EXTRACTION, PURIFICATION, PARTIAL CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF A PROTEASE INHIBITOR FROM *Albizia niopoides* SEEDS

Dávia Guimarães-Pompeu[1], Lívio Guimarães-Pompeu[1], Flávia-Cristina Policarpo-Tonelli[1], Daniel Moreira-dos-Santos[2], José-Antônio da-Silva[1] and Paulo Afonso Granjeiro[1*]

[1] Campus Centro Oeste, Universidade Federal de São João Del-Rei, Divinópolis, MG 35501296, Brazil

[2] Biochemistry, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270901, Brazil

Abstract

The trypsin serine protease inhibitor from *Albizia niopoides* seeds was purified, characterized, and had its antimicrobial activity tested against different bacteria and fungi strains. The purification was performed in three chromatographic steps. The molecular mass estimated by polyacrylamide-Tricine-SDSPAGE-gel was confirmed by mass spectrometry as 19,479.071Da. Its biochemical characterization by the inhibition curve indicated the inhibition stoichiometry as 1:1(inhibitor/Enzyme). The Ki was calculated to be 4.5×10^{-9}M. In the stability test, the inhibitor activity remained above95% at pH2.0 to 12.0. The *A.niopoides* trypsin inhibitor(AnTI) also showed high thermostability up to 60°C, with a gradual reduction in temperatures above 70ºC. In the test of resistance against DTT, this protease inhibitor showed resistance to all three of the tested concentrations of the reducing agent(1mM, 10mM and 100mM), with a slight loss of residual activity(96%) at a concentration of 100mM DTT after 60min and also at concentrations of 10mM and 100mM DTT after 120min(94% and 85%, respectively). In the isothermal titration calorimetry test, the ΔH was calculated at -4029.33Kcal/mol and ΔS at 16.20J/K, and confirmed the stoichiometric balance. The AnTI showed antimicrobial activity against *Candida tropicalis* ATCC28707 at a concentration of 200μg/mL, thus being a promising protein as an antifungal agent.

Keywords: purification, protease inhibitors, Albizia niopoides, seeds, antimicrobial activity.

1. Introduction

Protease Inhibitors are one of the most numerous types of protein present in species from the simplest living forms such as bacteria and fungi to the highest complex organisms (Machado et al., 2013). The inhibitors can be classified according to their specificity against the different mechanistic classes of proteolytic enzymes inhibited such as serine protease. The inhibitors of serine proteases have been classified into families according to their primary homology, location and number of disulfide bonds, the position of the reactive site and molecular weight (Oliva et al., 2011).

They are categorized into a number of families, including Kunitz protease inhibitor family (Chan et al, 2013). The proteins of this class of inhibitors are usually composed of two polypeptide chains, one heavy chain of about 13-16kDa and a light chain

with a molecular weight near 5-6kDa, both subunits linked by one or two disulfide bridges due to the low presence of cysteine residue disulfide bonds. In general, they have one or two reactive sites (Machado et al., 2013).

Many studies have shown a wide applicability for the proteases inhibitors, for instance antimicrobial activity (Silva et al., 2015). Researches aimed at the discovery of new antimicrobial agents of plant origin that may present promising features and serve as a source of biological models for drug development have become increasingly common, since the widespread use of the currently available antimicrobials agents in the therapy has resulted in the increased incidence of microbial infection due to the emergence of resistant strains (Ibal et al., 2013).

Albizia niopoides is a medium height tree. The species appears in areas of Hawaii and the Americas, where its presence extends from southern Mexico, throughout all of Central America towards South America from the Amazon region to the Brazilian coast. It is planted mainly in pasture and environment regeneration areas, because of its ability to fix nitrogen and to improve the ground quality (Carvalho, 2009). In the present work, the trypsin inhibitor of serine protease from *Albizia niopoides* seeds was purified, partial characterized and tested for its antimicrobial activity against different strains of bacteria and fungi.

2. Materials and Methods

The seeds of *Albizia niopoides* were collected in Corumbá Ecological Station in Arcos/MG/Brazil.

