Study of the Proteolytic Activity of the Tropical Legume Crotalaria spectabilis

Juliana da Silva Pacheco and Raquel Elisa da Silva-López*

Departamento de Química de Produtos Naturais, Farmanguinhos, FIOCRUZ, Av. Brazil 4365, 21045-900, Rio de Janeiro, RJ, Brazil. Fax: +55 21 3977 2422. E-mail: rlopez@far.fiocruz.br

* Author for correspondence and reprint requests

Z. Naturforsch. 67c, 495-509 (2012); received September 20, 2011/July 24, 2012

The characterization of legume proteases contributes to the understanding of the physiology of plants and their interaction with the environment. Thirteen extracts from various parts of *Crotalaria spectabilis* were made using different extraction systems. The highest protein content was found in seeds, and the most pronounced proteolytic activity was observed in leaf extracts, with an optimal pH value in the alkaline range. Proteases in extracts from roots, stems, and flowers were active in various pH ranges. Proteases in all extracts were maximally active between 30 °C and 60 °C and were thermostable (24 h, 60 °C). Hemoglobin, bovine serum albumin, casein, and gelatin were hydrolyzed by *C. spectabilis* extracts in different ways. The highest serine protease activity was found in leaves. Seeds contained high levels of serine proteases and low levels of cysteine proteases. Flowers, roots, and stems contained different levels of serine, aspartic, and metalloproteases, respectively. The proteolytic activities in extracts were modulated by cations and oxidants to various degrees. *C. spectabilis* proteases are differentially expressed in distinctive organs, and their stability against heat and oxidants makes this plant an important source of stable proteases.

Key words: Crotalaria spectabilis, Proteolytic Activity

Introduction

Legumes of the genus *Crotalaria* contain approximately 550 species dispersed throughout the world, the great majority well adapted to the tropical climate. Only a limited number occurs in temperate regions. They are annual erect herbs or woody shrubs, originated from India, Africa, and Madagascar, and have been used in the paper and animal feed industry as a source of fibers and silage, in green manure for nitrogen fixation, particularly where control of nematodes is important, as trap plants in endemic regions of dengue, and serve as a nurse species during early reforestation, protecting against soil erosion, and as covers for control of weeds (Pacheco and Silva-López, 2010). Despite the high toxicity of seeds due to the presence of the alkaloid monocrotaline, protease inhibitors, and other compounds which are important weapons in the chemical defence of *Crotalaria*, there are descriptions of *C. spectabilis* and *C. juncea* being used in small communities in South America and India to treat various diseases (Maregesi *et al.*, 2007).

Plants produce a diverse array of compounds that have ecological functions and adaptive interactions, such as in defence against predators and pathogens (Croteau *et al.*, 2000). Peptidases, also known as proteases, play crucial roles in plant defence and are commonly expressed in species of the Leguminosae family (van der Hoorn and Jones, 2004; Mosolov and Valueva, 2006). These enzymes may be classified as: exopeptidases which act on the ends of protein substrates and are designated

Abbreviations: BSA, bovine serum albumin; CS, Crotalaria spectabilis; CS-CA, aqueous stem extract from CS; CS-CPVPP, polyvinylpolypyrrolidone stem extract from CS; CS-EA, aqueous leaf extract from CS; CS-ED, detergent leaf extract from CS; CS-FA, aqueous flower extract from CS; CS-FPVPP, polyvinylpolypyrrolidone flower extract from CS; CS-P, phosphate leaf extract from CS; CS-PVPP, polyvinylpolypyrrolidone leaf extract from CS; CS-RA, aqueous root extract from CS; CS-RPVPP, polyvinylpolypyrrolidone root extract from CS; CS-SP, phosphate seed extract from CS; CS-ST, Tris seed extract from CS; CS-T, Tris leaf extract from CS; E-64, L-trans-epoxysuccinyl-leucylamido-(4guanidino) butane; EC, enzyme classification; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; L-TAME, N-p-tosyl-L-arginine methyl ester; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; U, enzymatic activity.

amino- or carboxypeptidases, respectively, and endopeptidases acting on the interior of protein substrates. Peptidase classification is based on the type of functional group at the active site. The hydroxy group of serine proteases (EC 3.4.21) and the sulfhydryl group of cysteine proteases (EC 3.4.22) are the nucleophile in catalysis, while activated water is the nucleophile for aspartic proteases (EC 3.4.23) and metalloproteases (EC 3.4.24). Proteases are ubiquitously found in all living beings from unicellular to higher organisms and are encoded by approximately 2% of all genes of an organism (Rawlings et al., 2010). Plant genomes encode hundreds of proteases which represent dozens of unrelated families and are responsible for protein metabolism. The biological role of these enzymes is mostly unknown, but molecular studies have provided phenotypes for a growing number of proteases (van der Hoorn and Jones, 2004). Proteolysis in plants, similar to other organisms, is not limited to protein turnover to provide a source of amino acids or of carbon and nitrogen necessary for the synthesis of new molecules and new proteins, but they are important mediators of a striking variety of biological processes, since they cleave specific peptide bonds in key proteins and enzymes and thus are involved in the regulation of growth and development (Kurepa et al., 2009). Proteolytic events control gene expression that is responsible for cell growth, differentiation, division or reproduction, as well as senescence, meiosis, gametophyte survival, epidermal cell fate, stomata development, chloroplast biogenesis, removal of damaged or improperly folded proteins, processing and targeting of proteins, zymogens and peptide hormones through digestion of signal peptides, participate in programmed cell death, and local and systemic defence responses (Kurepa et al., 2009; Palma et al., 2002). The functional diversity correlates with the molecular data: Proteases accumulate in different subcellular compartments (Janská et al., 2010).

