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Original Article

A cysteine protease from the latex of *Ficus benjamina* has *in vitro* anthelmintic activity against *Haemonchus contortus*

Atividade anti-helmíntica *in vitro* de uma protease cisteínica do látex de *Ficus benjamina* contra *Haemonchus contortus*

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Abstract

Haemonchus contortus is a gastrointestinal nematode that is responsible for high mortality rates in ruminant herds. The resistance of nematodes to synthetic anthelmintics is widespread and requires a continuous search for new bioactive molecules, such as proteins. The objective of this study was to evaluate the anthelmintic potential of a protease purified from the latex of *Ficus benjamina* against *H. contortus*. Fresh latex was collected from plants via small incisions in the green stems, the rubber was removed by centrifugation, and the latex protein extract (LPE) was obtained. After LPE fractionation with ammonium sulfate and chromatography of the fraction containing the highest proteolytic activity on CM-cellulose, a cysteine protease (FbP) was purified. FbP has a molecular mass of approximately 23.97 kDa, and its proteolytic activity was stable between pH 6.0 and pH 10 and over a broad temperature range, with optimum activity at 60 °C. FbP inhibited both the development and exsheathment of *H. contortus* larvae, with 50% effective concentrations of 0.26 and 0.79 mg/mL, respectively. We conclude that this cysteine protease from *F. benjamina* latex with anthelmintic activity against *H. contortus* could be a promising alternative for the development of products for use in parasite control programmes.

Keywords: Protease, nematode, cysteine protease, parasite control, small ruminant.

Resumo

Haemonchus contortus é um nematoide gastrintestinal, responsável por altas taxas de mortalidade em rebanhos de pequenos ruminantes. A resistência dos nematoides aos anti-helmínticos sintéticos está generalizada e requer uma busca contínua por novos compostos bioativos, como as proteínas. O objetivo deste trabalho foi avaliar o potencial anti-helmíntico da protease purificada do látex de *Ficus benjamina* contra *H. contortus*. O látex fresco foi coletado das plantas por pequenas incisões nas hastes verdes e o extrato proteico de látex (EPL) foi obtido. Após o fracionamento do EPL com sulfato de amônio e cromatografia da fração contendo a maior atividade proteolítica da CM-Celulose, uma protease cisteínica (FbP) foi purificada. A FbP tem massa molecular de cerca de 23,97 kDa, a atividade proteolítica foi estável entre pH 6,0 e pH 10 e ao longo de uma ampla faixa de temperatura, com atividade ótima a 60 °C. A FbP inibiu tanto o desenvolvimento quanto o desembainhamento das larvas de *H. contortus*, com 50% de inibição nas concentrações de 0,26 e 0,79 mg/mL, respectivamente. Concluímos que esta protease cisteínica do látex de *F. benjamina*, com ação anti-helmíntica contra *H. contortus*, pode ser uma alternativa promissora para o desenvolvimento de produtos a serem utilizados em programas de controle de parasitos.

Palavras-chave: Protease, nematoide, protease cisteínica, controle de parasitos, pequenos ruminantes.

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Introduction

Haemonchus contortus infection is one of the most important health problems that affects small ruminant productivity worldwide, especially in the tropics and sub-tropics (TAYLOR et al., 2007; LAMBERT et al., 2017). Resistance to synthetic chemical compounds has prompted the development of new treatments and alternative control strategies (PAPADOPOULOS, 2008; CRISTEL et al., 2017). As an alternative, many plant-derived natural compounds have been identified as sources of potential anthelmintic products against *H. contortus* (BEHNKE et al., 2008; SQUIRES et al., 2011; HOSTE et al., 2015; KLONGSIRIWET et al., 2015).

Proteases have been shown to be promising alternatives for nematode control (STEPEK et al., 2015). In plants, proteases are responsible for controlling various biological processes. Proteases from different plant sources present anthelmintic activity against gastrointestinal nematodes, although important aspects regarding the stability of these proteases at different pH values and temperatures have not been addressed (DOMSALLA & MELZIG, 2008; LUOGA et al., 2015).