2.1 Effect of extraction media on trypsin/chymotrypsin inhibitor extraction:

Distilled water and the solutions 0.15M NaCl, 0.30M NaCl and 0.01M NaOH were used in this process. The protein content in the extract and the trypsin inhibitory activity were determined. The best extraction media determined was chosen for further extraction analyses steps.

2.2 Effect of extraction time on trypsin/chymotrypsin inhibitor extraction:

The mixtures of the seed flour with 0.3M NaCl at the ratio of 1:5 (w/v) were submitted to extraction under stirring for 1, 2, 3, 4 and 5h. After the designated time, the mixtures were then centrifuged at 10,000g for 30min. The specific trypsin inhibitory activities were obtained and the best extraction time considering the highest specific trypsin inhibitory activity was selected for further studies.

2.3 Effect of extraction temperature on trypsin/chymotrypsin inhibitor extraction:

The best temperature (25/37°C) for extraction was tested centrifuging the extracts at 10,000g for 10min at 4°C. From the supernatant the specific activity of the trypsin inhibitor was monitored and the supernatant with the highest specific activity chosen.

2.4 Purification of trypsin inhibitor:

The CE presenting the best results from the extraction was then suspended and fractionated by ammonium sulfate precipitation into three fractions(40, 60, and 80% saturation). Each fraction (precipitates and supernatants) was dialyzed and lyophilized. The fraction with the highest activity against trypsin was selected for the following purification steps. This fraction was then subjected to gel-filtration chromatography using Sephadex-G-75 resin under isocratic condition using 0.2M Ambic-buffer. The sample with inhibitory activity underwent ion exchange chromatography on DEAE-Sepharose equilibrated with 50mM Tris-HCl-buffer pH7.6 and eluted with a 50mM Tris-HCl/1M+NaCl-buffer pH7.6 gradient. Samples of the lyophilized DEAE-Sepharose product with inhibitory activity were applied to the affinity chromatography in Sepharose-4B-trypsin equilibrated with 300mM phosphate-buffer pH7.6 and eluted with 100mM HCl pH2.0. Samples of the inhibitory fraction from the affinity chromatography were suspended in 0.1%TFA and analyzed with a HPLC by reverse phase chromatography on Luna C18column using a 0.1%TFA solution and a linear gradient of acetonitrile 66.5% TFA 0.1% under constant flow of 0.5ml/min for 50min. Proteins were detected by monitoring the absorbance at 280nm.

2.5 Protein Quantification:

The total proteins were quantified by the Bradford method (1976). A standard curve was obtained by the known concentrations of BSA. Protein samples (50mL) were incubated for 20 minutes at room temperature (25°C) with 2.5ml of Bradford reagent. The protein concentrations were measured at 595nm in a spectrophotometer.

2.6 Inhibitory Activity Assays:

The samples were incubated with the enzymes trypsin and chymotrypsin for 10min at 37°C, in 50mM Tris-HCl-buffer pH7.6, before the reaction. Hereafter, 1mM BAPNA or 20mM BTPNA was added and reacted for 15min. The reaction was interrupted by the addition of 30%

International Journal of Advanced Scientific Research and Management, Vol. 1 Issue 11, Nov 2016.

www.ijasrm.com

ISSN 2455-6378

(v/v) acetic acid. The chromophore p-nitroanilide was measured at 405nm for both substrates.

2.7 Polyacrylamide Gel Electrophoresis:

In the SDS-PAGE the protease inhibitor of *A.niopoides* samples (10μg) was dissolved in Tris buffer. The electrophoresis was performed at room temperature with the cathode buffer and the anode buffer and ran for 3h at 25mA. The gel was stained with 0.25% Coomassie Blue solution R-250 in methanol, acetic acid and water and the discoloration of the gel was performed with a mixture of methanol, acetic acid and water.

2.8 Mass Spectrometry:

The *A.niopoides* inhibitor sample was mixed with the alpha-cyano-4-hydroxycinnamic acid matrix. Aliquots of 1μL of this solution (1μg/mL) were applied in the mass spectrometer Bruker Daltonics - Autoflex III SmartBeam. Operating in the positive reflector mode, the detection of ions was achieved by the associated mass espectrometric techniques matrix-assisted laser desorption/ionization and time-of-flight. External calibration was performed using bovine serum albumin.

