow in R). This retained fraction, which is correspond to the *Vu*-Defr fused with the thioredoxin (Trx) and histidine (His) tags, was treated with enterokinase (EK) that recognize a specific cleavage site located between the histidine (His) tag and *Vu*-Defr. After

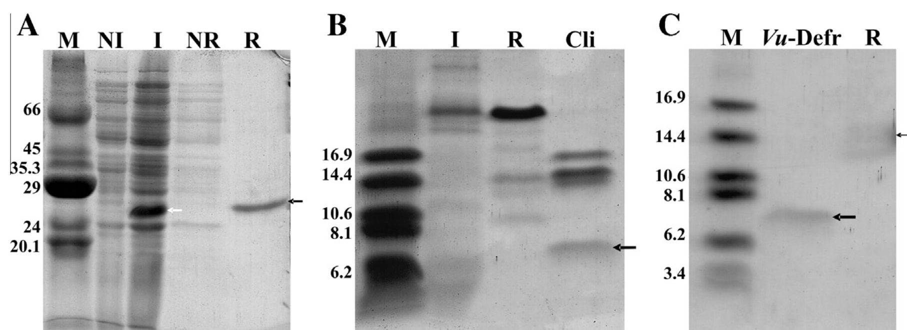

Fig. 2. Electrophoretic visualization of the different steps of the purification of the *Vu*-Defr. (A) The first step purification of *Vu*-Defr from the soluble bacterial extract after the Ni^+-NTA agarose column in SDS–PAGE. NI, total non-induced bacterial extract. I, induced total bacterial extract. The white arrow indicates *Vu*-Defr expressed after induction. NR, non-retained fraction from the Ni^+-NTA agarose column. R, retained fraction from the Ni^+-NTA agarose column and that eluted with 500 mM of imidazole. The arrow indicates *Vu*-Defr after purification. (B) Electrophoretic visualization of the cleavage of the Trx and His tags from *Vu*-Defr by tricine–SDS–PAGE. I, total bacterial extract induced (used as control). R, retained fraction in the Ni^+-NTA agarose column and that eluted with 500 mM of imidazole, used as cleavage control. The tricine–SDS-PAGE showed contaminants of low molecular mass that were not observed in the SDS–PAGE. Cli, represents the retained fraction in the Ni^+-NTA agarose column (R) treated with enterokinase, the arrow indicates *Vu*-Defr. (C) Electrophoretic visualization by tricine–SDS–PAGE of purification of *Vu*-Defr after cleavage. *Vu*-Defr, non-retained fraction in the Ni^+-NTA agarose column after cleavage, this fraction now corresponds to *Vu*-Defr (bold arrow). R, retained fraction in the Ni^+-NTA agarose, this fraction now corresponds to the Trx and His tags. M, molecular mass marker in kDa. Gels stained with Coomassie.

the treatment the *Vu*-Defr is liberated from the tags and it can be recuperated after new chromatographic round in the Ni^{+}-NTA-agarose as described in item 2.5. However this time the interested fraction was the non-retained which corresponded to the *Vu*-Defr that lost affinity to the coupled Ni^{+} of the column because it is no more fused to the His tag. The retained fraction corresponds now to the tags. Analysis in tricine–SDS–PAGE of these fractions demonstrated that after EK cleavage the band of approximately 27 kDa disappear and new bands appear, among them is one of approximately of 7 kDa which corresponds to the *Vu*-Defr (Fig. 2B, Cli). Low molecular weight contaminants are observed in the R sample before the EK treatment (Fig. 2B, R) that were not seen in the SDS–PAGE (Fig. 2A, R). The fraction (Cli) containing *Vu*-Defr separated from the tags (Trx and His) was subjected to same column and yielded *Vu*-Defr (Fig. 2C) and the tags (Trx and His), labeled R in Fig. 2C. The purification of *Vu*-Defr was completed by running it on a C18 reversed-phase column (Fig. 4). The natural *Vu*-Def, purified from extract of *V. unguiculata* seeds, is shown in Fig. 3 and it presented the same molecular mass in tricine–SDS–PAGE that *Vu*-Defr.

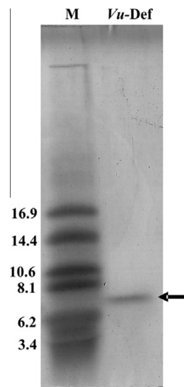

Fig. 3. Electrophoretic visualization by tricine–SDS–PAGE of purification of the natural defensin (*Vu*-Def, bold arrow) from *Vigna unguiculata* seeds. M, molecular mass marker in kDa. Gel stained with Coomassie.