Species of the Fabaceae family, part of the Leguminosae, store large amounts of proteins in their seeds as a consequence of an extensive protein metabolism, which is more expressive in comparison with plants from other families. In order to regulate the protein metabolism, plants express high levels of proteolytic enzymes and of their inhibitors (Silva-López, 2009; Gomes *et al.*, 2011). Surprisingly, these enzymes are poorly

studied in plants. So, the aim of this study was to characterize the proteolytic activity of extracts of different *Crotalaria spectabilis* organs.

Material and Methods

Plant

Crotalaria spectabilis Roth organs were collected in the morning on sunny days from Campus Mata Atlântica of Fundação Instituto Oswaldo Cruz in the state of Rio de Janeiro, Brazil. A plant specimen was deposited in the Rio de Janeiro Botanical Garden, Rio de Janeiro, Brazil, under number RB-488.839.

Preparation of extracts

Fresh leaves and flowers were powdered using liquid nitrogen, and proteins were extracted using different systems for 2 h at room temperature (24 °C), with gentle stirring and centrifugation at 10,000 x g for 30 min at 4 °C. The supernatants were collected and lyophilized. The following extracts were obtained from leaves: an aqueous extract (CS-EA) using only distilled water; a detergent extract (CS-ED) using 1% Triton X-100; a phosphate extract (CS-P) using 50 mM sodium phosphate (pH 6.5); a CS-PVPP extract using 50 mM sodium phosphate (pH 6.5) and 5% polyvinylpyrrolidone (PVPP); a Tris extract (CS-T) using 50 mM Tris-HCl (pH 7.5). From flowers, only aqueous (CS-FA) and PVPP (CS-FPVPP) extracts were obtained. Fresh seeds, stems, and roots were homogenized in distilled water using a blender, and the supernatants obtained after centrifugation were lyophilized for the production of different extracts: From seeds CS-ST and CS-SP extracts were obtained using Tris-HCl and phosphate buffers, and from stems and roots the aqueous extracts CS-CA and CS-RA were obtained, and the PVPP extracts CS-CPVPP and CS-RPVPP, respectively, as described above.

Determination of optimal pH value, temperature, and heat stability

The assays for pH dependence determination were carried out incubating $10 \,\mu g$ of protein from the extracts for 15 min at room temperature with 0.125 mM *N*- ρ -tosyl-L-arginine methyl ester (L-TAME) with different buffers. L-TAME is a substrate that contains arginine at the P1 site and is suitable for serine proteases such as trypsin, thrombin, plasmin, and other protease classes (Silva-López and De Simone, 2004). The buffers used were as follows: 50 mM sodium citrate (pH 4.0–6.5), Tris-HCl (pH 7.0–8.5), and sodium carbonate/bicarbonate (pH 9.0–10.0).

To determine the temperature optimum of enzyme activity in the *C. spectabilis* extracts, L-TAME and 10 μ g of protein from the extracts were incubated with a 50-mM buffer of choice for 15 min at different temperatures ranging from 20 °C to 60 °C for all extracts and 20 °C to 90 °C only for seed extracts. Absorbance was monitored at 247 nm and each assay was performed in triplicate. Specific activity was expressed in μ mol min⁻¹ mg⁻¹ of protein.

For thermal stability assays, extracts were incubated prior to the assay with selected buffers at 60 °C for up to 24 h. The reaction was triggered by adding L-TAME at room temperature. The residual activity was calculated considering the protease activity (at 24 °C) without previous incubation as 100%. The results of each series were expressed as the mean value \pm SD (standard deviation).

Polyacrylamide gel electrophoresis and substrate gel electrophoresis

Protein profiles of extracts were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970); the gels were stained with Coomassie Blue R-250. For molecular mass characterization, Precision Plus Protein Standards (250–10 kDa) from Bio-Rad (Berkeley, CA, USA) were used as molecular standards. Enzymatic activity of the extracts was first analysed by gelatin substrate gel electrophoresis carried out under reducing conditions as previously reported by Silva-López and De Simone (2004). After electrophoresis the gel was washed with 2.5% Triton X-100 for 1 h to remove SDS and incubated overnight at room temperature in different 50-mM buffers to allow proteolysis. The next day, gels were stained with 0.1% amide black and destained in methanol/acetic acid/distilled water (3:1:6 v/v/v).

Enzyme assays with protein substrates

Protein substrates (0.1%, w/v) of high grade [hemoglobin, bovine serum albumin (BSA), and ovalbumin, from Sigma (St. Louis, MO, USA)] were dissolved in buffer and incubated with gentle agitation for 30 min at room temperature with 10 μ g of protein from the extracts. The reactions were stopped by addition of 500 μ l of 10% (v/v) trichloroacetic acid. The tubes were kept on ice for 15 min and centrifuged at 12,000 x g for 10 min. Absorbance of the supernatants was measured at 280 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme required to cause an absorbance increase of 0.1 under standard conditions. The specific activity was defined as U min⁻¹ mg⁻¹ protein. The results of each series were expressed as the mean value ± SD.

Effect of protease inhibitors, cations, and an oxidant on protease activity of C. spectabilis extracts

The types of C. spectabilis proteases were investigated using specific inhibitors for the known protease classes with L-TAME as substrate. Different concentrations of protease inhibitors dissolved in water [iodoacetamide, benzamidine, and ethylenediaminetetraacetic acid (EDTA)], or in dimethyl sulfoxide [phenylmethylsulfonyl fluoride (PMSF) and L-trans-epoxysuccinyl-leucylamido-(4-guanidino) butane (E-64)], in methanol (pepstatin), or in ethanol [N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and 1,10-phenanthroline] were incubated with $10 \,\mu g$ of protein from the extracts for 15 min at room temperature. The reaction was started by addition of the substrate (0.125 mm L-TAME) in the selected buffer at 24 °C for 15 min, and the activity was measured as described above. Appropriate controls were carried out in parallel using the same enzyme solutions without inhibitors. Inhibition was expressed as the percentage of the appropriate control activity. All inhibitors were purchased from Sigma.