2.9 Stability studies

In pH stability, the purified AnTI (50μg) in final concentration of 1mg/ml was incubated with buffers of pH range (2-12). Have been used as buffers 0.1M of glycine–HCl (pH 2 and 3), acetate (pH 4 and 5), phosphate (pH 6 and 7), Tris–HCl (pH 8 and 9) and glycine–NaOH (pH 10, 11 and 12). At pH 8.0 the inhibitory activity against trypsin was determined after 30min of incubation. In thermal stability studies, AnTI (50μg) in 50mM Tris–HCl buffer pH 8.0 was tested in various temperatures of incubation (20-100°C) during 30min, then kept on ice for 15min, centrifuged, supernatants taken for determination of residual inhibitory activity against trypsin and measured as described above.
To evaluate the property of reducing agents on inhibitory activity against trypsin purified AnTI (50μg) was incubated at different final concentrations of DTT (1, 10 and 100mM) using different times (15, 30, 60 and 120min) at 37°C. By adding the double of amount of iodoacetamide for each concentration of DTT the reaction can stopped and the inhibitory activity against trypsin was measured.

2.10 Isothermal Titration Calorimetry:

The solution containing the enzyme trypsin from bovine pancreas type I (2.57×10^{-5}M) was titrated against the solution of protease inhibitor of *A.niopoides* (7.37×10^{-4}M) in a VP-ITC calorimeter MicrocalTM.

2.11 Antimicrobial activity:

The minimal inhibitory concentration (MIC) methodology was performed to detect the antimicrobial activity of the *A.niopoides* trypsin inhibitor. Different concentrations of the test sample (180μl in liquid growing medium) were incubated in a microtiter plate containing the microbial inoculum (20μl). The optical density was measured at 490nm. The test was performed against the bacterial strains of *Staphylococcus aureus*-ATCC29213, *Escherichia coli*-ATCC4238 and against the fungal strains of *Candida albicans*-ATCC14053, *Candida krusei*-ATCC34135, *Candida globata*-ATCC2001, and *Candida tropicalis*-ATCC28707, using the protease inhibitor at concentrations of 100, 150 and 200μg/mL. The fungal positive control contained miconazol (500μg/ml) and the bacterial positive control contained streptomycin (900μg/ml). The negative controls were: Yeast Malt Agar for fungi and Mueller Hinton for bacteria+microorganism; the growth medium, the microorganism and 50mM Tris-HCl-buffer pH7.6.

2.12 Statistical Analysis:

The statistical analysis was performed using ANOVA and Tukey's test.

3. Results and Discussion

3.1 Extraction of the inhibitor:

To analyze the best extraction media, CE from the *A.niopoides* seeds was submited in differents conditions(Table1). The most efficient method of extraction was observed for the NaCl 0.3M with 64.9131±0.4093 of specific trypsin inhibitor activity. The best extraction time and temperature were 5h and 25°C(data no shown), respectively.

3.2 Purification of the inhibitor:

The first purification step where the crude extract of *A.niopoides* seeds was achieved involved ammonium sulfate fractioning. Among the fractions obtained, the highest activity against trypsin was seen on the

precipitate of the 80% ammonium sulfate saturation(PIII).

Table 1 A) Analysis of different extraction media for the tripsin ihibtor from *Albizia niopoides* seeds. B) The extraction time variation for the selected media with the highest trypsin inhibitory activity. C) The best extraction temperature for the selected media.