Historically, plant latex, which is rich in proteases, was used to treat worms in humans and dogs (BEHNKE et al., 2008). However, the efficacy of this approach depends on the protease type and the nematode target (STEPEK et al., 2006; STEPEK et al., 2007a,b). The anthelmintic action of cysteine proteases has been evaluated for parasites of different regions of the digestive tract in experiments using small rodent nematode models and *Trichuris suis* in pigs (STEPEK et al., 2006; STEPEK et al., 2007a,b; BEHNKE et al., 2008; LEVECKE et al., 2014). Ficin is a protease with a molecular mass of approximately 24 kDa that is extracted from plants of the *Ficus* genus and is member of a group of enzymes that includes papain and bromelain (GAUGHRAN, 2008). *Ficus glabrata* latex was shown to exhibit anti-helminthic activity against human worms (HANSSON et al., 1986), and the protease ficin is the only active compound that has been identified in the latex.

Ficus benjamina, of the tribe Ficeae in the Moraceae family, is also known as the weeping fig tree. This species is a medicinal plant in Indonesia but is widespread worldwide due to its ornamental value as an indoor plant and its use in the urban afforestation of public spaces (HASTI et al., 2014; MORO et al., 2014). Although *F. benjamina* latex possesses high proteolytic activity, the protease remains poorly understood and has not been purified to homogeneity to date. The purification is indispensable for confidently testing the bioactive properties of a potential molecule. We have developed a protocol for purifying a protease from *F. benjamina* latex to facilitate its characterization and to assess its anthelmintic potential against the *H. contortus* contributing to an alternative, effective, and sustainable agent for the control of this pathogen.

Materials and Methods

Latex collection in the field and protein extraction

The latex of *F. benjamina* was obtained from adult plants in São Luís, Maranhão, Brazil (2°33'13" S and 44°18'20" W). The plant material was deposited in the Herbarium of Maranhão (MAR) of the Federal University of Maranhão, Brazil (registration number 1457). The latex was harvested from incisions made at the apex of the plant branches and collected in a tube containing sodium phosphate buffer (75 mM, pH 7.0) in an ice bath. To remove the rubber, the latex was initially centrifuged at 12,000 × *g* for 15 min at 4 °C. The supernatant was dialyzed against distilled water (14 kDa cut-off) for 24 h at 4 °C and subsequently centrifuged under the same conditions described above. The resulting supernatant was termed latex protein extract (LPE) and maintained at -20 °C for subsequent analysis.

Purification of the Ficus benjamina latex protease (FbP)

LPE was fractionated with ammonium sulfate via sequential precipitation at 0-30% (F0-30), 30-60% (F30-60), and 60-90% (F60-90). Briefly, in an ice bath with gentle stirring, ammonium sulfate was slowly added to LPE; the suspension was allowed to stand for 12 h and was then centrifuged at 15,000 × *g* for 30 min at 5 °C. The precipitate obtained was resuspended in sodium phosphate buffer (75 mM, pH 7.0) and dialyzed against distilled water (14 kDa cut-off) for two days with five changes per day to remove the ammonium sulfate. The resultant supernatant was used for the next step of salt precipitation.

The ammonium sulfate fraction containing the highest proteolytic activity was applied to an ion exchange column (2.0 × 13.0 cm) (CM-cellulose from GE-Healthcare) that was previously equilibrated with sodium acetate buffer (50 mM; pH 5.2) and eluted at a flow rate of 0.8 mL/min. The unadsorbed proteins (FNR) were washed out with the equilibration buffer. The bound proteins (FbP and F100) were eluted with the above buffer containing 50 mM and 100 mM NaCl, respectively. The samples were dialyzed against distilled water (14 kDa cut-off) for 24 h at 4 °C with five changes, lyophilized, and stored at 4 °C.