3.2. Biological activity of Vu-Defr

To evaluate the functional expression of *Vu*-Defr we compared circular dichroism (CD) spectra and activity against *L. amazonesis* promastigotes with *Vu*-Def (the natural defensin purified from the *V. unguiculata* seed). The CD spectra of both peptides presented good superimposition, with a maximum at 196 nm and a mini-

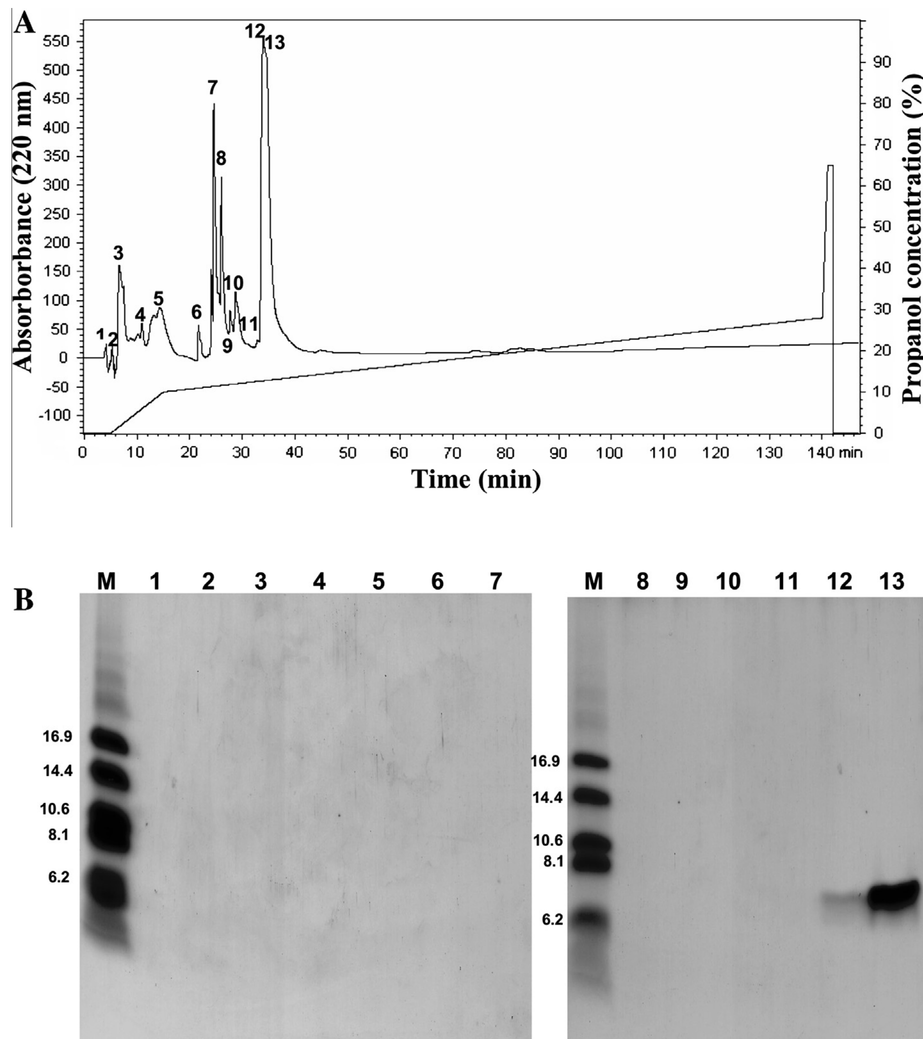

Fig. 4. Final step in purification of *Vu*-Defr. (A) Chromatogram of the non-retained fraction from the Ni^{+}-NTA agarose column after cleavage loaded onto a C18 reversed-phase column in HPLC. Thirteen peaks were obtained, one not retained (P1) and 12 (P2–P13) retained and eluted with a linear propanol gradient. (B) Electrophoretic visualization in tricine–SDS–PAGE of the 13 different peaks of the C18 column. *Vu*-Defr was obtained in the last peak. M, molecular mass marker in kDa. Gel stained with Coomassie.

mum at 217 nm, indicating that both peptides present very similar secondary structures and that the *Vu*-Defr was correctly folded during the recombinant production (Fig. 5).