Extraction media	Trypsin inhibitor (units/g seeds)	Protein (mg/g seed)	Specific activity (units/mg protein)
Distilled water	2,3360 ± 0,005	0,0985 ± 0,005	23,6958 ± 0,0508
0.15 M NaCl	4,2733 ± 0,186	0,0658 ± 0,0039	64,9131 ± 0,0735
0.30 M NaCl	0,5935 ± 0,241	0,04230 ± 0,00172	29,9150 ± 0,0455
0.01 M NaOH	2,0732 ± 0,073	0,0693 ± 0,002	29,9150 ± 0,0455

This fraction was submitted to the gel-filtration chromatography technique with Sephadex-G-75. At this stage, the PIII fraction was fractionated into four protein peaks (Fig.1A). Only the second and third peaks showed inhibitory activity against trypsin and no peaks presented the inhibition of chymotrypsin. For this study, only the second peak was selected and subjected to subsequent separation processes due to the small amount of material that was obtained for peak3. Peak2 from gel-filtration chromatography was then subjected to anion-exchange chromatography using DEAE-Sepharose (Fig.1B). The first peak eluted presented TI activity and was selected for the next purification step, affinity chromatography on Sepharose-4B-trypsin-gel (Fig.1C). As expected, the second peak eluted in the process had inhibitory activity, indicating that this protein possesses binding properties to the target enzyme used in the process. After that, this peak was subjected to hydrophobic RP-HPLC chromatography, an important technique used for the assessment of purity and quantity of protein isoforms of interest. Resulting in two major peaks with inhibitor of protease activity. The peak one was denominated AnTI(Fig.1D). The methodology promoted a yield of 1.68% purification after all of the procedures(Table2).
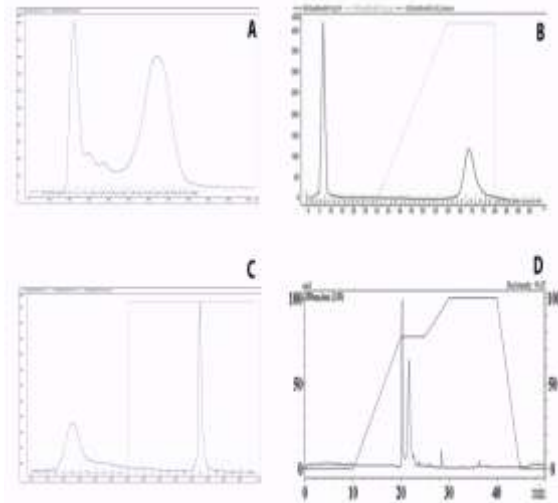

Fig. 1 A) Gel filtration chromatography of the AnTI with Superdex G-50 on FPLC system. The peak 2 has inhibitory activity. B) Ion exchange chromatography of the AnTI with DEAE-Sepharose on FPLC system. The peak 1 has inhibitory activity. C) Affinity chromatography of the AnTI with Sepharose 4B-trypsin on FPLC system. The peak 2 has inhibitory activity. D) Elution profiles for RP-HPLC analysis of the AnTI

Table 2 Purification of trypsin inhibitors from *Albizia niopoides*

Stage	Total Protein (mg)	Inhibitory activity (IU g-1)	Specific activity (IU mg-1)	Purification (x)	Yield (%)
Crude Extract	231.66	486.81	2.10	1.00	100.00
Sephadex G-75	10.08	126.10	12.52	5.96	25.90
DEAE Sepharose	0.83	77.60	93.27	44.41	15.94
4B-Sepharose-Trypsin	0.06	8.20	144.37	68.74	1.68

3.3 Polyacrylamide Gel Electrophoresis:

Fig.2A shows the test from the third step of purification by affinity chromatography. Using the relative molecular mass electrophoretic markers in the lane MW ranging from 18.4kDa to 116.0kDa, the sample showed an estimated molecular mass value of 19kDa. It can be observed that the electrophoretic profile of this last step shows high purity.