Proteolytic activity assay

Proteolytic activity was measured as previously described (XAVIER-FILHO et al., 1989), using azocasein as a substrate. Sample aliquots (0.2 mL) were incubated with 0.3 mL of sodium phosphate buffer (25 mM, pH 6.0), 0.002 mL of dithiothreitol (DTT, 1 M), and 0.2 mL of azocasein (1%) for 60 min at 37 °C. The reaction was stopped by the addition of 0.3 mL of trichloroacetic acid (TCA, 20% m/v). The mixture was centrifuged (10,000 × *g*, 10 min, room temperature), and 0.5 mL of the supernatant obtained was mixed with an equal volume of NaOH (2 M). The absorbance was read at 420 nm. A variation in absorbance of 0.01 was considered to be a unit of proteolytic activity and was expressed as units of activity per milligram of protein (AU/mgP). The assays were carried out in triplicate.

Protein content determination

The protein content was determined following the Bradford (1976) method, using known concentrations of bovine serum albumin (BSA) as the standard.

Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to Laemmli (1970), and protein bands were visualized with silver nitrate (GROMOVA & CELIS, 2006). In every run, protein molecular mass markers [phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20.1 kDa)] were used.

Determination of the optimum pH and temperature for FbP activity

The optimum pH was determined at 37 °C by measuring the proteolytic activity of FbP at pH values ranging from 6.0 to 12.0 (azocasein is insoluble at pH values below 5.0). The following buffer solutions were used at 50 mM concentrations: sodium phosphate (pH 6.0); Tris-HCl (pH values 8.0 and 10); and glycine-NaOH (pH 12) (POST et al., 2012; COÊLHO et al., 2016). The optimum temperature for FbP activity was determined in a temperature range from 10 to 80 °C under standard assay conditions at pH 6.0. Three different experiments were performed for both assays.

Molecular mass determination and FbP identification

The native mass of FbP was carried out according Ponce-Soto et al. (2007) with modifications. Briefly, an FbP aliquot was desalted using C4 ZipTip micro-columns (Millipore), co-crystallized with an alpha-cyano-4-hydroxycinnamic acid matrix, and analysed in a MALDI TOF/TOF 5800 mass spectrometer (AB Sciex, Canada) in positive linear mode. To identify FbP by similarity, reversed-phase nanochromatography coupled with nanoelectrospray high-resolution mass spectrometry was performed after tryptic digestion of the protein. For each sample, 4 µL of desalted tryptic peptide digest was initially applied to a 2-cm-long (100-µm internal diameter) trap column packed with 3 µm of 200 A Magic C18 AQ matrix (Michrom Bioresources, USA), followed by separation on a 20-cm-long (75-µm internal diameter) separation column that was packed with the same matrix directly on an empty self-pack 15-µm PicoFrit column (New Objective, USA). Chromatography was carried out on an EASY-nLC II instrument (Thermo Scientific, USA). The samples were loaded onto the trap column at 2000 nL/min, and chromatographic separation occurred at 200 nL/min. Mobile phase A consisted of 0.1% (v/v) formic acid in water. Mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. The gradient conditions were as follows: 2 to 40% B for 168 min and up to 80% B for 4 min. This concentration was maintained for 2 additional min before the column was re-equilibrated. The eluted peptides were directly applied to an LTQ XL/Orbi/Trap MS (Thermo, USA) for analysis. The voltage source was set to 1.9 kV, the capillary temperature was set to 200 °C, and the tube lens voltage was set to 100 V. The full ion trap value and the MSn AGC target value were 30,000 and 10,000, respectively. The MS1 spectra were acquired on an Orbitrap analyser (300 to 1,700 *m/z*) at a 60,000 resolution (for *m/z* 445.1200). For each spectrum, the 10 most intense ions were subjected to CID fragmentation (minimum signal required of 10,000; isolation width of 2.5; normalized collision energy of 35.0; activation Q of 0.25 and activation time of 30 s), followed by MS2 acquisition on a linear trap analyser. The dynamic exclusion option was enabled. The parameter settings were as follows: repeat count = 1; repeat duration = 30 s; exclusion list size = 500; exclusion duration = 45 s; and exclusion mass width = 10 ppm.

The peptide mass profiles were analysed using Peaks Studio 8.0. Searches were performed using the NCBI and UniProt databases. The search parameters for monoisotopic peptide masses allowed one missed enzymatic cleavage and accepted the carbamidomethylation of cysteine residues and the oxidation of methionine as modifications.