The inhibition of *L. amazonensis* promastigotes growth was also evaluated with both defensins with the main intent to verify if the *Vu*-Defr after the recombinant production was as biologically active as its natural counterpart. *Vu*-Defr eliminated 54.3% and 46.9% of the protozoa growth at 24 and 48 h, respectively, and *Vu*-Def eliminated 50% and 54.8% of the protozoa growth at 24 and 48 h, respectively, both defensins at the concentration of 100 μg/mL (Fig. 6). Statistical analysis of *Vu*-Defr and *Vu*-Def elimination percentage demonstrated that they are not significantly different confirming that *Vu*-Defr retained full biological activity after the recombinant production.

The effect of *Vu*-Defr on *L. amazonensis* promastigotes cell growth has been accompanied at 0, 8, 16, 24, 32 and 48 h at 100 μg/mL. In the graphic of Fig. 7 is observed that from 0 to 8 h the number of the protozoan cells per mL drop from 1.375×10^6 to 0.975×10^6, it means that 30.1% protozoan cells were eliminated by the *Vu*-Defr. In regard to the control of 8 h, there was an elimination of 33.3%. Moving to the next time the number of *L. amanzonensis* cells per mL treated with the *Vu*-Defr remains almost constant, being 0.925×10^6, however as the control growth 21.4% in regard to the control of 8 h, and so the elimination of the protozoan by *Vu*-Defr rose to 47.9% to this time point. This reasoning can be applied to the other times. These data from 8 and 16 h means that the *L. amazonensis* cells treated with *Vu*-Defr were not growing during 16 h. At 24 h after the initial contact with the *Vu*-Defr the number of the *L. amazonensis* cells per mL (1.2×10^6) start to rise in comparison to the cell number at 0 h. This increasing in cell number per mL of the treated samples is observed from this time on. In regard to the controls of each time the growth inhibition was 45.6%, 40.0%, 41.6% for 24, 32 and 48 h, respectively. The protozoan cells treated with the reference drug, amphoterecin B, at the same concentration of the *Vu*-Defr were completed eliminated since the time of 8 h (Fig. 7).

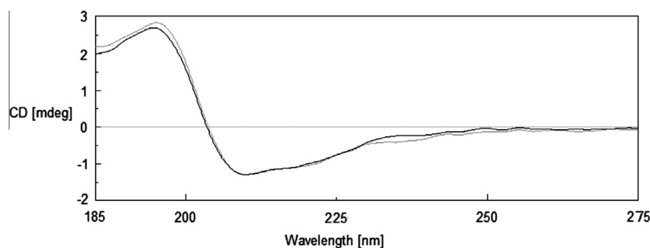

Fig. 5. Superimposition of circular dichroism spectra of the natural *Vu*-Def (black line) and *Vu*-Defr (gray line). Mdeg – millidegrees.

4. Discussion

The pervasive problem in recombinant protein expression in bacterial systems is insolubility, especially if the protein possesses Cys bonds, as the majority of AMPs do. In the prokaryotic system, the correct formation of the Cys bonds is an intrinsic problem that leads to incorrect folding and aggregation of the recombinant protein in precipitates called inclusion bodies. Misfolding also abrogates biological activity. Another problem is codon bias, which leads to low or absent recombinant protein synthesis, premature termination and misincorporation of amino acids into the recombinant protein (Gräslund et al., 2008).

To overcome the problem of disulfide bond formation, fusion proteins that are directed to the bacterial periplasmic space where they can be correctly processed with assistance from a disulfide isomerase have been used (Nakamoto and Bardwell, 2004). Another approach is based on the mutation of genes that alter the redox state of the bacterial cytoplasm and thus permit the correct formation of disulfide bonds (Derman et al., 1993). The problem of codon bias has been solved by the incorporation of a plasmid that supplies all rare codons used by eukaryotic system into the bacterial host (Baca and Hol, 2000).