International Journal of Advanced Scientific Research and Management, Vol. 1 Issue 11, Nov 2016.
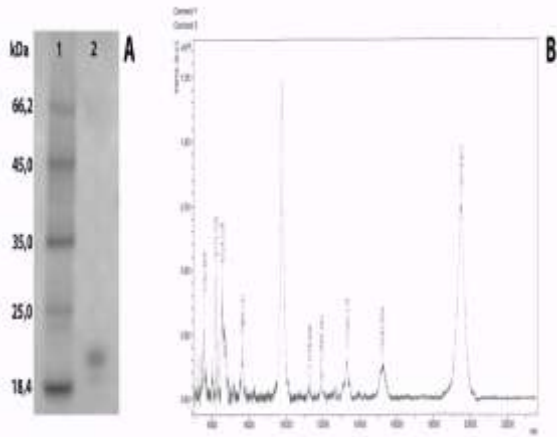
www.ijasrm.com

Fig. 2 A) Electrophoresis on Tricine-SDS-PAGE gel of AnTI from the affinity chromatography. The band had its value estimated near 19kDa. B) Mass spectrometry of AnTI. The sign of 19479.071Da is related to the inhibitor and the sign of 9768.006Da is generated by ionization of the inhibitor molecule with a mass-balance in half

3.4 Mass Spectrometry:

The analysis of AnTI by MADLI/TOF-MS revealed that the exact mass of this protein. In Fig.2B, there is a sign of 19,479.071Da, a value that is consistent with the value found in the Tricine-SDS-PAGE electrophoresis. Another important sign has a value of 9,768.006Da, which would probably be caused by ionization of the double-charged inhibitor molecule.

3.5 Inhibition curve and dissociation constant:

Extrapolating data from the inhibition curve, through the construction of a tangent to the curve, it is possible to assume that this reaction has an inhibition stoichiometry of 1:1 (inhibitor/enzyme)(Fig.3A). The dissociation constant (K_i) of 4.5×10^{-9}M for AnTI denote a strong inhibitor of trypsin. It is possible to infer that type of inhibition is competitive from the analysis of the Dixon plot(Fig.3B).
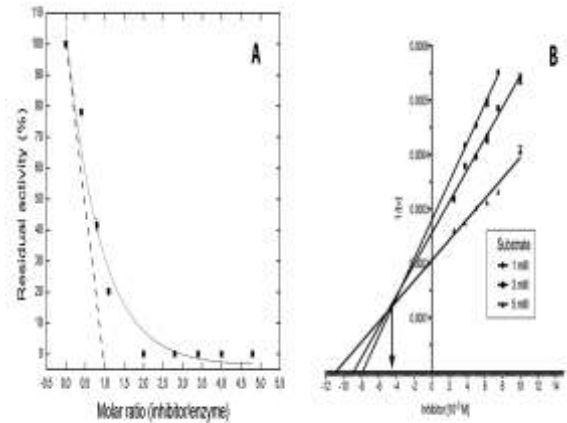

Fig. 3 A) Titration curves of AnTI against trypsin. B) Dixon plot with substrate concentrations of 1 mM, 3 mM and 5 mM. The K_i of 4.5×10^{-9}M shows high affinity of AnTI to the trypsin

3.6 Stability tests:

The inhibitory activity of the AnTI remained above 95% throughout the pH range; no statistically significant differences were observed(Fig.4A). The AnTI also demonstrated high thermal stability up to 60°C, presenting a small reduction of the inhibitory residual activity at 70°C. From 80°C, there was a sharp drop in activity (to about 10%) and the activity was practically nullified at 100°C(Fig.4B). The AnTI showed great stability when incubated with 1mM of DTT up to 120 minutes. At the concentration of 10mM DTT, however, there was a slight loss of activity to 96% after 120 minutes. When in contact with 100mM DTT, the AnTI had its activity reduced to 94% by 60 minutes and to 85% after 120min, demonstrating that the effect was time-dependent. Statistically significant differences were found(Fig.4C).
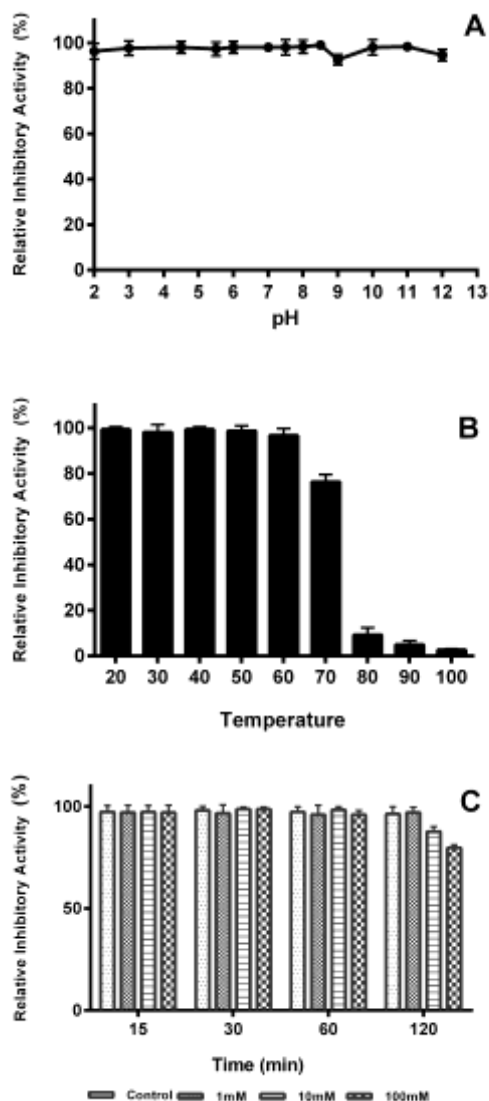
Fig. 4 A) Stability test against different pHs from 2.0 to 12.0. B) Stability test against different temperatures from 20 to 100°C. C) Stability test against different DTT concentration

