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Partial purification of trypsin inhibitors from *Parkia* seeds (Fabaceae)

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ABSTRACT - (Partial purification of trypsin inhibitors from *Parkia* seeds (Fabaceae)). Leguminous seeds (Fabaceae) have a high content of inhibitors of which serine protease inhibitors are the most widely studied. However, there are only a few studies related to the investigation of these proteins in tree species belonging to the Amazon flora. The protein content presented in seeds of four Amazonian Leguminosae species, *Parkia pendula*, *P. discolor*, *P. multijuga* and *P. Nitida*, was extracted by using NaCl 0.15 mol L^{-1} and then partially fractionated by using affinity chromatography performed on a trypsin-Sepharose 4B. These inhibitors presented different affinities between trypsin and chymotrypsin serine proteases, showing a higher inhibition to trypsin compared to chymotrypsin, except for *P. nitida*, which showed high inhibition against both enzymes. The SDS-PAGE analysis showed that the species from *Parkia* genus have a main band corresponding to partially purified trypsin inhibitors. The apparent molecular mass inhibitors (approximately 13-18 kDa) and the high specificity for trypsin suggest the occurrence of Bowman-Birk and Kunitz type inhibitors.
Keywords: Amazonia, Bowman-Birk inhibitor, Kunitz inhibitor, leguminous seeds, serine protease

RESUMO - (Purificação parcial de inibidores de tripsina de sementes de *Parkia* (Fabaceae)). Sementes de leguminosas (Fabaceae) apresentam alto conteúdo de inibidores, incluindo os inibidores de serinoproteinases que têm sido extensivamente estudados. Todavia, poucos estudos foram realizados quanto à investigação dessas proteínas em espécies arbóreas pertencentes à flora amazônica. As proteínas presentes nas sementes de quatro espécies de leguminosas da Amazônia, *Parkia pendula*, *P. discolor*, *P. multijuga* e *P. nitida*, foram obtidas pela extração usando NaCl 0.15 mol L^{-1} e, parcialmente purificadas usando a cromatografia de afinidade em tripsina-Sepharose 4B. Os inibidores exibiram afinidades diferentes entre a tripsina e a quimotripsina, exceto para *P. nitida*, a qual apresentou alta inibição contra as duas enzimas. A análise em SDS-PAGE mostrou que as espécies do gênero *Parkia* contém uma banda principal correspondendo aos inibidores de tripsina parcialmente purificados. As massas moleculares aparentes determinadas para os inibidores (aproximadamente, 13 a 18 kDa) e a alta especificidade pela tripsina sugerem a ocorrência de inibidores do tipo Bowman-Birk e Kunitz.
Palavras-chave: Amazônia, Inibidor Bowman-Birk, Inibidor Kunitz, sementes de leguminosas, serinoproteinase

Introduction

Fabaceae seeds are a rich source of protease inhibitors (PIs). These proteins are produced either constitutively or by induction in vegetative and reproductive plant tissues composing approximately 5% to 15% of the total protein content (Shee & Sharma 2008, Chan *et al*. 2013).

PIs play an important role in many functions of different plant tissues, regulating proteolytic

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events through specific interactions with proteolytic enzymes, which fulfill an important function during the seed germination process by preventing premature hydrolysis of storage proteins. Furthemore, those molecules as a source of sulfur amino acids, and/or have been associated with defense against insect and pathogen attack (Klomklao *et al.* 2011, Martínez *et al*. 2012, Praxedes-Garcia *et al*. 2012).

These PIs have been isolated, extensively characterized and grouped according to their structural and functional characteristics. Most of PIs show an affinity with serine, cysteine, aspartic or metalloproteases (Macedo *et al.* 2007, Marra *et al*. 2009). Currently, plant PIs are grouped into Bowman-Birk, Kunitz, Potato I and II, Pumpkin, Cereal, Ragi I-2 or Thaumatin families (Chan *et al*. 2013). PIs belong to the Bowman-Birk and Kunitz families and are found in high concentrations in Fabaceae seedsas well as other plant tissues (Mosolov & Valueva 2005, Chevreuil *et al*. 2011, Ruan *et al.* 2011).