To choose the best system to produce *Vu*-Defr and account for these problems, we initially scrutinized some previously published reports on the production of AMPs in both prokaryotic and eukaryotic systems (Alves et al., 1994; Chen et al., 2005; Elmorjani et al., 2004; Kristensen et al., 1999; Lullien-Pellerin et al., 1999; Song et al., 2005), and we also consulted the technical manuals of some cloning system manufacturers. From these analyses, we chose the pET32 EK/LIC vector from Novagen (see description in the materials and methods section) and the Rosetta-gami 2 DE_3 pLysS strain as the bacterial host. The combination of these two systems alleviated the aforementioned problems and generated soluble and biologically active *Vu*-Defr (Fig. 2) as compared with its natural counterpart, *Vu*-Def, obtained directly from *V. unguiculata* seeds (Fig. 3 and 4). Additionally, the CD spectra of *Vu*-Defr and Vu-Def

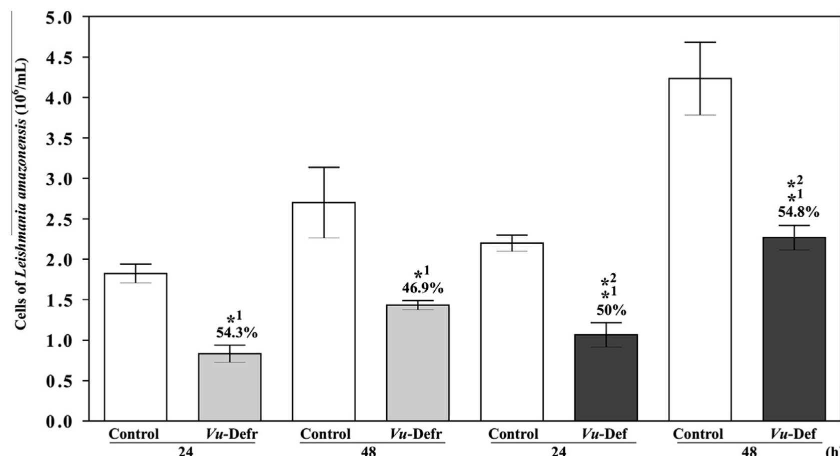

Fig. 6. Growth inhibition assay of *Leishmania amozonensis* in the absence (control) and presence of 100 μg/mL of both recombinant (*Vu*-Defr) and natural (*Vu*-Def) defensins. Growth was observed after 24 and 48 h. The percent of protozoan elimination in regard to their respective controls is shown above the gray bars. ($*^1$) indicates significance by the Tukey test ($P < 0.01$) among the experiments and their respective controls. ($*^2$) indicates no significance by the Tukey test ($P < 0.01$) between *Vu*-Defr 24 h and *Vu*-Def 24 h and between *Vu*-Defr 48 h and *Vu*-Def 48 h. Experiments were done in triplicate and the results presented are average with standard deviation.

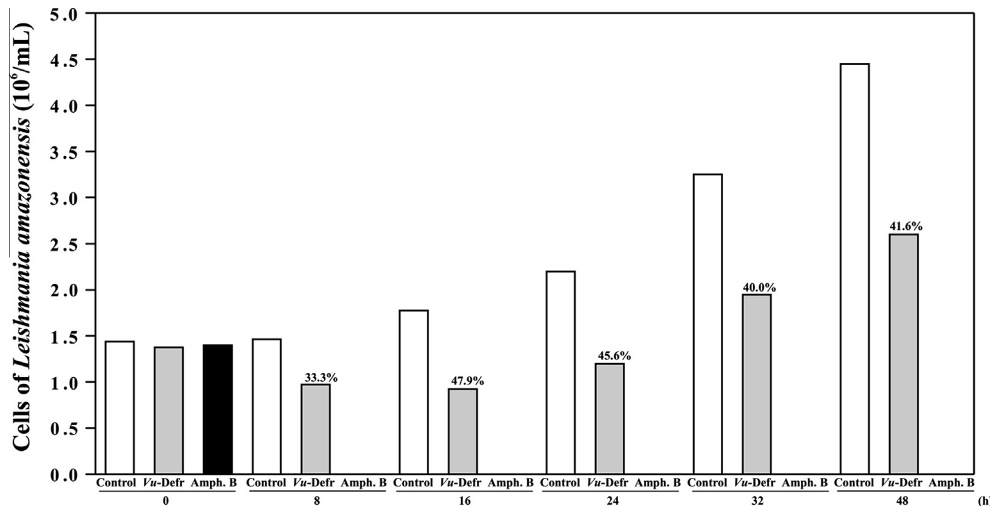

Fig. 7. Time course of growth inhibition assay of *Leishmania amozonensis* promastigotes in the absence (control) and presence of 100 μg/mL of recombinant defensin (*Vu*-Defr) and amphotericin B (AmphB). Growth was observed at 0, 8, 16, 24, 32 and 48 h. The percent of protozoan elimination in regard to their respective controls is shown above the gray bars. To the time of 0 h, immediately after the parasite has been added to the medium containing the *Vu*-Defr and amphotericin B, the culture was homogeneous mixed and counted. The protozoan cells treated with the reference drug, amphoterecin B, were completed eliminated since the time of 8 h.

were not significantly different. These spectra indicate that both peptides have the same secondary structure and that they are principally composed of β-strand secondary structure (Fig. 5), which is characteristic of plant defensins (Fant et al., 1998; Liu et al., 2006).