3.7 Isothermal Titration Calorimetry:

The isothermal titration calorimetry generated the result shown in Fig.5A. It is possible to observe in this graphic that the initial injection promotes a calorimetric change of -0.35μcal/sec and the heat exchange was stabilized after 20min, indicating the saturation of interactions between the Inhibitor and Enzyme. The signs generated by the heat flow as a function of time in fig.5A.In fig.5B, it is possible to observe that from a molar ratio of 1.0, there is a tendency to stabilize the calorimetric change in kcal/mol injected at 1:1 stoichiometry (inhibitor/enzyme). The software has calculated the values of entropy and enthalpy of reaction. A ΔH of -

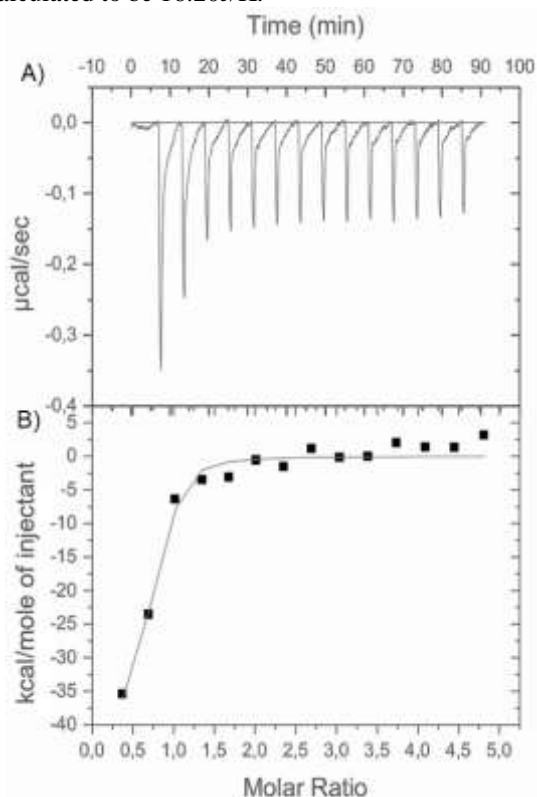4,029.33Kcal/mol was observed and the ΔS was calculated to be 16.20J/K.

Fig. 5 A) Result of isothermal titration calorimetry from the measurements of the inputs corresponding to each of the injections of the inhibitor in μcal/sec versus time in minutes. B) The integrated values analyzed by the MicroCal Origin software generated the calorimetric variation graph Kcal per mole injected versus the molar ratio (inhibitor/enzyme)

3.8 Antimicrobial activity:

The AnTI only showed antimicrobial activity against *C. tropicalis*-ATCC28707 at a concentration of 200μg/mL, as evidenced by the absence of turbidity in the medium (Table3).