Anthelmintic assays

Sheep were artificially infected with a monospecific population of *H. contortus*. The experiments were performed in accordance with the guidelines established by the Brazilian College of Animal Experimentation and were approved (23115018061/2011-01 protocol number) by the Ethics Committee for Animal Experimentation of the Federal University of Maranhão, Brazil. *H. contortus* eggs and larvae were collected in accordance with Coles et al. (1992) and Ueno & Gonçalves (1998), respectively.

Haemonchus contortus development assay

The *H. contortus* larval development assay was performed according to Hubert & Kerboeuf (1992). Approximately 100 eggs were placed in 24-well plates and kept in a controlled environment chamber (BOD, 27 °C, RH ≥ 80%) for 24 h to obtain the first-stage larvae (L_1). Nutritional medium (containing freeze-dried *Escherichia coli* (ATCC 9637, Sigma), yeast extract, and balanced salt solution from Earle), amphotericin B, and serially diluted protein samples were added to the wells. FbP was solubilized in sodium/potassium phosphate buffer (pH 7.2) containing 125 mM NaCl. This buffer was used as the negative control. Pre-standardized FbP concentrations (2.0, 1.0, 0.5, 0.25 and 0.125 mg/mL) were used in quadruplicate. The plates were further incubated (27 °C, RH ≥ 80%), and after 5 days, Lugol's iodine was added to each well to stop larval development. L_1 and L_3 were counted, and larval development inhibition (%) was calculated using the following equation: $L_3 \times (L_3 + L_1)^{-1} \times (100)$. Efficacy was calculated using the following formula: (the larval development of the negative control [%]) – (the larval development during FbP treatment [%]) × (negative control development)$^{-1}$ × (100).

Larval exsheathment inhibition assay

This assay was carried out according to the technique described by Bahuaud et al. (2006). Viable third-stage (L_3) *H. contortus* larvae were placed in 96-well plates containing FbP (protein concentrations of 2.0, 1.0, 0.5, 0.25 and 0.125 mg/mL) and incubated in a BOD (27 °C, RH ≥ 80%) for 3 h. The larvae were washed three times by centrifugation with distilled water (2540 × *g*, 3 min), and exsheathment was stimulated by the addition of sodium hypochlorite (2%, v/v). The exsheathment process was

interrupted every 20 min (up to 60 min) by the addition of 10 µL of Lugol's iodine. The percentages of larval exsheathment process were monitored by observation under an inverted microscope.

Statistical analysis

The experiments were performed in triplicate, and the data are expressed as the mean ± standard deviation. All data were tested by Shapiro-Wilks tests. Biochemical data were compared by one-way ANOVA test using the GraphPad Prism 6.0 at a 5% significance level. The 50% effective concentrations (EC_{50}) was calculated by probit analysis (LeOra Software Company PoloPlus 2.0) and expressed as the mean and confidence interval (95%). Values without overlapping confidence intervals were considered significantly different (RODITAKIS et al., 2005).

Results

Purification of FbP

Following harvest, the *F. benjamina* latex, which is a milky material, was processed to remove non-protein components, especially rubber. A translucent sample was obtained and denominated LPE. After LPE fractionation by ammonium sulfate, three protein fractions were obtained: F0-30; F30-60; and F60-90. F0-30 was discarded because it had a low protein recovery (data not shown), whereas F30-60 and F60-90 accounted for approximately 17% and 62% of the initial total latex protein content, respectively ($p = 0.02$) (Table 1).

Proteolytic activity was detected in all tested samples. LPE showed 43,471.1 AU/mgP (Table 1). After ammonium sulfate fractionation of LPE, the F60-90 fraction showed the highest proteolytic activity (57,857.5 ± 5,997.4 AU/mgP); therefore, this fraction was further fractionated by ion exchange chromatography on CM-cellulose. Amongst the three fractions obtained (FNR, FbP, and F100) in this step, FbP (abbreviation for *Ficus benjamina* protease), which was eluted with 50 mM NaCl, had the highest proteolytic activity (Table 1).

After SDS-PAGE, FbP appeared as the main protein band, with an estimated molecular mass of 26.472 kDa (Figure 1), which was close to the 23.972 kDa obtained by mass spectrometry analysis (data not shown). Moreover, the peptides generated by the tryptic hydrolysis of FbP showed similarity only to plant cysteine proteases (Table 2).