Bowman-Birk inhibitors have a molecular mass ranging from 6 to 16 kDa and often present a conserved pattern of five to seven disulfide bonds which form a compact and very stable tertiary structure with two opposite reactive loops (Qi *et al.* 2005, Kumar & Gowda 2013). Conversely, members of the Kunitz family have a molecular mass ranging from 18 to 26 kDa, with one or two polypeptide chains cross-linked by one to three disulfide bonds. These protease inhibitors usually have one reactive site, but recently a secondary reactive site has been observed (Oliva *et al*. 2010, Oliveira *et al*. 2012).

Considering that there are few studies related to the isolation of protease inhibitors (PIs) from Fabaceae seeds belonging to Amazonian *Parkia* species and also that scientific data about this protein can help us understand their physiological role in the protein metabolism during seed maturation, seed under germination or in storage. Furthermore, leguminous trees in the Amazon have been reported as other important source of protein molecules, such as lectins (Fernandes *et al*. 2011). Thus, this work was aimed at detecting and characterizing partially these proteins in tissue storage of seeds from *Parkia pendula*, *P. discolor*, *P. multijuga* and *P. nitida*.

Materials and methods

Plant material - Mature seeds of *Parkia pendula* were collected from the Adolpho Ducke Forest Reserve - INPA (Manaus-AM), *P. discolor* from the Anavilhanas National Park (Novo Airão and Manaus-AM), *P. nitida* from the Tarumã-Mirim settlement (Manaus-AM), and *P. multijuga* from the Timber Exporters Association of the Pará State (AIMEX). Seeds were ground into a powder.

Evaluation of total protein content - The determination of the total protein content was carried out by the micro Kjeldahl method using the factor 5.71 (Anderson & Ingram 1993, Ezeagu *et al.* 2002). After drying at 65 °C, 100 mg of powdered material was submitted to digestion in a block digester at 350 °C using tubes containing sodium selenite (0.25 g), copper sulphate (11.25 g), sodium sulphate (47.5 g), salicylic acid (1.25 g) and sulphuric acid (500 mL). After digestion and cooling, the digestion products were submitted to a distillation process with the addition of 20 mL of distilled water, 40% sodium hydroxide (15 mL) and a solution containing boric acid (10 mL). A solution of 0.01N sulphuric acid was used for titration.

Protein extraction - The powder (30 g) was homogenized with 300 mL of 0.15 M NaCl for two hours and centrifuged at 5.000 g for 20 minutes at 4 °C. The supernatant was dialysed with distilled water for 48 hours and lyophilized, resulting in the protein extract.

Protein quantification - The protein concentration in the protein extracts and chromatography fractions were estimated with 50 µL of the sample which was incubated for five minutes at room temperature with 2.5 mL of Bradford Reagent (BioAgency). The protein concentration was determined at λ = 595 nm using a spectrophotometer (Ultrospec 2100 pro, Armesham Biosciences).

Affinity chromatography - The resin was equilibrated with Tris-HCl 0.1 M pH 8.0 buffer containing 0.3 M NaCl. The protein extracts were applied and the retained material was eluted with 0.1 M HCl containing NaCl 0.3 M. The fractions with inhibitory activity were collected, dialysed and lyophilized. The monitoring of the chromatographic profile was carried out at λ = 280 nm using an Ultrospec 2100-pro spectrophotometer (Armesham Biosciences).

Trypsin inhibition assay - The inhibitory activity of the samples against trypsin was carried out by adding 250 µL Tris-HCl 0.05 M, pH 8.0 buffer; 50 µL bovine trypsin (EC 3.4.21.4, Serva) in 1 mM HCl; and 150 µL of the sample. The 2 mL solution was then preincubated for 10 minutes at 37 ºC. After this time, 1 mL the chromogenic substrate (0.5 mM) benzoyl arginine paranitroanilide (DL-BApNA, Sigma) was added and incubated for a further 20 minutes at 37 ºC. The reaction was halted by adding acetic acid 30%

(v/v). The product of the substrate hydrolysis was monitored at λ = 410 nm using an Ultrospec 2100-pro spectrophotometer (Armesham Biosciences). The calculation of the inhibitory activity was carried out by the trypsin residual activity.