Ten μ g of protein from each extract were incubated for 15 min at 24 °C with 10 mM of the chlorides of calcium, zinc, manganese, magnesium, and mercury before the substrate was added. The reactions were performed as described above. The percentage of inhibition was calculated considering the protease activity in the absence of added ions as 100%. Each assay was performed in triplicate, and the results were expressed as the mean value \pm SD.

The effect of an oxidizing agent on the *C*. *spectabilis* proteolytic activity was studied using hydrogen peroxide (H_2O_2) at 1, 5, and 10%. About 10 μ g of protein from the extracts were pre-incubated for 30 min at room temperature

with H_2O_2 , and the reactions were triggered as described above. The percentage of inhibition was calculated considering the protease activity in the absence of H_2O_2 as 100%. Each assay was performed in triplicate, and the results were expressed as the mean value \pm SD.

Protein determination

Protein content was determined by the method of Bradford (1976) in order to minimize the interference of plant alkaloids and polyphenols, using BSA as standard.

Results

Protein measurement and peptidase activity against L-TAME of C. spectabilis extracts

As shown in Table I, the various extracts from *C. spectabilis* have different protein contents and activities against L-TAME. As expected, the seed extracts exhibited higher protein levels compared to the extracts of other organs of the plant. On the other hand, lower levels of protein were found in leaf extracts and CS-PVPP, which had the lowest protein content, but demonstrated the highest peptidase activity. The phosphate buffer was more efficient in extracting proteins from leaves and seeds, and the phosphate buffer containing PVPP was ideal to extract proteins with proteo-

lytic activity, but only from leaves. Both systems, aqueous and phosphate buffer with PVPP, which were used to extract proteins from flowers, stems, and roots, were very effective in obtaining proteins with enzymatic activity, specially the aqueous system.

Effects of pH value and temperature on the enzymatic activity of C. spectabilis extracts

The pH dependence with L-TAME as substrate is demonstrated in Fig. 1. All pH curves present peaks of various peptidase activities in different pH ranges, indicating the proteolytic heterogeneity of the extracts. The maximum activity was around pH 9.5 for leaf extracts (Figs. 1A and 1B), but peaks were also observed at around pH 7.5 and pH 5.5 for CS-PVPP, CS-EA, and CS-ED, pH 8.0 for CS-T and pH 6.5 for CS-P. The two seed extracts showed similar pH curves with maximal activity at pH 8.5, minor peaks at pH 9.5, 7.5, 6.5, and a slight one at pH 5.0 (Fig. 1C). Differently from leaf and seed extracts, maximum activity in root and flower extracts was found in the acidic pH range (Figs. 1D to 1F). Although the root extracts exhibited curves with different shapes, the enzymatic activity was highest in the acidic range around pH 5.0–5.5 and good enzymatic activity was observed at pH 6.5, 8.0, 8.5, and 9.0 (Fig. 1D). In flower extracts, maximum activity was observed

Extract	Lyophilized extract	Total protein [g]	Protein content (%)	Specific activity $[\mu \text{mol min}^{-1} \text{mg}^{-1}]$	Recovered activity $[\mu \text{mol min}^{-1} \text{mg}^{-1}]$
	[g]			protein]	protein]
From leaves					
CS-EA	8.9	1.1	13.2	$3.9 \cdot 10^{-6}$	0.7
CS-ED	3.2	0.4	11.4	$3.7 \cdot 10^{-6}$	2.2
CS-PVPP	9.5	0.1	1.03	$7.8 \cdot 10^{-5}$	1.5
CS-T	5.9	0.7	12.0	$4.7 \cdot 10^{-6}$	1.7
CS-P	8.4	1.2	15.0	$3.1 \cdot 10^{-6}$	1.8
From seeds					
CS-ST	13.1	3.3	26.4	$4.5 \cdot 10^{-6}$	7.5
CS-SP	13.1	4.0	30.2	$1.7 \cdot 10^{-6}$	3.3
From flowers					
CS-FA	7.1	1.6	23.0	$6.1 \cdot 10^{-6}$	$9.9 \cdot 10^{-3}$
CS-FPVPP	7.1	1.8	25.4	$1.0\cdot10^{-6}$	$1.8 \cdot 10^{-3}$
From roots					
CS-RA	14.47	3.4	23.3	$8.6 \cdot 10^{-6}$	$2.9 \cdot 10^{-2}$
CS-RPVPP	14.47	2.6	17.7	$7.9 \cdot 10^{-6}$	$2.0 \cdot 10^{-2}$
From stem					
CS-CA	2.46	0.7	25.8	$9.2 \cdot 10^{-6}$	$6.0 \cdot 10^{-3}$
CS-CPVPP	2.46	0.5	21.1	$7.6 \cdot 10^{-6}$	$4.0 \cdot 10^{-3}$

Table I. Protein quantification and peptidase activity against L-TAME of C. spectabilis extracts.



Fig. 1. Effect of pH value on the peptidase activity of *C. spectabilis* (A) leaves, (B) PVPP, (C) seeds, (D) roots, (E) flowers, and (F) stems extracts. Ten μ g of protein from extracts and the substrate L-TAME (0.125 mM) were used. The following buffers (50 mM) were used: sodium citrate (pH 4.0–6.5), Tris-HCl (pH 7.0–8.5), and sodium bicarbonate (pH 9.0–10).

at pH 6.5 with secondary peaks at pH 5.5 and 9.0 for CS-FA and for CS-FPVPP, the highest activity being at pH 5.5 followed by that at pH 8.5.