In comparison to *Vu*-Def, *Vu*-Defr has an additional methionine at its N-terminus. This addition is an artifact of cloning the peptide into the pET-32 EK/LIC vector. However, this addition did not interfere with the global structure of *Vu*-Defr, as demonstrated by the CD analysis (Fig. 5), or with its antimicrobial activity, as demonstrated by the antimicrobial assay (Fig. 6). Other expression systems add extra amino acids to the expressed recombinant peptides, and these extra amino acids were not reported to interfere with the biological activity of the recombinant peptide (Chen et al., 2004; de Zélicourt et al., 2007).

The plant defensin *Vu*-Defr and its natural counterpart presented activity against *L. amazonensis* promastigotes. At 100 μg/mL, *Vu*-Defr and *Vu*-Def inhibited approximately 50% of the growth of the protozoan at 48 h of incubation time (Fig. 6). The concentration of 100 μg/mL of *Vu*-Def and *Vu*-Defr was chosen taking in consideration the difficulty in obtain more quantity of *Vu*-Def at the end of the purification processes. Berrocal-Lobo et al. (2009) tested the activity of four plant AMPs against *L. donovani* promastigotes. Among them, only the thionin and the PTH1 defensin were able to inhibit this protozoan. The PTH1 defensin, similar to *Vu*-Defr and *Vu*-Def, also inhibited approximately 50% of the protozoan proliferation with incubation time of 48 h, though its concentration was 5.4 μM (approximately 27 μg/mL). Other results also indicate that, depending on the identity of the AMP and protozoan, there is variability in the concentration necessary to inhibit growth (Bera et al., 2003; Kulkarni et al., 2006). The antimicrobial activity of plant defensins is mainly directed against fungi (Almeida et al., 2002; de Beer and Vivier, 2011; Osborn et al., 1995; Rogozhin et al., 2011; Segura et al., 1998; Terras et al., 1992; Wu et al., 2011), which most likely pose a stronger pressure upon plants during evolution, thereby molding or redirecting the activity of these plant peptides against them. However, even among fungi, the activity of plant defensins is dependent on the identity of the tested fungi and the plant defensin (Almeida et al., 2002; de Beer and Vivier, 2011; Osborn et al., 1995; Rogozhin et al., 2011; Segura et al., 1998; Terras et al., 1992; Wu et al., 2011). Reported concentrations range from 0.4 μg/mL for *Rs*-AFP_2 and *Pyricularia oryzae* (Terras et al., 1992) to higher than 100 μg/mL for Psd_1 and *Fusarium oxysporum* (Almeida et al., 2002). Such variation in the concentration used to cause inhibition can be explained by the small variability in the spatial structure of plant defensins dictated by the different primary structures presented by the molecules (Carvalho and Gomes, 2011).

Leishmania have developed methods to evade the activity of AMPs. Protozoa from the kinetoplastid order produces an anionic glycocalix, known in *Leishmania* as lipophosphoglican (LPG) (Descoteaux and Turco, 1999), which is supposed to act as an anionic barrier to AMPs. Proteases (Kulkarni et al., 2006; Mottram et al., 2004) known to have broad proteolytic activity against several proteins are also produced. In regard to LPG, studies comparing the activity of indolicitin and the seminalplasmin-derived peptides SPFK and 27RP on wild type *L. donovani* and a mutant strain lacking LPG demonstrated that the mutant strain is less susceptible to these peptides (Bera et al., 2003). In regard to proteases, the AMPs α-defensin cryptidin-4, human β defensing-3, cyclic defensin theta-II, cathelicidin protegrin-1 and pexiganan (a magainin analog originally obtained from the skin of the African clawed frog) presented a stronger activity on a mutant of *Leishmania major* that lacks the major surface metalloprotease leishmanolysin (Kulkarni et al., 2006). Our results demonstrate that these protection mechanisms do not affect the activity of the defensin from *V. unguiculata* seeds.

