Journal of Agricultural Science; Vol. 7, No. 6; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Identification of a Novel 2S Albumin with Antitryptic Activity from *Caryocar brasiliense* Seeds

Tiago Gonçalves Costa[1], Octávio Luiz Franco[1,2], Ludovico Migliolo[1,2] & Simoni Campos Dias[1]

[1] Centro de Análises Proteômicas e Bioquímicas, Universidade Católica de Brasília, Brasília, Brazil

[2] Universidade Católica Dom Bosco, Campo Grande, Mato Grosso do Sul, Brazil

Correspondence: Simoni Campos Dias, Universidade Católica de Brasília, SGAN 916 Módulo B Avenida W5, CEP: 70790-160, Brasília, DF, Brazil. Tel: 55-61-3448-7220. E-mail: si.camposdias@gmail.com

Received: March 5, 2015 Accepted: April 10, 2015 Online Published: May 15, 2015

doi:10.5539/jas.v7n6p197 URL: http://dx.doi.org/10.5539/jas.v7n6p197

Abstract

Proteinaceous and non-proteinaceous compounds with insecticidal activities have been isolated from plant sources. Here, protein extracts from Pequi seeds (*Caryocar brasiliense*) showed *in vitro* activity against trypsin-like enzymes. However, no activity was obtained against cancer cells, phytopathogenic fungi or human pathogenic bacteria. Further purification was performed by using trypsin-Sepharose affinity chromatography. Electrophoretic analysis revealed a single protein compound with molecular mass of approximately 14 kDa. Under reducing conditions, two peptides with smaller molecular masses were observed in SDS-PAGE, showing the presence of a heterodimeric peptide. MALDI-ToF/ToF sequencing was performed with a protein band removed from SDS-PAGE, and the sequence revealed high identity with the 2S albumin family. Purified peptide was challenged *in vitro* against bovine trypsin and *Spodoptera frugiperda* digestive enzymes by using BApNA as a substrate, showing high inhibitory activity in both cases. Data here reported suggest that a 2S albumin dimeric peptide could show biotechnological potential for *S. frugiperda* control. In summary, Cb2SA could be useful in controlling insect pests of agricultural interest.

Keywords: 2S albumins, proteinase inhibitor, plant defense, *Caryocar brasiliense*

1. Introduction

The fall armyworm (*S. frugiperda*) is one of the most difficult pests to control in field corn, being efficiently controlled only when larvae are small. Although fall armyworm feeds primarily on corn, this insect pest is also capable of feeding on many crops, including alfalfa, cotton, peanuts, grasses, tobacco and several others (Oakeshott et al., 2013). On the other hand, several insecticidal compounds have been isolated from plant sources (Becker-Ritt & Carlini, 2012; Coots, Lambdin, Grant, & Rhea, 2013). Plants are able to synthesize numerous compounds that include secondary metabolites and proteinaceous compounds, and many of these compounds may be synthesized in the plant's defense system. Among such defensive proteins, some classes are notable for their activity against proteolytic enzymes, also known as proteinase inhibitors (Gomes et al., 2005). In this large group there are common proteinase inhibitor families, including the Kunitz-type inhibitors (Jongsma & Bolter, 1997; Oliva et al., 2000), the Bowman-Birk class (Odani & Ikenaka, 1973), the cyclotides MCoTI-I and MCoTI-I (Felizmenio-Quimio, Daly, & Craik, 2001), thionins and vicilins, (Macedo, Andrade, Moraes, & Xavier-Filho, 1993; Melo et al., 2002) and also 2S albumin proteins commonly found in storage seeds (Berrocal-Lobo et al., 2002).

2S albumins are water-soluble proteins widely distributed in dicot and monocot seeds, being considered one of the largest protein groups found in seed tissues, along with globulin (Berrocal-Lobo, et al., 2002). This protein was first isolated from soy (*Glicine max*) (Odani, Koide, & Ono, 1987), and it is rich in cysteine, arginine, glutamine and asparagine (Koppelman et al., 2004; Monsalve, Villalba, Rico, Shewry, & Rodríguez, 2007). Besides presenting anti-proteolytic activity (Kelly & Hefle, 2000), 2S albumins can also present deleterious activity against fungi (Pelegrini et al., 2006; Ribeiro et al., 2011) and bacteria (Maria-Neto et al., 2011). Moreover, many of these proteins show biotechnological potential and might also work as emulsifiers (Burnett et al., 2002), as allergenics (Kelly & Hefle, 2000) and, by interfering in blood calcium levels, acting as a calmodulin-antagonist (Polya, Chandra, & Condron, 1993).

In terms of structure, 2S albumins may be composed of two polypeptide chains linked by two disulfide bridges, with short-chain of 3 to 5 kDa and long-chain of about 8 to 10 kDa, both being encoded by the same gene (Koppelman et al., 2004; Pantoja-Uceda et al., 2004). Mature 2S albumin may be yielded from a post-translational process that involves a pro-peptide cleavage and formation of disulfide bonds in the vacuole (Hara-Nishimura, Shimada, Hatano, Takeuchi, & Nishimura, 1998). Studies using *Bertholletia excelsa* demonstrated the presence of at least six 2S albumin isoforms for the longer chain (8.5 kDa) (Christophe et al., 1986).

In this context, this work aims to isolate and characterize a novel anti-proteolytic 2S albumin from *C. brasiliense* seeds with biotechnological potential for the control of insect pests. This compound may be used to inhibit digestive enzyme activities in the insect pest by using transgenic plants or applying directly on crops, leading to insect death by starvation.