The effect of temperature on the enzymatic activity of the *Crotalaria* extracts was analysed using different buffers, adjusted to the optimum pH value, and temperatures ranging from 20 °C to 60 °C for all extracts and 20 °C to 90 °C for

seed extracts (Fig. 2). Leaf proteases exhibited maximum activity at 50 °C, and a peak of activity around 20 °C, with the exception of CS-ED that showed a peak of activity at 30 °C and a rise in activity at 60 °C (Figs. 2A and 2B). Both extracts from seeds demonstrated similar temperature dependence, the maximum activity being observed at 60 °C, but drastically diminishing above 70 °C,



Fig. 2. Effect of temperature on the peptidase activity of *C. spectabilis* (A) leaves, (B) PVPP, (C) seeds, (D) roots, (E) flowers, and (F) stems extracts. Ten μ g of protein from extracts and the substrate L-TAME (0.125 mm) were used. The enzyme and L-TAME were incubated for 15 min at the respective temperature.

suggesting protein denaturation (Fig. 2C). Proteases from root extracts exhibited maximum activity at 30 °C, but at 60 °C they CS-RA activity increased while that of CS-RPVPP decreased (Fig. 2D). Below 60 °C both stem extracts exhibited similar temperature curves, showing peaks of activity at 50 °C and 30 °C (Fig. 2F). Flower extracts showed only a slight increment of activity at 20 °C and 60 °C (Fig. 2E).

Thermal stability assays demonstrated that proteases of *C. spectabilis* extracts might preserve all or none of their activity after pre-incubation for 24 h at 60 °C without L-TAME (Fig. 3). In general, activities of leaf extracts are thermally resistant, especially of CS-EA and CS-ED. While CS-PVPP had the highest peptidase activity, it also had the lowest thermal stability. Ambiguous results were obtained with the seed extracts: While CS-SP remained 100% active, CS-ST retained only 20% of its activity. The residual activity of root extracts was similar to that of seed extracts, the activity of CS-RA being completely abolished by this treatment, CS-RPVPP on the other hand preserved very good residual activity (85%). Taken together, stem and flower extracts presented the best thermal stability, retaining more than 80% of their activity.

Electrophoretic analysis of C. spectabilis extracts

Profiles of the more abundant proteins of C. spectabilis were similar, but presented particular differences. Profiles of leaves were very similar under both reducing and non-reducing conditions, suggesting that the abundant proteins could be single polypeptide chains (Fig. 4A). All extracts exhibited a major protein of about 31 kDa, and CS-ED (Fig. 4A, lane a) and CS-EA (Fig. 4A, lane b) had exclusive proteins of 120 kDa and 47 kDa, respectively. Two proteins of about 112 and 96 kDa were only present in CS-PVPP and CS-P. Proteins of 47-41 kDa were found in all extracts, except in CS-ED that contained an exclusive protein of 48 kDa. A high-molecular weight protein of about 180 kDa was observed in CS-PVPP, CS-T, and CS-P, but not in CS-ED and CS-

EA. The electrophoretic profiles of seeds were similar under both reducing and non-reducing conditions, but changed when β -mercaptoethanol was omitted, suggesting the presence of proteins with multiple polypeptide chains in the samples (Fig. 4B). In all extracts, a major protein of 65 kDa and other minor proteins with 79, 73, 58, 52, 36, 34, 26, 25, and 21 kDa were present, however, only under reducing conditions, and CS-SP showed two proteins of 95 and 38 kDa (Fig. 4B, lane b). Furthermore, both extracts contained two proteins of 55 and 28 kDa, respectively. Figs. 4C and 4D show protein profiles of root, flower, and stem extracts under reducing and non-reducing conditions, respectively. All extracts presented abundant proteins of 80, 44, 41, 40, and 36 kDa. Root and stem extracts showed very similar protein profiles under reducing and non-reducing conditions, but there was specifically a 27-kDa protein in all root extracts and two proteins of 22 and 18 kDa in stem extracts. On the other hand, profiles of flower extracts are distinct in the two gel conditions. In aqueous extracts, proteins of 92, 35, and 30 kDa, and a high-molecular weight protein above 250 kDa could be observed in both gels. Also, the flower PVPP extract showed exclusively 24-, 23-, and 22-kDa proteins in both gel conditions.

The proteolytic activity of *C. spectabilis* extracts was first evaluated using gelatin substrate SDS-PAGE (Fig. 5). Electrophoresis profiles from leaf extracts were very similar in both reducing and



Fig. 3. Thermal stability of peptidase activity of *C. spectabilis* extracts. The heat stability was determined after pre-incubating the extracts (10 μ g of protein) at 60 °C up to 24 h without substrate and then assaying the residual activity at room temperature (24 °C).



Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *C. spectabilis* extracts. About 30 μ g of protein from each extract were analysed by 12% SDS-PAGE. (A) Leaf extracts under reducing conditions: lane a, CS-ED; lane b, CS-EA; lane c, CS-PVPP; lane d, CS-T; and lane e, CS-P. (B) Seed extracts: lane a, CS-ST; and lane b, CS-SP; under reducing conditions. Lane c, CS-ST; and lane d, CS-SP; under non-reducing conditions. (C) Root, flower, and stem extracts under non-reducing conditions: lane a, CS-RA; lane b, CS-FA; lane c, CS-CA; lane d, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. (D) Root, flower, and stem extracts under non-reducing conditions: lane a, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. (D) Root, flower, and stem extracts under non-reducing conditions: lane a, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. (D) Root, flower, and stem extracts under non-reducing conditions: lane a, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. (D) Root, flower, and stem extracts under non-reducing conditions: lane a, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. (D) Root, flower, and stem extracts under non-reducing conditions: lane a, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. Standard proteins (M_t in kDa) are on the left side of the gels.