Table 3 Minimum inhibitory concentration (MIC) of the AnTI against the yeast and bacteria strains

Microorganism	MIC
Candida albicans ATCC 28707	-
Candida glabrata ATCC 2001	-
Candida krusei ATCC 34135	-
Candida tropicalis ATCC 14053	+ (200μg/mL)
Escherichia coli ATCC 4238	-
Staphylococcus aureus ATCC 29213	-

In contrast to other studies which use distilled water as the extractant (Klomlao et al., 2010), 0.3M NaCl presented the highest trypsin inhibitory activity

recovery of the *A.niopoides* trypsin inhibitor after 5h of stirring at 25°C.

Overall, trypsin inhibitors have a relatively low molecular weight in comparison with other proteins in seeds. With the final yield of 1.68% for purification, it is possible to verify that this value is higher than the yield for the purification of other inhibitors described in the literature (Ee et al., 2009)

The estimated AnTI molecular weight shows consistency with the other inhibitors described in the literature. The trypsin inhibitor from *Cajanus platycarpus* (Padmasree et al, 2016) seeds demonstrated molecular weight close to 20kDa. The molar mass found for AnTI, confirmed by mass spectrometry, is very close to the value that is commonly found in other protease inhibitors, such as in the study of Bhattacharyya and Babu (2009), who isolated a serine protease inhibitor from the seeds of *Derris trifoliata* with a molecular weight of 20,172.53Da. A 1:1 molar ratio with trypsin by stoichiometric studies was agreed well with other studies (Pompeu et al., 2014). The K_i value found for AnTI shows good affinity of this inhibitor with trypsin and is consistent and has a comparable value for other trypsin inhibitors (Macedo et al., 2010).

AnTI showed good stability against variations in pH, temperature and DTT; this characteristic is commonly found in the Kunitz family (Macedo et al., 2010). The AnTI stability results suggest the presence of disulfide bonds in the tertiary structure, as highlighted in studies such as that of Tetenbaum and Miller (2001), which explained the high resistance to changes in pH and temperature based on the disulfide bonds. However, as the inhibitory activity remains, even in the presence of DTT, there is a strong indication that these stabilizing structures may be distant from the reactive site, thus maintaining its functionality as the crystallographic data obtained by the trypsin inhibitor *Erythrina caffra* in experiments conducted by Onesti and coworkers (1991).

The 1:1 stoichiometry (inhibitor/enzyme) obtained by titration is consistent with the result found in the inhibition curve against trypsin.

The antimicrobial activity against the fungal strain of *C.tropicalis*-ATCC28707 at a concentration of 200μg/mL was likely to occur since there have already been some positive tests describing the inhibition of this fungi by protease inhibitors, while the antibacterial activity of these molecules is rarely reported (Wang and Ng, 2006). In 2006, Yang and coworkers (2006) showed a trypsin inhibitor isolated from *Psoralea corylifolia* seeds with antifungal effect against many fungi. Many pathogenic fungi are known to express proteinases to extracelluar environment and responsible to play essential function in the progress of diseases. In response to the attack of microorganism proteases, plants synthesize polypeptide inhibitors that can suppress enzymatic activities (Kim et al., 2005).

4. Conclusions

The trypsin serine protease inhibitor from *Albizia niopoides* seeds was purified and characterized, and its antimicrobial activity was tested against different bacteria and fungi strains. It has shown antimicrobial activity, thus being a promising source of biological models for drugs with the development of fungicidal activity. As the need for novel molecules with such antimicrobial features, high resistance to a wide range of pH, temperature and DTT, as well as a target interaction potential, has become increasingly important due to the emergence of resistant strains, this work contributes by the identification of a new protease inhibitor source in order to provide a good option for further studies to fight the increasing incidence of microbial infection worldwide.

Acknowledgments

References

[1] Bhattacharyya A and Babu CR, Purification And Biochemical Characterization Of A Serine Proteinase Inhibitor From *Derris Trifoliate* Lour. Seeds: Insight Into Structural And Antimalarial Features. Phytochemistry, 70:703–712, 2009.