2. Material and Methods

2.1 Extraction and Purification Procedures

C. brasiliense seeds were acquired in a local market and were extracted. Approximately 100 g of seed flour was macerated using a blender, followed by continuous magnetic stirring with cold absolute acetone (1:10, w:v) for 2 h at 4 °C to remove the oil (Li et al., 2012). Acetone was removed and seed flour was air-dried for 2 h at room temperature. Tris-HCl 50 mM pH 7.5 was subsequently added to the flour, and this mixture was placed in a magnetic stirrer for 12 h at 4 °C, then being centrifuged for 30 min at 12,000 g at 4 °C. The supernatant was collected and nominated CE (Crude Extract). CE was submitted to protein precipitation using acetone centrifuged for 30 min at 12,000 g at 4 °C, desalted with column PD-10 G-25M Sepharose (GE Healthcare), lyophilized and re-suspended in Tris-HCl 50 mM, pH 7.5. Subsequently, the fraction collected was concentrated in speed vacuum and submitted to trypsin affinity chromatography, using the resin Sepharose 4B (SIGMA ALDRICH ®), following the manufacturer's instructions. For each 3.5 mL of resin prepared, 1 g of Sepharose 4B was used. The resin was re-suspended in 50 ml of 1 mM HCl solution, washed, and vacuum filtered, and then added to 50 mM HCl and allowed to stand for 15 min. Trypsin was prepared by re-suspending 10 $mg \cdot mL^{-1}$ of resin in 100 mM sodium bicarbonate buffer, pH 8.3, 500 mM NaCl. The trypsin solution was then added to the resin, and this was allowed to stand for 16 h at 4 °C. Excess trypsin was removed by thoroughly washing the column with five volumes of sodium bicarbonate buffer pH 8.3 100 mM NaCl 500 mM. To block the remaining active groups, buffer was added to resin 100 mM Tris-HCl pH 8.0 for 2 h and then washed with five volumes of 100 mM HCl solution, followed by another five volumes of buffer 50 mM Tris-HCl, pH 7.5. The resin was then placed in a 10 mL vessel and equilibrated using the same buffer. During the chromatography, the absorbance was measured at 280 nm. Subsequently, the retained protein fractions were collected into the 1.5 mL fraction and lyophilized for later *in vitro* assays. The Bradford method was used for protein quantification of all purification steps and assays, and bovine serum albumin (BSA) was used as protein standard (Bradford, 1976).

2.2 Polyacrylamide Gel Electrophoresis

Denaturant sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 15% as described by Laemmli (Laemmli, 1970) at room temperature and also stained with silver nitrate (Blum, Beier, & Gross, 1987).

2.3 Preparation of the Insect Gut Proteinases

S. frugiperda larvae were obtained from Centro Nacional de Recursos Genéticos e Biotecnologia (CENARGEN/EMBRAPA), Brasília, Brazil. Digestive proteinases were obtained after dissection and extraction of the guts (Terra, Ferreira, & De Bianchi, 1977). The guts were surgically removed from the organisms and placed in an iso-osmotic saline (150 mM NaCl) solution. Gut tissue was stirred and centrifuged at 10,000 *g* at 4 °C, for 10 min. The supernatants were then recovered and used for *in vitro* assays. Additionally, all insect gut homogenates were prepared in Tris-HCl 50 mM, pH 7.5. The inhibitory activity was carried out according to the section below.

2.4 Trypsin inhibitory Activity Assays

Trypsin inhibitory activity was evaluated by using N-alpha-benzoyl-DL-arginine-p-nitroanilide BapNA (Erlanger, Kokowsky, & Cohen, 1961). Aliquots of 10 µL solution of bovine trypsin (0.3 $mg \cdot mL^{-1}$ in 50 $mM \cdot L^{-1}$ Tris-HCl buffer, pH 7.5) were pre-incubated for 10 min at 37 °C with 120 µL of 2.5 $mM \cdot L^{-1}$ HCl solution and 365 µL of 50 $mM \cdot L^{-1}$ Tris-HCl buffer, pH 7.5; a volume of 5 µL containing different concentrations of fraction obtained from affinity chromatography was added (151.3, 115.7, 80.1, 62.3, 44.5, 35.6, 26.7, 17.8, 8.9, 5.34, 1.78 µg). The reaction was started by adding 500 µL of 1.25 $mM \cdot L^{-1}$ BApNA. The reaction was continued for 15 min at

37 °C and finished by adding 120 µL of 30% acetic acid (v:v). The 4-nitroaniline generation was monitored in an OD 410 nm. The values obtained allowed the IC50 (Inhibitory concentration for 50% of enzymatic activity) to be identified. Positive controls were accomplished with the same method as described above, except without the addition of substrate. All assays were performed in triplicate. The calibration curve was obtained using diverse enzyme concentrations (2.5, 5.0, 7.5, 10, 12.5, 15, 17.5 and 20 $\mu g \cdot mL^{-1}$).