Fig. 5. Gelatin substrate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE-gelatin) of *C. spectabilis* extracts. The proteolytic activity of about 50 μ g of protein from the extracts was analysed by 10% SDS-PAGE. (A) Leaf extracts under reducing conditions: lane a, CS-EA; lane b, CS-ED; lane c, CS-PVPP; lane d, CS-T; and lane e, CS-P. (B) Seed extracts: lane a, CS-ST; and lane b, CS-SP; under reducing conditions. Lane c, CS-ST; and lane d, CS-SP; under non-reducing conditions. (C) Root, stem, and flower extracts under reducing conditions: lane a, CS-RA; lane b, CS-FA; lane c, CS-CA; lane d, CS-RPVPP; lane e, CS-FPVPP; and lane f, CS-CPVPP. Standard proteins (M_r in kDa) are on the left side of the gels.

non-reducing conditions, and all of them showed proteolytic activity against gelatin, except CS-ED. Proteins of about 180, 130, and 66 kDa in CS-PVPP (Fig. 5A, lane c), 110 kDa in CS-EA and CS-T (Fig. 5A, lanes a and d), and 180, 110, and 72 kDa in CS-P (Fig. 5A, lane e) exhibited significant proteolytic activity. Some of these proteins, such as the 180-kDa protein found in CS-PVPP, CS-T, and CS-P, the 110-kDa protein of CS-EA, and the 130-kDa protein that was found exclusively in CS-PVPP, were detected by SDS-PAGE (Fig. 4A, lane c). Both seed extracts preserved proteolytic activity only under reducing conditions, and of them CS-ST was the more active (Fig. 5B). These results corroborate the previous observation that CS-ST had also exhibited higher peptidase activity (Table I). Distinct bands of proteolytic activity were absent, because these proteases hydrolyzed the gelatin during electrophoresis down to the region of about 92 kDa of the gel, suggesting high activity. Only PVPP extracts from flowers, stems, and roots continued to exert proteolytic activity down to 68 kDa suggesting hydrolysis during electrophoresis (Fig. 5C). In CS-RPVPP five bands of activity can be observed: two proteases above 250 kDa, and three proteases of 230 and 240 kDa, and a 120-kDa protease, which was the most active (Fig. 5C, lane a). A proteolytic activity giving a clear band at about 180 kDa was present in the stem extract (Fig. 5C, lane b) but clear bands could not be detected in the flower extract where there were some regions of protease activity with about 170, 110, and 68 kDa (Fig. 5C, lane c). Notably, proteins of C. spectabilis extracts with proteolytic activity were not the major proteins revealed by SDS-PAGE.

Proteolytic activity of C. spectabilis extracts

C. spectabilis extracts exhibited peptidase activity with L-TAME as substrate (Table I) and proteolytic activity against gelatin (Fig. 5), as well as when they were assayed using other proteins such as hemoglobin, BSA, and casein (Fig. 6). All leaf extracts failed to show activity against BSA, but they did have activity against hemoglobin and casein, especially CS-EA and CS-PVPP. Seed extracts hydrolyzed all protein substrates but exhibited the lowest activity among all extracts. Extracts from flowers, stems, and roots showed strong activity against protein substrates and, generally, BSA was the best substrate, with exception of CS-FA that exhibited the highest activity against casein.

Effect of protease inhibitors, cations, and an oxidant on protease activity of C. spectabilis extracts

Various substances were able to inhibit or modulate the activity of *C. spectabilis* extracts towards L-TAME hydrolysis. The enzymatic activity of leaf extracts was strongly inhibited by serine protease inhibitors such as TPCK, iodoacetamide, and PMSF. An inhibition of the CS-P activity of about 40% by EDTA, a metalloprotease inhibitor, was observed (Table II). TPCK, a chymotrypsinlike serine protease inhibitor, completely inhibited the enzymatic activity of all leaf extracts, except CS-EA, in which TPCK abolished more than 50% of its activity. The inhibition profile of seed extracts suggested the presence of both serine and cysteine proteases, since the activity was inhibited by all serine protease inhibitors, and slightly by E-64, a cysteine protease inhibitor. CS-RA was inhibited by PMSF, but still more complete by EDTA. On the other hand, CS-RPVPP showed strong serine and aspartic protease activity, since it was significantly affected by iodoacetamide and benzamidine, and by pepstatin. Flower extracts, especially CS-FPVPP, were significantly inhibited by serine protease inhibitors and slightly inhibited by EDTA and pepstatin. Furthermore, stem extracts were strongly inhibited by the trypsin-like serine protease inhibitor benzamidine and also by EDTA. These studies are essential for knowing which kinds of proteases are found in each extract and to work out strategies to purify them.

Modulation of the proteolytic activities of the C. spectabilis extracts was examined for divalent cations such as Mg²⁺, Mn²⁺, Ca²⁺, Zn²⁺, and Hg²⁺ (Fig. 7). With many of the ions, leaf proteases were negatively modulated, with the exception of CS-P whose activity was enhanced. Mn²⁺ enhanced the activity of all extracts about 1.5 to 2.0 times, with the exception of CS-PVPP. Ions exerted different effects on seed proteases, however, Mn²⁺ and Hg²⁺ decreased the activity of both extracts, while Zn²⁺ increased it. Ca²⁺ modulated positively the activity of CS-ST, but decreased the activity of CS-SP, and vice versa does Mg²⁺. The activity of the aqueous flower extract was positively modulated by these cations, but the PVPP extract was not significantly affected. The root extract activity was strongly modulated negatively by all ions, with the exception of CS-RA which was activated by Zn²⁺. On the other hand, stem extracts were not significantly affected by these ions, except in the case of Hg²⁺ which decreased the activity of CS-CPVPP by about 50%.

The proteolytic activity of all extracts was affected by hydrogen peroxide (Fig. 8). Proteases from leaves were the most resistant to the treatment with H_2O_2 , because even at 10% peroxide all extracts exhibited activity against L-TAME, except CS-T which had excellent activity at 1 and



Fig. 6. Proteolytic activity of *C. spectabilis* extracts on the protein substrates. Ten μ g of protein from the extracts were incubated with 0.1% BSA, casein, or hemoglobin for 30 min at room temperature. The reactions were stopped, and absorbances of the supernatants were measured at 280 nm. One unit of enzymatic activity (U) was defined as the amount of enzyme required to cause an absorbance increase of 0.1 under standard conditions. The specific activity was defined as U min⁻¹ mg⁻¹ protein.