Table 1. Protein content and proteolytic activity of the protein samples obtained from the latex of *Ficus benjamina*.

Protein sample	Total protein (mg)	Proteolytic activity (AU / mgP)
LPE	121.6 ± 4.6^{A}	$43{,}471.1 \pm 4{,}760.2^{A,B}$
F30-60	21.2 ± 0.7^{B}	$13{,}245.9 \pm 3{,}217.0^{B}$
F60-90	$75.7 \pm 1.0^{A,B}$	$57{,}857.5 \pm 5{,}997.4^{A}$
FbP	1.2 ± 0.3^{C}	$36{,}393.8 \pm 6{,}878.3^{A,B}$

Means followed by the different letters in a column differ significantly at $p < 0.01$ by the Tukey test. p value was 0.003. LPE: latex protein extract; F30-60, F60-90%: fractions obtained by precipitation of LPE at 30-60% and 60-90% saturation with ammonium sulfate, respectively; FbP: *F. benjamina* protease obtained after chromatographic fractionation of F60-90 on CM-Cellulose, eluted with 50 mM NaCl.

Optimum pH and temperature and biological activity

The proteolytic activity of FbP was stable in the pH range between 6 and 10 (Figure 2A) after incubation at 37 °C for 60 min. Although the enzymatic activity was remarkably reduced at pH 12, it remained high (6,167 AU/mgP). Furthermore, FbP maintained high proteolytic activity in the temperature range between 40 and 70 °C, with the maximum activity reached at 60 °C (104,978 AU/mg/P) ($p \leq 0.05$) (Figure 2B).

Anthelmintic assays

FbP inhibited the larval development and larval exsheathment of *H. contortus*, with EC_{50} values of 0.22 (CI 0.212-0.228) and 0.79 (0.736-0.853) mg/mL, respectively (Figure 3).

Discussion

Plant latexes are complex mixtures of important bioactive compounds (AGRAWAL & KONNO, 2009). In this study, we report for the first time the isolation and partial characterization of a protease from *F. benjamina* latex with characteristics similar

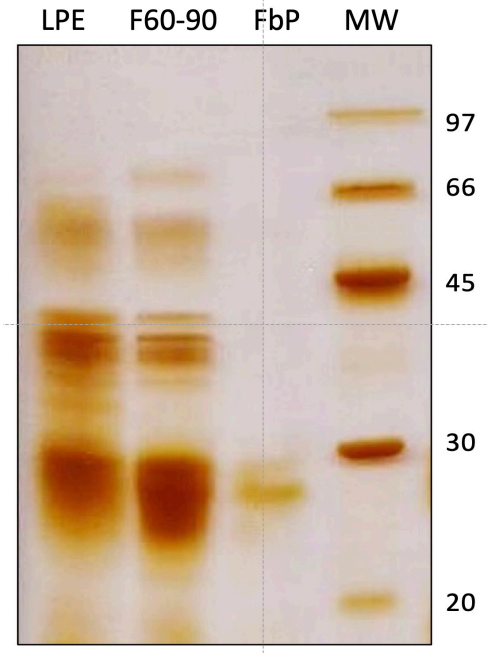

Figure 1. SDS-PAGE (12%) of the protein fractions from *Ficus benjamina* latex. LPE: latex protein extract; F60-90: fraction obtained by the precipitation of LPE at 60-90% saturation with ammonium sulfate; and FbP: *Ficus benjamina* protease obtained after the chromatographic fractionation of F60-90 on CM-cellulose, eluted with 50 mM NaCl. MW: Molecular-weight size marker.

Table 2. Similarity of FbP to other plant cysteine proteases.