2.5 Assays against Colorectal and Breast Cells

Heterogeneous human epithelial colorectal adenocarcinoma (CACO-2), human breast cancer (MCF-7) and human colorectal carcinoma (HCT-116) cells were acquired from the Cell Bank in Rio de Janeiro (CR108). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM Gibco), supplemented with 10% fetal serum bovine, penicillin (100 $U \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$), and maintained at 37 °C in CO_2 atmosphere 5% (Invitrogen, Burlington, ON, Canada). Evaluation of Cb2SA effect against the tumor cells described above was assayed with 15 $\mu g \cdot mL^{-1}$ final concentration. An MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide) cytotoxicity test was used at 1 $mg.mL^{-1}$ to analyze the cell viability after incubation with samples for periods of 24, 48 and 72 h. The cell culture assays were performed in triplicate.

2.6 Antifungal Assays

The MICs of Cb2SA were determined by using the medium microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) M27-S3 (Wikler, Cockerill, Bush, Dudley, & Eliopoulos, 2009) with RPMI 1640 medium. Stock Cb2SA solutions were dissolved in RPMI 1640 broth. The final concentrations ranged from 0.25 to 264 $\mu g \cdot mL^{-1}$. Briefly, a standard inoculum of *Fusarium oxysporum* (Identification number 1042), *Botrytis cinerea* (Identification number 358), and *Collestotrichum acutatum* (Identification number 1933), *Gilbertella persicaria* (Identification number 1145), and *Penicillium lividum* (Identification number 244) were obtained from the phytopathogenic fungi collection of the Catholic University of Brasilia. Cellular density was adjusted at 530 nm wavelength to yield a fungal stock of 1×10^6 $CFU \cdot mL^{-1}$. Further dilutions were performed with RPMI 1640 broth, resulting in a final inoculum of approximately 0.5×10^3 to 2.5×10^3 $cells \cdot mL^{-1}$. Furthermore, 100 µL of fungal suspension was incubated at 35 °C and 100 µg of the Cb2SA was placed in the wells of the microdilution tray. End points were visually read after 72 h. The MIC of Cb2SA was considered as the lowest concentration that caused complete growth inhibition (100%) when compared to control tube growth. Each antifungal test was carried out in triplicate.

2.7 Antibacterial Assays

Staphylococcus aureus ATCC 25923 and *Escherichia coli* ATCC 8739 were used for antimicrobial assays. The bacterial species were cultured in 1.0 mL LB broth for 2 h, at 37 °C, in accordance with guidelines from the CLSI, 2009. The Cb2SA was incubated with 5×10^6 $CFU \cdot mL^{-1}$ for each bacterial species for 4 h, at 37 °C. The positive and negative assay controls were several dilutions of chloramphenicol and bacteria in LB medium, respectively. Bacterial growth was evaluated at 595 nm, every hour within the incubation period, carried out according to protocols described by the National Committee for Clinical Laboratory Standards (NCLS) guidelines. All antibacterial experiments were carried out in triplicate. In addition, to determine the minimum inhibitory concentration (MIC), Cb2SA was serially diluted from 0.25 to 264 $\mu g \cdot mL^{-1}$ in LB medium. MIC was determined as the lowest concentration that produced complete growth inhibition (100%) in comparison to the negative control. In an individual well of a 96-well polypropylene plate, 100 µL of each dilution (medium + peptide) and 10 µL of cell suspension of bacteria were added (approximately 5×10^6 CFU of bacteria). The plates were kept for 12 h at 37 °C. During this period, the absorbance was read in a plate reader (Bio-Rad 680 Microplate Reader) at 595 nm every 30 min.

2.8 MALDI-ToF de novo Sequencing

The partial sequence of Cb2SA was determined by using MALDI-ToF MS/MS analysis (AutoFlex, Bruker Daltonics, Billerica, MA). The fraction corresponding to the Cb2SA in SDS-PAGE 15% (see above) was digested in gel by porcine trypsin (*Trypsin Gold Mass Spectrometry Grade* - Promega®). To the dried gel pieces, a solution of trypsin was added (0.033 $\mu g \cdot \mu L^{-1}$) in a minimal volume to cover the gel, and these suspensions were kept in an ice bath for 30 min. A 40 µL volume of ammonium carbonate (50 $mM \cdot L^{-1}$) solution was added, and the system was incubated for 19 h at 37 °C (Shevchenko, Wilm, Vorm, & Mann, 1996). For MALDI-MS analysis, α-cyano-4-hydroxycinnamic acid (CHCA) at 50 $mM \cdot L^{-1}$ in 0.3% aqueous acetonitrile was employed as a matrix. The peptides obtained by Cb2SA hydrolyses were mixed with ACHC (α-cyano-hydroxycinnamic acid) in a proportion of 1:3 (v:v) and deposited on to an AnchorChip™ target (Bruker Daltonics, Bilerica, USA) and allowed to crystallize at room temperature. The ionization was performed in the positive reflected mode. Data were recorded in the m/z range from 600 to 4,000. Peptide fragmentation was conducted by LIFT methodology

(Suckau et al., 2003). Spectrum interpretation and peptide sequencing were manually performed by using FlexAnalysis 3.4 software (Bruker Daltonics, Billerica, USA).

2.8.1 *In silico* Alignment Analyses

The peptide sequence generated was examined and compared to database protein sequences deposited in the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) using the BLASTp search tool (Altschul et al., 1997) with a set to search in a database of plants (taxid: 3193), using the program defaults. The sequences obtained were compared by using the multiple alignment program ClustalW, with matrix BLOSSUM (Thompson, Higgins, & Gibson, 1994) standard default (Weight Matrix - Blossum; Penalty for opening GAP - 10.0; penalty extension GAP - 0.2, hydrophobic residues - GPSNDQERK; percentage identity for delay - 30 and distance separation GAP - 8).