Chymotrypsin inhibition assay - The inhibitory activity of the samples against chymotrypsin was carried out by adding 500 µL of Tris-HCl 0.05 M, pH 8.0 buffer; 150 µL bovine chymotrypsin (EC 232.671.2, Sigma) in 1 mM HCl; and 100 µL of the sample. The solution (2 mL) was then preincubated for 10 minutes at 37 ºC. After this time, 50 µL the chromogenic substrate (0.1 M) benzoyl tyrosine paranitroanilide (BTpNA, Sigma) was added and incubated for a further 20 minutes at 37 ºC. The reaction was halted by the addition of acetic acid 30% (v/v). The product of the substrate hydrolysis was monitored at λ = 410 nm using an Ultrospec 2100-pro spectrophotometer (Armesham Biosciences). The calculation of the inhibitory activity was carried out by the chymotrypsin residual activity.

Polyacrylamide gel electrophoresis - Polyacrylamide gel was produced from the stacking gel (5%) was prepared with Tris-HCl 0.5 M buffer, pH 6.8. The resolving gel (20%) was prepared with Tris-HCl 1.5 M buffer, pH 8.8. SDS 20% was added to both. Polymerization was carried out by the addition of TEMED and PSA (10%). Samples were dissolved in Tris-HCl 0.0625 M buffer, pH 6.0, containing SDS (1%), ß-mercaptoethanol (1%), glycerol (10%) and bromophenol blue (0.1%), and then immersed in boiling water for 10 minutes. Electrophoresis was carried out with running buffer Tris-HCl 0.025 M, glycine (0.192 M) and SDS (0.1%) at 200 volts, 15 mA, for two hours. A molecular weight marker ranging from 10 to 225 kDa (Promega) was used. The gels were stained with Coomassie brilliant blue, acetic acid (0.1%), methanol and de-ionized water at a ratio of 1:4:5 for two hours and then discoloured in a solution containing glacial acetic acid, methanol and deionized water at a ratio of 1:4:5.

Results

The total protein content measured by the micro Kjeldahl method for the seeds of *P. pendula* *P. discolor*, *P. multijuga* and *P. nitida* ranged from 9.4% (*P. multijuga*) to 17.2% (*P. pendula*) of the dry weight, and showed an average of 13.4% (table 1). Conversely, the amount of soluble protein estimated by the Bradford method for the saline protein extract was much smaller, ranging from 0.448% (*P. pendula*) to 0.825% (*P. nitida*) of the seed dry weight, with an average of 0.63% (table 1), this represents a reduction which was about 21 times lower than the total protein content. The protein content related to trypsin inhibitors separated from saline extract using affinity chromatography (figure 1) revealed that the amount of soluble proteins related to trypsin inhibitors varied from 8.1% for *P. multijuga* to 68.9% for *P. nitida* (table 1). This result indicates that the role of trypsin inhibitors could differ depending on the species of *Parkia*.

The protein extracts and fractions pooled and analyzed by Coomassie Blue-stained SDS-PAGE indicated that a large amount of proteins were removed by affinity chromatography resulting in a main electrophoretic band corresponding to the partially purified inhibitor. The molecular weight (MW) of the inhibitors was determined by the mobility (Rf) in gel electrophoresis using a plot of log (MW) vs Rf. Different inhibitors with affinity for trypsin, isolated from the seed species were *Parkia* molecular weight ranging from 9 to 18 kDa (figure 2).

The partially purified inhibitors exhibited different affinities towards the serine proteases, with a predominant high inhibition against trypsin, when compared with chymotrypsin. However, the *P. nitida* inhibitors showed a potent inhibition activity for both

Table 1. Seed protein content measured for Amazon *Parkia* species, Brazil.

Species	Protein Content (% of the seed dry weight)		
	Total*	Protein Extract**	Inhibitory Fraction** (% of the protein extract weight)
Parkia pendula	17.2 ± 1.36	0.448 ± 0.021	0.042 ± 0.003 (9.4)
Parkia discolor	13.8 ± 0.83	0.708 ± 0.035	0.120 ± 0.014 (17.0)
Parkia multijuga	9.4 ± 0.29	0.539 ± 0.013	0.044 ± 0.017 (8.2)
Parkia nitida	13.4 ± 0.80	0.825 ± 0.031	0.566 ± 0.037 (68.6)

* Estimated by the Kjedahl method.
** Estimated by the Bradford method.

enzymes, while *P. pendula* inhibitors has not shown any activity against chymotrypsin (figure 3).