[2] Bradford MM, A Rapid And Sensitive Method For Quantitation Of Microgram Quantities Of Protein Utilizing The Principle Of Dye Binding. Anal. Biochemistry, 72:248-254, 1976.

[3] Carvalho PER, Espécies Arbóreas Brasileiras. Brasília: Embrapa Informação Tecnológica. Colombo: Embrapa Florestas, 1-8, 2009.

[4] Chan YS, Zhang Y and Ng TB, Brown Kidney Bean Bowman–Birk Trypsin Inhibitor Is Heat And pH Stable And Exhibits Anti-Proliferative Activity. Appl Biochem Biotechnol, 169:1306–1314, 2013.

[5] Ee KY et al., Purification And Characterization Of A Kunitz-Type Trypsin

Inhibitor From *Acacia Victoriae* Bentham Seeds. J Agric Food Chem, 57:7022–702, 2009.

[6] Ibal J et al, A Simple Assay To Screen Antimicrobial Compounds Potentiating The Activity Of Current Antibiotics. Biomed Research International, 1:1-4, 2013.

[7] Kim JY et al., Antimicrobial Activity Studies On A Trypsin–Chymotrypsin Protease Inhibitor Obtained From Potato. Biochem Biophys Res Commun, 330:921–927, 2005.

[8] Klomklao S et al., A Heat-Stable Trypsin Inhibitor In Adzuki Bean (Vigna Angularis): Effect Of Extraction Media, Purification And Biochemical Characteristics. International Journal Of Food Science And Technology, 45:163–169, 2010.

[9] Macedo MLR et al., A Trypsin Inhibitor From *Sapindus Saponaria* L. Seeds: Purification, Characterization, And Activity Towards Pest Insect Digestive Enzyme. The Protein Journal, 30:9–19, 2010.

[10] Machado RJA et al., Characterization And Pharmacological Properties Of A Novel Multifunctional Kunitz Inhibitor From *Erythrina Velutina* Seeds. Plos One, 8(5):E63571, 2013.

[11] Oliva MVL et al., Structural And Functional Properties Of Kunitz Proteinase Inhibitors From Leguminosae: A Mini Review. Current Protein And Peptide Science, 12:348-357, 2011.

[12] Onesti S, Brick P, Blow DM, Crystal Structure Of A Kunitz-Type Trypsin Inhibitor From *Erythrina Coffra* Seeds. Journal Of Molecular Biology, 217(1):153-176, 1991.

[13] Padmasree K et al., Purification And Partial Characterization Of Trypsin-Specific Proteinase Inhibitors From Pigeonpea Wild Relative *Cajanus Platycarpus* L. (*Fabaceae*) Active Against Gut Proteases Of Lepidopteran Pest*helicoverpa Armigera*. Frontiers In Physiology, 7 (388): 1-13, 2016.

[14] Pompeu DG et al., Anti-Nutritional Factors And *In Vitro* Digestibility Of Leaves Of *Pereskia Aculeata* Miller. Biochemistry And Biotecnology Reports, (1):1-9, 2014.

[15] Silva JA et al., Primary Structure Of A Trypsin Inhibitor (Copaifera Langsdorffii Trypsin Inhibitor-1) Obtained From C. Langsdorffii Seeds. Journal Of Biomolecular Techniques, 26: 90–102, 2015.

[16] Tetenbaum J and Miller LMA, New Spectroscopic Approach To Examining The Role Of Disulfide Bonds In The Structure And Unfolding Of Soybean Trypsin Inhibitor. Biochemistry, 40(40):12215-12219, 2001.

[17] Wang HX and Ng TB, Concurrent Isolation Of A Kunitz Type Trypsin Inhibitor With Antifungal Activity And A Novel Lectin From *Pseudostellaria Heterophylla* Roots. Biochem Biophys Res Commun, 342:349–353, 2006.

[18] Yang X et al., Psc-Afp, An Antifungal Protein With Trypsin Inhibitor Activity From *Psoralea Corylifolia* Seeds. Peptides, 27:1729-1731, 2006.