3. Results

3.1 2S Albumin Isolation

Initially, the seed proteins were extracted and submitted to G-25M column P10 desalting to minimize the presence of carbohydrates and other interfering compounds (data not shown). The protein fraction was applied onto an affinity chromatograph using a trypsin-Sepharose column. The 2S albumin bound to immobilized trypsin, being released after elution buffer application. CE was separated in non-retained and retained fractions, and the latter were eluted with 100 mM HCl (Figure 1A). These fractions were pooled and evaluated by 15% SDS-PAGE (Figure 1B). Gel was performed by using a sample buffer containing β-mercaptoethanol and another without β-mercaptoethanol. In the absence of β-mercaptoethanol a single band with 14 kDa was observed (Figure 1B, Lane 1). In contrast, β-mercaptoethanol treatment caused a dissociation of the proteinaceous compound in two smaller polypeptide chains (probably 9 and 5 kDa) (Figure 1B, Lane 2). The inhibitor was ineffective against fungi and bacteria, not demonstrating deleterious activities against microbial growth. Moreover, no activity against cancer cells was noted.

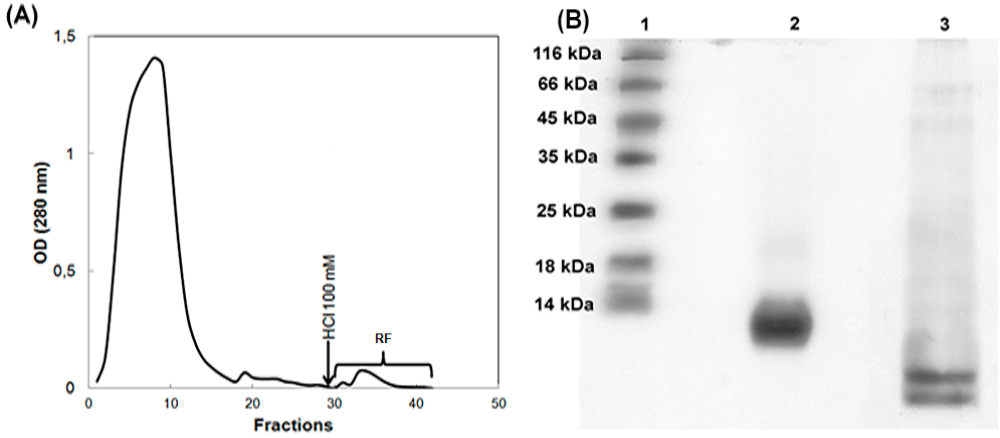

Figure 1. **(A)** Affinity chromatography of crude extract of Pequi using trypsin-sepharose resin. As a buffer, 50 mM Tris-HCl pH 7.5 was used, flow rate of 5 mL·min^{-1}. Absorbance was monitored at 280 nm. After reaching the absorbance values of 0.002, 100 mM HCl was used as eluant. RF indicates the retained fraction collected. **(B)** Gel 15% SDS-PAGE using 50 µg of the fraction retained from the trypsin-Sepharose column. (2) Fraction prepared on reducing condition, using sample buffer with β-mercaptoethanol. (3) fraction prepared on non-reducing condition, using sample buffer without β-mercaptoethanol. The gel was stained using Coomassie Blue. (1) indicates the molecular marker

3.2 de novo Sequencing by MALDI-ToF/ToF

Mass spectrometry analyses were performed by using trypsinized protein bands from SDS-PAGE 15% (Shevchenko, Tomas, Havlis, Olsen, & Mann, 2006). The spectrum analysis indicated various proteinaceous fragments obtained by tryptic digestion, and a fragment obtained after Cb2SA proteolysis was chosen for *de novo* sequencing. This fragment presented a major ion of 1395.62 Da (Figure 2) selected due to high intensity. Through FlexAnalysis v3.4 software, the fragment was partially sequenced, and the y and b series were

identified. The primary sequence obtained from Met-Ala-Glu-Asp-(Ile/Leu)-Pro-Ser from sequencing was compared with the NCBI protein database.

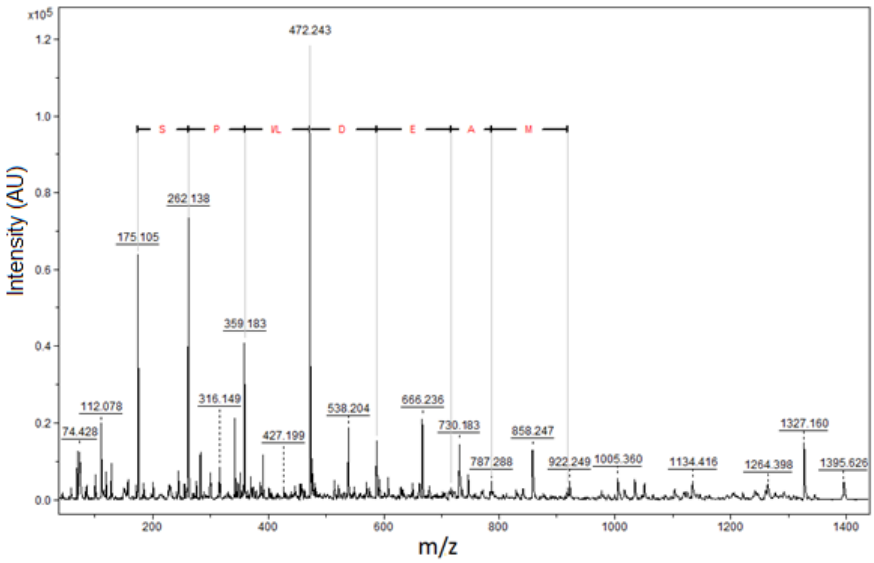

Figure 2. *De novo* sequencing of peptide was generated by mass spectrometer LIFT after SDS-Page trypsinization band. The fragment observed represents the mass 1395.62 Da and the series of *y* was determined by FlexAnalysis 3.6 version