Fig. 7. Effects of cations on the peptidase activity of *C. spectabilis* extracts. Extracts ($10 \mu g$ of protein) were preincubated with solutions of each ion (10 mM) for 15 min at room temperature. Values are the residual activity of extracts as percentage of the activity on L-TAME without ions, and represent the average of 3 separate experiments carried out in duplicate.

5%, but not at 10%. CS-ED proteases preserved about 45% of their activity in the presence of H₂O₂, even at 10%. Seed extracts also exhibited very good peptidase activity after H₂O₂ pre-treatment. Surprisingly, the activities of proteases from stems, roots, and flowers were greatly decreased in the presence of the oxidant. Similar assays were performed using Triton X-100 as detergent and β -mercaptoethanol as reducing agent on the *C. spectabilis* activity, but the results were not reproducible, despite several repetitions.

Discussion

Previous studies of proteases in Leguminosae have been practically limited to seed extracts, and there is little information available on these enzymes in other plant parts (Demartini *et al.*, 2007). The present investigation reports the proteolytic activity of enzymes found in the different organs of *C. spectabilis* for the first time. These organs express distinctive types of proteases showing significantly different biochemical and

Tris-HCI buff	er (pH 9.5) for 30	min at room te	emperature. The r	emaining activi	ty was assayed by	/ incubation with 0	.125 mm l-TAM	IE.
Extract				Residual	activity (%) ^a			
		Sei	rine		Cysteine	Aspartic acid	Metallo	proteases
	Iodoacetamide, $100 \mu M$	TPCK, 100 μM	Benzamidine, $10 \mu_{\rm M}$	PMSF, 1 mM	E-64, 10 µM	Pepstatin, $10 \mu M$	EDTA, 100 mM	1,10-Phenanth- roline, 10 mM
CS-EA	100.0 ± 0.3	49.5 ± 0.1	100.0 ± 0.0	74.3 ± 0.9	100.0 ± 0.0	100.0 ± 0.0	90.2 ± 0.5	100.0 ± 0.0
CS-ED	100.0 ± 0.0	0.0 ± 0.0	100.0 ± 4.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
CS-PVPP	34.5 ± 0.4	0.0 ± 0.0	100.0 ± 1.2	86.7 ± 1.5	100.0 ± 0.7	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
CS-P	46.4 ± 1.6	0.0 ± 0.0	100.0 ± 3.5	100.0 ± 0.4	100.0 ± 3.8	92.0 ± 0.3	60.6 ± 1.9	100.0 ± 0.0
CS-T	21.9 ± 0.3	0.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.9	100.0 ± 0.5	100.0 ± 5.7	100.0 ± 0.7
CS-ST	59.5 ± 1.3	27.9 ± 2.0	82.7 ± 2.8	76.2 ± 2.5	79.2 ± 2.1	100.0 ± 0.0	100.0 ± 0.8	100.0 ± 5.9
CS-SP	76.4 ± 1.2	67.6 ± 1.7	100.0 ± 0.0	58.8 ± 3.4	89.5 ± 0.0	100.0 ± 0.0	100.0 ± 2.5	100.0 ± 9.2
CS-FA	90.6 ± 0.5	100.0 ± 0.0	42.8 ± 2.4	100.0 ± 0.8	100.0 ± 2.7	100.0 ± 0.0	82.2 ± 3.4	100.0 ± 0.3
CS-FPVPP	93.0 ± 0.2	55.0 ± 1.6	64.3 ± 3.6	100.0 ± 6.0	100.0 ± 0.5	71.1 ± 1.1	90.3 ± 0.9	100.0 ± 0.0
CS-RA	100.0 ± 0.1	100.0 ± 0.0	100.0 ± 5.8	81.7 ± 2.2	94.8 ± 3.1	100.0 ± 0.5	24.7 ± 0.4	100.0 ± 0.8
CS-RPVPP	55.4 ± 0.0	100.0 ± 2.7	28.2 ± 0.5	97.8 ± 0.7	78.0 ± 0.0	0.0 ± 0.0	100.0 ± 7.8	100.0 ± 7.2
CS-CA	85.7 ± 0.5	100.0 ± 5.6	19.5 ± 0.2	100.0 ± 4.6	100.0 ± 2.6	100.0 ± 1.0	87.9 ± 8.4	100.0 ± 3.3

Values are the remaining activity of serine protease as percentage of the activity on L-TAME without inhibitor, measured as described in "Materiout in duplicate. carried of 3 separate experiments the average represent als and Methods", and

 100.0 ± 0.0

 87.9 ± 8.4 70.7 ± 1.2

 100.0 ± 1.0 91.3 ± 0.5

 100.0 ± 2.6 100.0 ± 6.5

 19.5 ± 0.2 37.0 ± 0.8

 100.0 ± 5.6 100.0 ± 3.3

 85.7 ± 0.5 100.0 ± 0.2

CS-CPVPP

 0.0 ± 0.0

kinetic properties which are probably important for the plant's physiology and its interaction with the environment. Although the leaf extracts had the lowest protein content, a leaf extract showed the highest proteolytic activity. In contrast, seed extracts had a high protein content, but low proteolytic activity, possibly associated with a higher content of protease inhibitors (Oliva et al., 2010). Important proteolytic activity was also observed in roots and stem. The root system is responsible for the acquisition of resources, anchorage, and storage (Patel et al., 2010). Besides the intracellular proteases, plants also secrete these enzymes by way of the roots which are engaged in the nitrogen assimilation process that involves proteolysis of soil proteins (Adamczyk et al., 2010).