Accession[a]	Coverage (%)[b]	Avg. Mass (kDa)[c]	Description
_	_	23.972	FbP - *Ficus benjamina* Protease
gi|186516984	7	41.639	Cysteine proteinase1 [*Arabidopsis thaliana*]gi|15290508|gb|AAK92229.1| cysteine proteinase [*Arabidopsis thaliana*]gi|332661313|gb|AEE86713.1| cysteine proteinase1 [*Arabidopsis thaliana*]
gi|297802228	7	41.547	Cysteine proteinase [*Arabidopsis lyrata* subsp. *lyrata*]gi|297314834|gb|EFH45257.1| cysteine proteinase [*Arabidopsis lyrata* subsp. *lyrata*]
gi|587864551	6	39.860	Germination-specific cysteine protease 1 [*Morus notabilis*]
gi|470105671	5	42.599	Predicted: cysteine proteinase RD21a-like, partial [*Fragaria vesca* subsp. *vesca*]
gi|422001787	15	25.524	Germination-specific cysteine protease 1, partial [*Raphanus sativus*]
gi|2511689	3	40.474	Cysteine proteinase precursor [*Phaseolus vulgaris*]

FbP: *F. benjamina* protease obtained after chromatographic fractionation of F60-90 on CM-Cellulose, eluted with 50 mM NaCl; [a]NCBI access code; [b]the percentage of the protein sequence covered by identified peptides; [c]Average mass in kDa (Avg. Mass).

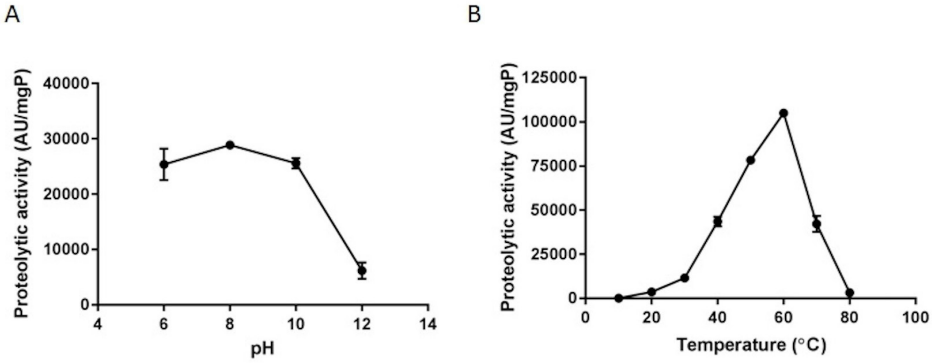

Figure 2. Determination of the optimum pH (A) and temperature (B) for FbP activity. The pH assay was performed at 37 °C using the following buffers at 50 mM concentrations: sodium phosphate (pH 6.0); Tris-HCl (pH 8.0 and 10); and glycine-NaOH (pH 12). The ideal temperature was determined by assays conducted at temperatures ranging from 10 to 80 °C at the pH of the standard test (pH 6.0).

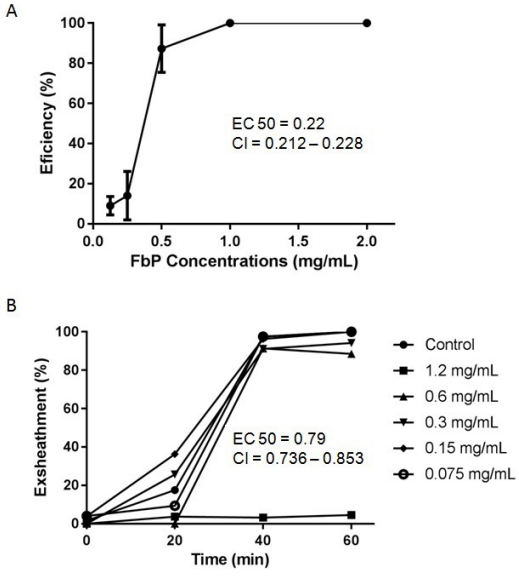

Figure 3. Efficiency (% ± standard deviation) of FbP in inhibiting the larval development (A) and exsheathment (B) of *Haemonchus contortus* after treatment with different FbP concentrations. EC_{50}: effective concentration (the dose required to achieve a 50% response); CI: confidence interval (95%). FbP: *Ficus benjamina* protease obtained after the chromatographic fractionation of F60-90 on CM-cellulose, eluted with 50 mM NaCl.