The sequence achieved from MS showed higher identity with the peptide motif of 2S albumin proteins isolated from *B. excelsa* (ACI70206.1) (Table 1). This peptide obtained from *C. brasiliense* was named Cb2SA.

Table 1. Multiple alignment among different isoforms of 2S albumin with storage and allergenic function. Letters in red and green are considered identical and similar amino acids, respectively

Accession Number	Alignment	Species	Identity%	Function	Reference
BAA96554.1	RRMMRLAENIPSRCNLSPM	*B. excelsa*	88	Storage and allergenic	(Yamauchi, 2002)
ACI70207.1	RRMMRLAENIPSRCNLSPM	*B. excelsa*	88	Storage and allergenic	(Altenbach et al., 1992)
Cb2SA	----------MAEDLPSR----------	*C. brasiliense*	-	Storage and defense	This report
ACI70206.1	RMMMRMAENLPSRCNLSPQ	*B. excelsa*	100	Storage and allergenic	(Altenbach et al., 1992)

3.3 2S Albumin Functional Characterization

After identification, the 2S albumin-rich fraction was evaluated against fungi, bacteria, tumor cells and erythrocytes, not showing any activity (data not shown). However, purified Cb2SA was able to inhibit bovine trypsin completely at a concentration of 15 $\mu g \cdot mL^{-1}$ (Figure 2). Moreover, Cb2SA was capable of completely inhibiting digestive trypsin-like enzymes from *S. frugiperda* by using 17 $\mu g \cdot mL^{-1}$ (Figure 3). The values obtained allowed us to identify the IC50, a value of 11 $\mu g \cdot mL^{-1}$.

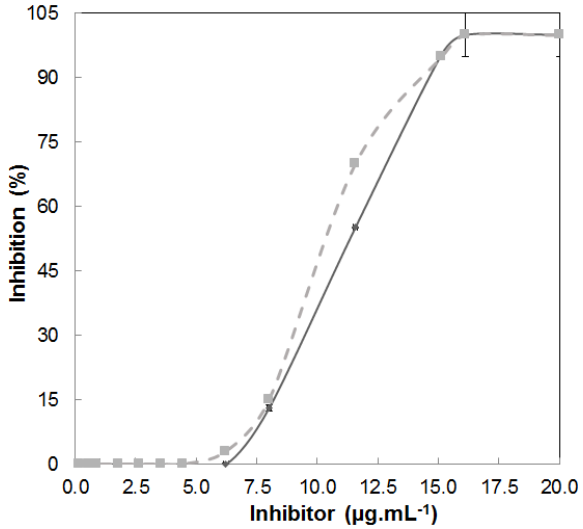

Figure 3. Inhibition curve of Cb2SA against bovine trypsin using BApNA as substrate (Continuous line) and inhibition curve of trypsin-like enzyme of *S. frugiperda* using BApNA as substrate (Dashed line). Values of 0.18, 0.53, 0.89, 1.78, 2.67, 3.56, 4.45, 6.23, 8.01, 11.57, 15.13, 16.13 and 20 µg·mL^{-1} of Cb2SA were used in both assays. The inhibition of bovine trypsin and trypsin-like enzyme of *S. frugiperda* started at 13.07 and 6.23 µg·mL^{-1} respectively. Vertical bars indicate the standard deviation

4. Discussion

Sequencing and electrophoretic analysis using sample buffer with and without β-mercaptoethanol corroborated the identification of Cb2SA as a 2S albumin. This class of protein is commonly synthesized as a single-chain precursor and undergoes the post-translational process in the vacuole. This process consists of pro-peptide cleavage, disulfide bond formation within and between chains and cleavage of the polypeptide chain, yielding two polypeptide chains linked by two disulfide bridges (Pantoja-Uceda et al., 2004). These results converge with other 2S albumin protein isolates from *Passiflora alata* (Ribeiro et al., 2011) and *Hellianthus annus* (Pantoja-Uceda et al., 2004).

Although several 2S albumins have been isolated, only a few members of this group have shown inhibitory activity toward proteolytic enzymes (Duarte, Pereira, Souza, & Conceição, 2010; Mandal, Kundu, Roy, & Mandal, 2002).

Studies using 2S albumin isolated from black beans (*Phaseolus vulgaris* L.) showed their ability to inhibit bovine trypsin (Duarte et al., 2010). Furthermore, in assays against bovine trypsin using specific synthetic substrate TAME (N-α-p-Tosyl-L-Arginine Methyl Ester), Mandal et al. (2002) determined that the activity of the precursor BjTI, isolated from *Brassica juncea*, was able to inhibit trypsin activity completely. Proportionately, the Cb2SA albumin isolated in this work was able to inhibit bovine trypsin of the same mass using 6 µg of protein, with the specific BApNA as substrate.