Defensins have therapeutic potential because they are less toxic than other AMPs. Plant AMPs such as thionins are very toxic and present activity against many cell types (Florack and Stiekema, 1994). According to the Berrocal-Lobo et al. (2009) the appropriate inhibitory concentration is very similar for *L. donovani* and other organisms. This general toxicity and very similar inhibitory concentrations for pathogens and mammalian cells suggests that thionins should not be used as possible therapeutic substances. In contrast, plant defensins presented very low toxicity to mammalian cells, which is only observed at higher concentrations of plant defensins (Tavares et al., 2008; Terras et al., 1992). Additionally, *Rs*-AFP_2 has been injected intravenously into mice experimentally infected with *Candida albicans*, and diminution of the burden and low toxicity to the mice that survived was observed (Tavares et al., 2008).

The Fig. 7 demonstrated that the used concentration of 100 μg/mL of *Vu*-Defr is enough to eliminate 30.1% of the protozoan cells after 8 h of contact. The survived cells to the exposition of *Vu*-Defr

in the culture, which correspond to 69.9% of the original cell number at 0 h, start to rise again at 24 h. Plant defensins, in regard to the fungicidal and fungistatic effect, present the fungicidal effect as already demonstrated to plant defensins isolated from *Heuchera sanguinea* (coral bells, *Hs*-AMP1), *Raphanus sativus* (radish, *Rs*-AFP2) and *Dahlia merckii* (dahlia, *Dm*-AMP1) (Thevissen et al., 2007) and *Nicotiana alata* (*Na*D1) (van der Weerden et al., 2010). In the aforementioned examples the cell viability assay clearly indicated the punctual concentration of the plant defensins used are enough to kill some cells of the total population while others still stay viable. In this sense is particularly interesting the result of the method of colony forming units of the viability assay described in Thevissen et al. (2007), which demonstrated that the exposed cells to plant defensins can restart growth. The outcome of the interaction of the plant defensins with microbial cells have been described as multifaced phenomenon. The interaction can result in ion flux across and membrane depolarization (Thevissen et al., 1996), membrane permeabilization (Thevissen et al., 1999; van der Weerden et al., 2010) and induction of reactive oxygen species and oxide nitric (Aerts et al., 2007; Mello et al., 2011). It is also known that plant defensins, exemplified by *Ps*D1 (a defensin isolated from *Pisum sativum* seeds) (Lobo et al., 2007) and *Na*D1 (van der Weerden et al., 2010) enter in the cytoplasm of fungal cells. Inside the cell the peptide has the possibility to interact with intracellular targets. In the specific case of *Pv*D1, this interacts with cyclin F from *Neurospora grassa* and interferes with fugal growth and with interkinetic nuclear migration in rat retina, a model for cell cycle (Lobo et al., 2007). Although these effects have been described to fungi, they have been also demonstrated to other AMPs in regard to protozoan inhibition. Mangoni et al. (2005) have demonstrated the effect of temporins (AMP isolated from the skin secretion of the amphibian *Rana temporaria*) causes loss of viability of some cell population, membrane depolarization and membrane permeabilization in *L. donovani*. Kulkarni et al. (2009) have demonstrated the intracellular localization of pexiganan (an analog of the AMP magainin2 from skin secretion of *Xenopus laevis*) in *L. major*. As the toxic effect is concentration dependent, the fate of the cell is determined by its ability to surmount the pleotropic effects of AMPs on its physiology.

In conclusion, we demonstrated the correctly folded and biologically active *E. coli* overexpression of a plant defensin originally obtained from seeds of *V. unguiculata*, *Vu*-Defr. Additionally, we demonstrated the activity of the recombinant defensin against *L. amazonensis* promastigotes and that the effect of *Vu*-Defr on *L. amazonensis* is concentration dependent and that at 100 μg/mL effect is lethal to the some *L. amazonensis* cell population. Future studies will be done to unravel the mechanism of action of *Vu*-Defr on *L. amanzonensis*.

Acknowledgments

This project was supported by the Brazilian agencies CAPES/PNPD, CNPq, FAPERJ and Universidade Estadual do Norte Fluminense. The authors would like to thank Luis C.D. Souza for technical assistance. The authors declare no conflict of interest.

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