In general, protein profiles of extracts from the same organ were similar, however, specific proteins gave particular biochemical and enzymatic characteristics for each extract. The determination of the optimal pH range is useful in protease classification since each class of proteases has its particular optimal pH range and this provides clues to the enzymes' location in the cell and their function in vivo. Overall, aspartic proteases have a pH optimum between 2.5 and 4.5, cysteine proteases between 5.5 and 7.5, serine and metalloproteases in the neutral to alkaline range, but serine proteases are also active at higher pH values (Barret, 1994). The pH curves from all extracts showed many peaks of proteolytic activity in different pH ranges, suggesting the presence of proteases belonging to various protease classes. Extracts of leaves, seeds, and stems exhibited maximum activity in the alkaline pH range, possibly due to the presence of serine proteases which are the most abundant proteases in all living organisms, including plants (Antão and Malcata, 2005). Peaks of activity at other pH values can suggest the presence of cysteine, aspartic, and metalloproteases, respectively. On the other hand, root and flower extracts exhibited maximum activity in the acidic pH range suggesting the presence of cysteine and aspartic proteases in these organs.

Temperature affects the velocity of enzymatic reactions, and raising the temperature increases the velocity as long as the native structure of a protein is preserved. However, high temperatures lead to the denaturation of enzymes mainly by breaking hydrogen bonds (Vogt et al., 1997). Generally, enzymes of C. spectabilis extracts exhibited very good proteolytic activity at high temperatures, be-

Table II. Effects of different types of protease inhibitors on *C. spectabilis* peptidase activity. The extracts were pre-incubated with each inhibitor in



Fig. 8. Effects of H_2O_2 on the peptidase activity of *C. spectabilis* extracts. Extracts (10 µg of protein) were preincubated with H_2O_2 at the indicated contents, for 15 min at room temperature. Values are the residual activity of extracts as percentage of the activity on L-TAME without ions, and represent the average of 3 separate experiments carried out in duplicate.

tween 50 °C and 60 °C, in contrast to proteases from most other species of the Fabaceae family, such as the high molecular weight serine protease from Caesalpinia bonducella seeds (Khan et al., 2010), the 30-kDa cysteine protease from horse gram cotyledons (Jinka et al., 2010), and the aminopeptidase from soybean cotyledons (Asano et al., 2010), all having maximum activity at 40 °C. On the other hand, the 41-kDa serine protease of Canavalia ensiformis seeds had a temperature optimum of 60 °C (Oshikawa et al., 2000). Thus proteases of C. spectabilis possess a thermal stability not observed for other plant proteases (Demartini et al., 2007). It could be a climatic adaptation since C. spectabilis is endemic in tropical areas of the world (Pacheco and Silva-López, 2010).

C. spectabilis proteolytic enzymes are versatile because they hydrolyze both peptide and protein substrates. *C. spectabilis* proteases hydrolyzed gelatine, hemoglobin, BSA, and casein differentially according to the source organs of the plant, suggesting that each organ has a particular group of proteases that are responsible for specific functions in the plant's physiology.

The effects of inhibitors on the protease activity provide the most reliable information concerning their catalytic mechanism, however, analysis of the sequence of the active site of the respective protein is necessary for classification into families and clans (Rawlings *et al.*, 2010). Serine proteases are the largest class of proteolytic enzymes in plants and display functions in the entire life history of plants (Janská *et al.*, 2010; Antão and Malcata, 2005). In all organs of C. spectabilis, particularly in leaves, seeds, and flowers, highest inhibition of proteolytic activity was observed with serine protease inhibitors. Seeds and flowers appeared to have both trypsin- and chymotrypsin-like serine protease activities, while leaves had only chymotrypsin activity and roots and stems only trypsinlike proteases. These differences in serine proteases expression in plant organs certainly have biological and/or ecological importance for C. spectabilis, and the relevant activity of serine proteases in leaves could represent a crucial role in plant defence, since this type of protease is known to interact with phytopathogenic microorganisms (Mosolov and Valueva, 2006). Many plant cysteine proteases are involved in apoptosis, regulate epidermal cell fate, flowering time or embryo development (Shindo and van der Hoorn, 2008), and their expression in C. spectabilis was observed in both seeds and roots. Despite the discrete activity of cysteine protease in roots, it is known that roots secrete this type of protease involved in N assimilation (Adamczyk et al., 2010). Also the highest inhibition by aspartic and metalloprotease inhibitors was observed in root extracts. In general, these two types of proteases in plants could be involved in nodulation, plastid differentiation, thermotolerance, plant senescence, and stress responses (van der Hoorn, 2008; Simões and Faro, 2004). Further studies are necessary to describe the types of proteases of *C. spectabilis* in more detail.

The peptidase activity of all *C. spectabilis* extracts was affected by calcium, zinc, manganese, magnesium, and mercury ions in different ways, indicating that the availability or the fluctuation of these ions in the soil or in the surroundings could modulate the activity of proteases in each organ of the plant in response to variations in the environment. Unfortunately, studies of ion modulation have not been carried out for other plant proteases.