to those of ficin. Proteases have previously been purified from latexes of other species of the genus *Ficus*, such as the serine protease of *F. religiosa*, the aspartic protease of *F. racemosa*, the serine collagenolytic protease of *F. carica*, various ficins and cysteine proteases from *F. carica*, and a new ficin from *F. johannis* (DEVARAJ et al., 2008; KUMARI et al., 2010; RASKOVIC et al., 2014; BAEYENS-VOLANT et al., 2015; HOMAEI et al., 2017). Proteases are enzymes that play a variety of physiological and defence roles in plants (SCHALLER, 2004). These enzymes act as control agents for herbivorous insects (MOHAN et al., 2006) and present nematicidal potential against several parasitic nematodes of animals that inhabit different regions of the digestive tract (ROBBINS, 1930; BERGER & ASENJO, 1940; BEHNKE et al., 2008).

FbP was identified by spectrometry as a cysteine protease of 23.972 kDa. Other plant proteases have similar molecular weights, such as those purified from *F. carica* latex (24.29 kDa) (BAEYENS-VOLANT et al., 2015), *F. johannis* latex (25 kDa) (HOMAEI et al., 2017), and *Pergularia extensa* latex (23.35 kDa) (SHIVAPRASAD et al., 2010). Moreover, FbP displays protease activity at a broad range of pH values (6-10) and temperatures (Figure 2). The high activity of the cysteine protease isolated from the latex of *Ficus microcarpa* at higher temperatures was previously described. The purified enzyme showed maximum activity at 70 °C (MNIF et al., 2015). Regarding pH stability,

FbP could remain active in the abomasum of ruminants, which is the *H. contortus* habitat. The abomasum corresponds to the glandular stomach of ruminants; under normal conditions, the abomasum has an acidic pH (2-3), but *H. contortus* infection can raise the abomasal pH to values close to neutral (6-7) (NICHOLLS & LEE, 1989; BUTTLE et al., 2011), which is the optimal range of FbP performance (Figure 2A). Moreover, FbP has the potential to display anthelmintic activity against other nematodes. These characteristics should be taken into account during the development of new anthelmintic products.

Previous *in vitro* and *in vivo* experiments demonstrated the efficient action of proteases against adult gastrointestinal nematodes (BUTTLE et al., 2011; STEPEK et al., 2006; STEPEK et al., 2007a,b; LEVECKE et al., 2014). Similarly, FbP had inhibitory effects on the development and exsheathment of *H. contortus* larvae, with EC_{50} values of 0.22 and 0.79 mg/mL, respectively (Figure 3). Hydrolysis of the peptide bonds of proteins may be responsible for the anthelmintic properties of the cysteine protease FbP. This assumption is based on previous microscopic observations that cysteine proteases from several plant species act on the protective cuticle of adult nematodes, degrading this structure and rendering it weak enough to be ruptured under the internal hydrostatic pressure of the parasite (STEPEK et al., 2006; STEPEK et al., 2007a,b). For instance, the oral administration of *C. papaya* latex in pigs reduced the *T. suis* load, with an efficiency similar to those of some commercial anthelmintics (LEVECKE et al., 2014). Similar treatment of infected sheep efficiently controlled *H. contortus* (abomasum habitat) but not *Trichostrongylus colubriformis* (small intestine habitat), possibly due to the physiologic characteristics of the habitat organ and the location of this parasite, which has part of its body inserted in the mucosa. In this latter case, the enzyme must be in contact with the cuticle to exert any deleterious effects (BUTTLE et al., 2011). In the abovementioned studies, the nematicidal efficiency of the latex was attributed to the presence of cysteine proteases. Blocking the anthelmintic action of latex *in vitro* via the use of cysteine protease inhibitors reinforces the hypothesis that proteases are the active components (STEPEK et al., 2007a,b).

In conclusion, we present herein a cysteine protease that interferes with the development and exsheathment of larvae of the gastrointestinal nematode *H. contortus*. Further studies are needed to assess the mechanism of action of this protease and to develop formulations that aim to increase proteolytic activity under the pH and temperature conditions of the abomasum and to increase enzyme stability. Nevertheless, FbP is a potential alternative for the development of products for use in control programmes for *H. contortus* in place as a coadjuvant for currently used drugs.

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