Cb2SA showed significant results in *in vitro* assays. The results allowed the IC50 of Cb2SA against *S. frugiperda* proteinases and bovine trypsin to be defined as 11 µg·mL^{-1} for both enzymes. These values approximate those of other proteins with anti-tryptic activity. For example, SKTI, a trypsin inhibitor belonging to the Kunitz family isolated from soybeans, showed IC50 of 8 µg·mL^{-1} against bovine trypsin (Ribeiro, Cunha, Fook, & Sales, 2010). The percentage inhibition using Cb2SA exceeds the potential of inhibiting *Cicer arietinum* trypsin inhibitor (CaTI), a trypsin inhibitor isolated from peas, which caused 73% inhibition of bovine trypsin and 78% inhibition of the digestive enzymes of *A. grandis* (Gomes et al., 2005).

S. frugiperda is responsible for severe losses in grain production. In Brazil, it is estimated that this species is responsible for losses of about 25% in corn, and millions of dollars are spent each year in chemical control (Melo et al., 2006). The results achieved in this study indicate that the possible application of Cb2SA on plantations, using transgenic plants or directly applying on crops, would result in a more efficient way to control *S. frugiperda*, requiring lower concentrations than CaTI (Gomes et al., 2005). This may lead to lower control costs, as well as minimizing any side effects from the use of chemical compounds.

The use of 2S albumins in *in vivo* insecticidal assays is not very common despite their clear and potent activities.

Currently, chemical compounds are widely used to control agricultural pests. The possibility of employing albumins in the control of such pests demands research into their use in transgenic plants, via gene expression or direct soil application, before it can become applicable in the field. Among albumins, the chain of albumin PA1 b, isolated from pea, presented *in vivo* activity in assays against *Culex pipiens* and *Aedes aegyptii* (Gressent, Da Silva, Eyraud, Karaki, & Royer, 2011). Moreover, the BjTI isolated from mustard, when expressed in the plant, improved resistance to *S. litura* (Mandal et al., 2002). The results obtained using Cb2SA against microorganisms and cancer cells did not show positive results, although other studies have indicated 2S albumins may have biotechnological potential against fungi and bacteria (Duarte et al., 2010; Maria-Neto et al., 2011; Pelegrini et al., 2006). A 2S albumin with similarity to napin isolated from *Brassica rapa* showed deleterious activity against *Bacillus cereus*, *Bacillus subtilis* and *Mycobacterium phlei* (Ngai & Ng, 2004). Another 2S albumin (To-A1) isolated from *Taraxacum officinale* showed activity against the fungus *Phytophthora infestans* (Odintsova et al., 2010). The results obtained in our study indicate a specific activity of Cb2SA to inhibit trypsin-like enzymes.

In summary, data here reported show a novel 2S albumin isolated from *C. brasiliense* with trypsin inhibitory activity against *S. frugiperda* digestive enzymes. These results represent a potential source of knowledge for future studies and alternatives in controlling insects of agricultural interest, eg. transgenic plants with resistance to pests or powerful new pesticides without adverse effects.

References

Altenbach, S. B., Kuo, C. C., Staraci, L. C., Pearson, K. W., Wainwright, C., Georgescu, A., & Townsend, J. (1992). Accumulation of a Brazil nut albumin in seeds of transgenic canola results in enhanced levels of seed protein methionine. *Plant Mol Biol, 18*(2), 235-245. http://dx.doi.org/10.1007/BF00034952

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389-3402. http://dx.doi.org/10.1093/nar/25.17.3389

Becker-Ritt, A. B., & Carlini, C. R. (2012). Fungitoxic and insecticidal plant polypeptides. *Biopolymers, 98*(4), 367-384. http://dx.doi.org/10.1002/bip.22097

Berrocal-Lobo, M., Segura, A., Moreno, M., Lopez, G., Garcia-Olmedo, F., & Molina, A. (2002). Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. *Plant Physiology, 128*(3), 951-961. http://dx.doi.org/10.1104/pp.010685

Blum, H., Beier, H., & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis, 8*(2), 93-99. http://dx.doi.org/10.1002/elps.1150080203

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248-254. http://dx.doi.org/10.1016/0003-2697(76)90527-3

Burnett, G. R., Rigby, N. M., Mills, E. N., Belton, P. S., Fido, R. J., Tatham, A. S., & Shewry, P. R. (2002). Characterization of the emulsification properties of 2S albumins from sunflower seed. *Journal of Colloid and Interface Science, 247*(1), 177-185. http://dx.doi.org/10.1006/jcis.2001.8093

Christophe, A., Jozef, D., Luiz, A. B. C., Maria José, A. M. S., Marc, M., & Joël, V. (1986). The amino-acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (*Bertholletia excelsa*). *European Journal of Biochemistry, 159*(3), 597-601. http://dx.doi.org/10.1111/j.1432-1033.1986.tb09926.x

Coots, C., Lambdin, P., Grant, J., & Rhea, R. (2013). Spatial and temporal distribution of residues of imidacloprid and its insecticidal 5-hydroxy and olefin and metabolites in eastern hemlock (Pinales: Pinaceae) in the southern appalachians. *Journal of economic entomology, 106*(6), 2399-2406. http://dx.doi.org/10.1603/ec13142

Duarte, M. S. L., Pereira, C. A. S., Souza, E. C. G., & Conceição, L. L. (2010). *Determination of the in vitro activity of trypsin inhibitors in beans (Phaseolus vulgaris L.) black, albumin and globulin* (Vol. 21). Araraquara: Alimentos e Nutrição.