Hydrogen peroxide significantly affects enzymatic activities of plants, and its reaction with proteins results in a rapid oxidation of multiple methionine residues (Navrot *et al.*, 2011). Leaf and seed proteases demonstrated very good activity after treatment with high concentrations of this peroxide, suggesting that *C. spectabilis* proteases are resistant to environmental and physiological oxidative stress. The characterization of the pro-

- Adamczyk B., Smolander A., Kitunen V., and Godlewski M. (2010), Proteins as nitrogen source for plants: A short story about exudation of proteases by plant roots. Plant Signal. Behav. 5, 817–819.
- Antão C. M. and Malcata F. X. (2005), Plant serine proteases: biochemical, physiological and molecular features. Plant Physiol. Biochem. 43, 637–650.
- Asano M., Nakamura N., Kawai M., Miwa T., and Nio N. (2010), Purification and characterization of an N-terminal acidic amino acid-specific aminopeptidase from soybean cotyledons (*Glycine max*). Biosci. Biotechnol. Biochem. **74**, 113–118.
- Barret A. J. (1994), Classification of peptidases. Methods Enzymol. 244, 1–15.
- Bradford M. M. (1976), A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
- Croteau R., Kutchan T. M., and Lewis N. G. (2000), Natural products (secondary metabolites). In: Biochemistry and Molecular Biology of Plants (Buchanan B., Gruissem W., and Jones R., eds.). American Society of Plant Physiologists, Rockville, MD, USA, pp. 1250–1268.
- Demartini D. R., Wlodawer A., and Carlini C. R. (2007), A comparative study of the expression of serine proteinases in quiescent seeds and in developing *Canavalia ensiformis* plants. J. Exp. Bot. **58**, 521–532.
- Gomes M. T., Oliva M. L., Lopes M. T., and Salas C. E. (2011), Plant proteinases and inhibitors: an overview of biological function and pharmacological activity. Curr. Protein Pept. Sci. 12, 341–347.
- Janská A., Marsík P., Zelenková S., and Ovesná J. (2010), Cold stress and acclimation – what is important for metabolic adjustment? Plant Biol. 12, 395–405.
- Jinka R., Ramakrishna V., Rao1 S. K., and Rao R. P. (2010), Purification and characterization of cysteine

teolytic activities of *C. spectabilis* improves our knowledge of plant physiology, and the plant's interaction with environmental factors, microorganisms, parasites, and insects, and also opens the possibility for application of these enzymes. The results obtained here are a prerequisite for the purification and the elucidation of biochemical properties of *C. spectabilis* proteolytic enzymes.

Acknowledgements

This study was supported by grants from Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ). We are grateful to Valério Morelli for *C. spectabilis* supply and Sérgio Monteiro for botanical advice. This manuscript was revised by Dr. Benjamin Gilbert, senior researcher of natural products at FIOCRUZ.

protease from germinating cotyledons of horse gram. BMC Biochem. **10**, 28–38.

- Khan H., Ali I., Khan A. U., Ahmed M., Shah Z., Saeed A., Naz R., Mustafa M. R., and Abbasi A. (2010), Purification and biochemical characterization of alkaline serine protease from *Caesalpinia bonducella*. Nat. Prod. Commun. 5, 931–934.
- Kurepa J., Wang S., Li Y., and Smalle J. (2009), Proteasome regulation, plant growth and stress tolerance. Plant Signal. Behav. 4, 924–927.
- Laemmli U. K. (1970), Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
- Maregesi S. M., Ngassapa O. D., Pieters L., and Vlietinck A. J. (2007), Ethnopharmacological survey of the Bunda district, Tanzania: Plants used to treat infectious diseases. J. Ethnopharmacol. **113**, 457–470.
- Mosolov V. V. and Valueva T. A. (2006), Participation of proteolytic enzymes in the interaction of plants with phytopathogenic microorganisms. Biochemistry (Moscow) **71**, 838–845.
- Navrot N., Finnie C., Svensson B., and Hägglund P. (2011), Plant redox proteomics. Proteomics **74**, 1450–1462.
- Oliva M. L. V., Silva M. C. C., Sallai R. C., Brito M. V., and Sampaio M. U. (2010), A novel subclassification for Kunitz proteinase inhibitors from leguminous seeds. Biochimie **92**, 1667–1673. Oshikawa K., Aoki K., Yoshino Y., and Terada S. (2000),
- Oshikawa K., Aoki K., Yoshino Y., and Terada S. (2000), Purification and characterization of a basic amino acid-specific peptidase from seeds of jack bean (*Canavalia ensiformis*). Biosci. Biotechnol. Biochem. 64, 2186–2192.
- Pacheco J. S. and Silva-López R. E. (2010), Genus Crotalaria L. (Leguminoseae). Rev. Fitos 5, 43–52.
- Palma J. M., Sandalio L. M., Javier Corpas F., Romero-Puertas M. C., McCarthy I., and del Río L. A. (2002),

Plant proteases, protein degradation, and oxidative stress: role of peroxisomes. Plant Physiol. Biochem. **40**, 521–530.

- Patel S., Ricucci D., Durak C., and Tay F. (2010), Internal root resorption: A review. J. Endod. 36, 1107–1121.
- Rawlings N. D., Barrett A. J., and Bateman A. (2010), MEROPS: the peptidase database. Nucleic Acids Res. 38, 227–233.
- Shindo T. and van der Hoorn R. A. (2008), Papain-like cysteine proteases: key players at molecular battlefields employed by both plants and their invaders. Mol. Plant Pathol. 9, 119–125.
- Silva-López R. E. (2009), Protease inhibitors originated from plants: Useful approach for development of new drugs. Rev. Fitos 4, 108–119.

- Silva-López R. E. and De Simone S. G. (2004), A serine protease from a detergent-soluble extract of *Leish-mania* (*Leishmania*) amazonensis. Z. Naturforsch. 59c, 590–598.
- Simões I. and Faro C. (2004), Structure and function of plant aspartic proteinases. Eur. J. Biochem. 271, 2067–2075.
- van der Hoorn R. A. L. (2008), Plant proteases: From phenotypes to molecular mechanisms. Annu. Rev. Plant Biol. **59**, 191–223.
- van der Hoorn R. A. L. and Jones J. D. (2004), The plant proteolytic machinery and its role in defence. Curr. Opin. Plant Biol. **7**, 400–407.
- Vogt G., Woell S., and Argos P. (1997), Protein thermal stability, hydrogen bonds, and ion pairs. J. Mol. Biol. **269**, 631–643.