Erlanger, B. F., Kokowsky, N., & Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics, 95*(2), 271-278. http://dx.doi.org/10.1016/0003-9861(61)90145-x

Felizmenio-Quimio, M. E., Daly, N. L., & Craik, D. J. (2001). Circular proteins in plants: solution structure of a novel macrocyclic trypsin inhibitor from *Momordica cochinchinensis*. *Journal of Biological Chemistry, 276*(25), 22875-22882. http://dx.doi.org/10.1074/jbc.M101666200

Gomes, A. P. G., Barbosa, A. E., Macedo, L. L., Pitanga, J. C., Moura, F. T., Oliveira, A. S., ... Sales, M. P. (2005). Effect of trypsin inhibitor from Crotalaria pallida seeds on Callosobruchus maculatus (cowpea weevil) and Ceratitis capitata (fruit fly). *Plant Physiology and Biochemistry, 43*(12), 1095-1102. http://dx.doi.org/10.1016/j.plaphy.2005.11.004

Gomes, A. P. G., Dias, S. C., Bloch, C., Jr.;, Melo, F. R., Furtado, J. R. Jr., Monnerat, R. G., ... Franco, O. L. (2005). Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 140*(2), 313-319. http://dx.doi.org/10.1016/j.cbpc.2004.10.013

Gressent, F., Da Silva, P., Eyraud, V., Karaki, L., & Royer, C. (2011). Pea Albumin 1 subunit b (PA1b), a promising bioinsecticide of plant origin. *Toxins Basel Journal, 3*(12), 1502-1517. http://dx.doi.org/10.3390/toxins3121502

Hara-Nishimura, I., Shimada, T., Hatano, K., Takeuchi, Y., & Nishimura, M. (1998). Transport of storage proteins to protein storage vacuoles is mediated by large precursor-accumulating vesicles. *Plant Cell, 10*(5), 825-836.

Jongsma, M. A., & Bolter, C. (1997). The adaptation of insects to plant protease inhibitors. *Journal of Insect Physiology, 43*(10), 885-895. http://dx.doi.org/10.1016/s0022-1910(97)00040-1

Kelly, J. D., & Hefle, S. L. (2000). 2S methionine-rich protein (SSA) from sunflower seed is an IgE-binding protein. *Allergy, 55*(6), 556-559. http://dx.doi.org/10.1034/j.1398-9995.2000.00498.x

Koppelman, S. J., Nieuwenhuizen, W. F., Gaspari, M., Knippels, L. M. J., Penninks, A. H., Knol, E. F., ... de Jongh, H. H. J. (2004). Reversible denaturation of brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin. *Journal of Agricultural and Food Chemistry, 53*(1), 123-131. http://dx.doi.org/10.1021/jf0491355

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature, 227*(5259), 680-685. http://dx.doi.org/10.1038/227680a0

Li, X., Du, Y., Wu, G., Li, Z., Li, H., & Sui, H. (2012). Solvent extraction for heavy crude oil removal from contaminated soils. *Chemosphere, 88*(2), 245-249. http://dx.doi.org/10.1016/j.chemosphere.2012.03.021

Macedo, M. L. R., Andrade, A., L. B. S., Moraes, R. A., & Xavier-Filho, J. (1993). Vicilin variants and the resistance of cowpea (*Vigna unguiculata*) seeds to the cowpea weevil (*Callosobruchus maculatus*). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 105*(1), 89-94. http://dx.doi.org/10.1016/0742-8413(93)90063-q

Mandal, S., Kundu, P., Roy, B., & Mandal, R. K. (2002). Precursor of the inactive 2S seed storage protein from the Indian mustard *Brassica juncea* is a novel trypsin inhibitor. Charaterization, post-translational processing studies, and transgenic expression to develop insect-resistant plants. *The Journal of Biological Chemistry, 277*(40), 37161-37168. http://dx.doi.org/10.1074/jbc.M205280200

Maria-Neto, S., Honorato, R. V., Costa, F. T., Almeida, R. G., Amaro, D. S., Oliveira, J. T., ... Franco, O. L. (2011). Bactericidal activity identified in 2S albumin from sesame seeds and in silico studies of structure-function relations. *The Protein Journal, 30*(5), 340-350. http://dx.doi.org/10.1007/s10930-011-9337-x

Melo, E. P. d., Fernandes, M. G., Degrande, P. E., Cessa, R. M. A., Salomão, J. L., & Nogueira, R. F. (2006). Spatial distribution of plants infested with Spodoptera frugiperda (J. E. Smith) (Lepidoptera: Noctuidae) on corn crop. *Neotropical Entomology, 35*, 689-697.

Melo, F. R., Rigden, D. J., Franco, O. L., Mello, L. V., Ary, M. B., Grossi de Sa, M. F., & Bloch, C. Jr. (2002). Inhibition of trypsin by cowpea thionin: characterization, molecular modeling, and docking. *Proteins, 48*(2), 311-319. http://dx.doi.org/10.1002/prot.10142

Monsalve, R. I., Villalba, M., Rico, M., Shewry, P. R., & Rodríguez, R. (2007). The 2S albumin proteins. *Plant Food Allergens*. Blackwell Publishing Ltd.

Ngai, P. H., & Ng, T. B. (2004). A napin-like polypeptide from dwarf Chinese white cabbage seeds with translation-inhibitory, trypsin-inhibitory, and antibacterial activities. *Peptides, 25*(2), 171-176. http://dx.doi.org/10.1016/j.peptides.2003.12.012

Oakeshott, J. G., Farnsworth, C. A., East, P. D., Scott, C., Han, Y., Wu, Y., & Russell, R. J. (2013). How many genetic options for evolving insecticide resistance in heliothine and spodopteran pests? *Pest Management*

Science, 69(8), 889-896. http://dx.doi.org/10.1002/ps.3542

Odani, S., & Ikenaka, T. (1973). Studies on Soybean Trypsin Inhibitors. *Journal of Biochemistry, 74*(4), 697-715.

Odani, S., Koide, T., & Ono, T. (1987). Amino acid sequence of a soybean (Glycine max) seed polypeptide having a poly(L-aspartic acid) structure. *The Journal of Biological Chemistry, 262*(22), 10502-10505.

Odintsova, T. I., Rogozhin, E. A., Sklyar, I. V., Musolyamov, A. K., Kudryavtsev, A. M., Pukhalsky, V. A., ... Egorov, T. A. (2010). Antifungal activity of storage 2S albumins from seeds of the invasive weed dandelion Taraxacum officinale Wigg. *Protein and Peptide Letters, 17*(4), 522-529. http://dx.doi.org/10.2174/092986610790963591

Oliva, M. L. V., Souza-Pinto, J. C., Batista, I. F. C., Araujo, M. S., Silveira, V. F., Auerswald, E. A., ... Sampaio, C. A. M. (2000). *Leucaena leucocephala* serine proteinase inhibitor: primary structure and action on blood coagulation, kinin release and rat paw edema. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1477*(1-2), 64-74. http://dx.doi.org/10.1016/s0167-4838(99)00285-x

Pantoja-Uceda, D., Palomares, O., Bruix, M., Villalba, M., Rodriguez, R., Rico, M., & Santoro, J. (2004). Solution structure and stability against digestion of rproBnIb, a recombinant 2S albumin from rapeseed: relationship to its allergenic properties. *Biochemistry, 43*(51), 16036-16045. http://dx.doi.org/10.1021/bi048069x

Pantoja-Uceda, D., Shewry, P. R., Bruix, M., Tatham, A. S., Santoro, J., & Rico, M. (2004). Solution structure of a methionine-rich 2S albumin from sunflower seeds: relationship to its allergenic and emulsifying properties. *Biochemistry, 43*(22), 6976-6986. http://dx.doi.org/10.1021/bi0496900

Pelegrini, P. B., Noronha, E. F., Muniz, M. A., Vasconcelos, I. M., Chiarello, M. D., Oliveira, J. T., & Franco, O. L. (2006). An antifungal peptide from passion fruit (*Passiflora edulis*) seeds with similarities to 2S albumin proteins. *Biochimica et Biophysica Acta, 1764*(6), 1141-1146. http://dx.doi.org/10.1016/j.bbapap.2006.04.010

Polya, G. M., Chandra, S., & Condron, R. (1993). Purification and sequencing of radish seed calmodulin antagonists phosphorylated by calcium-dependent protein kinase. *Plant Physiology, 101*(2), 545-551. http://dx.doi.org/10.1104/pp.101.2.545

Ribeiro, J. K., Cunha, D. D., Fook, J. M., & Sales, M. P. (2010). New properties of the soybean trypsin inhibitor: Inhibition of human neutrophil elastase and its effect on acute pulmonary injury. *European journal of pharmacology, 644*(1-3), 238-244. http://dx.doi.org/10.1016/j.ejphar.2010.06.067

Ribeiro, S. M., Almeida, R. G., Pereira, C. A., Moreira, J. S., Pinto, M. F., Oliveira, A. C., ... Franco, O. L. (2011). Identification of a *Passiflora alata* Curtis dimeric peptide showing identity with 2S albumins. *Peptides, 32*(5), 868-874. http://dx.doi.org/10.1016/j.peptides.2010.10.011

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., & Mann, M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature protocols, 1*(6), 2856-2860. http://dx.doi.org/10.1038/nprot.2006.468

Shevchenko, A., Wilm, M., Vorm, O., & Mann, M. (1996). Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical Chemistry, 68*(5), 850-858. http://dx.doi.org/10.1021/ac950914h

Suckau, D., Resemann, A., Schuerenberg, M., Hufnagel, P., Franzen, J., & Holle, A. (2003). A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Analytical and Bioanalytical Chemistry, 376*(7), 952-965. http://dx.doi.org/10.1007/s00216-003-2057-0

Terra, W. R., Ferreira, C., & De Bianchi, A. G. (1977). Action pattern, kinetical properties and electrophoretical studies of an alpha-amylase present in midgut homogenates from Rhynchosciara americana (Diptera) larvae. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry, 56*(2), 201-209. http://dx.doi.org/10.1016/0305-0491(77)90049-9

Wikler, M., Cockerill, F., Bush, K., Dudley, M., & Eliopoulos, G. (2009). *Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically*. Clinical and Laboratory Standards Institute.

Yamauchi, D. (2002). Brazil nut 2S albumin was synthesized in a transgenic French bean seed with a promoter of the gene for canavalin, 7S globulin from Canavalia gladiata. *Plant Biotechnology Journal, 17*, 4.