Discussion

Legume seeds are important sources of plant protein, in which the protein content can reach concentrations up to 40% (Shee & Sharma 2008). In this work *Parkia* species seeds had protein contents ranging from 9.4% to 17.2%, another study with *P. pendula* seeds have revealed protein contents from 12.9% (Gonçalves *et al.* 2002).

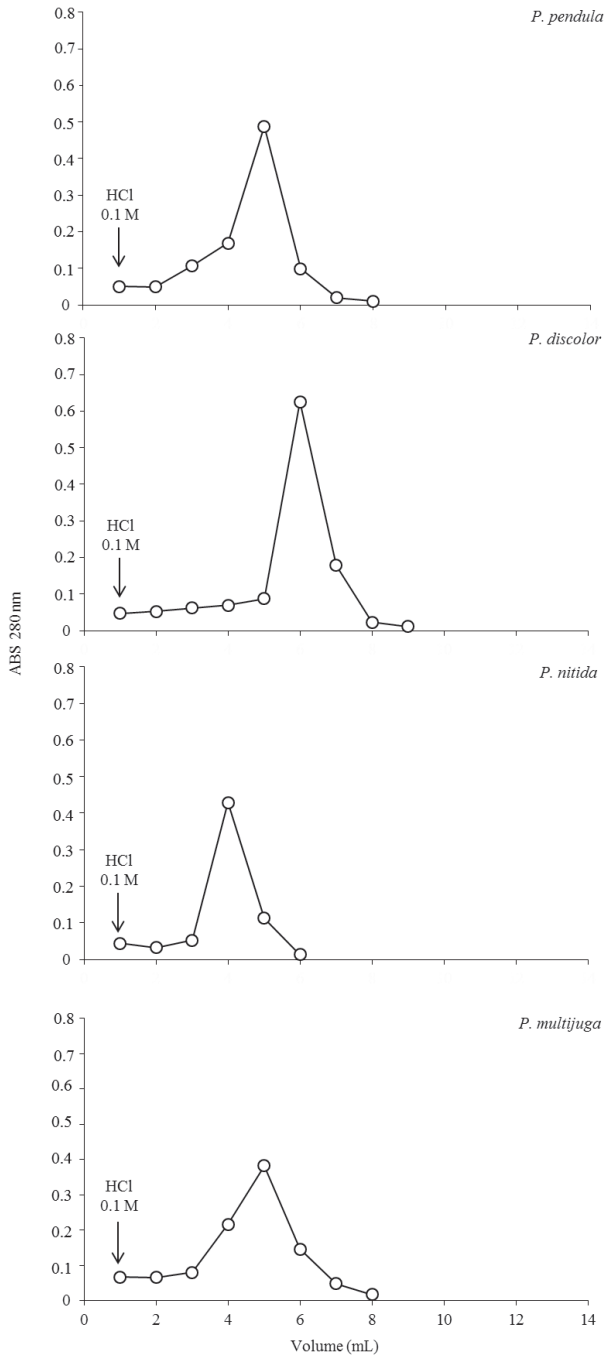

Figure 1. Affinity chromatography performed on a trypsin-Sepharose 4B of Amazon *Parkia* species, Brazil. The trypsin inhibitors were eluted with 0.1 M HCl containing 0.3 M NaCl.

Several proteolytic inhibitors in the seeds of different species are isolated and purified using molecular exclusion, ion exchange, and affinity as well as reversed-phase chromatographies as obligatory steps. Among these techniques, the affinity columns with immobilized proteinase are a very effective method in the purification of these proteins, although the possibility of limited digestion of proteinases inhibitors by immobilized trypsin during purification cannot be ruled out (Prasad *et al.* 2010, Chan *et al*. 2013).

Trypsin inhibitors purified from seeds of *Albizzia kalkora*, *Entada acaciifolia* and *Adenanthera pavonina*, respectively, using the technique of affinity chromatography on trypsin-Sepharose, molecular exclusion on Sephadex G-75 and ion exchange on DEAE-Sepharose (Zhou *et al.* 2008, Oliveira *et al*. 2012, Silva *et al*. 2012). However, Garcia *et al*. (2004) used in addition to these techniques, chromatography on Sephadex G-100 and HPLC column μ-Bondapak C18 in order to obtain a fully trypsin inhibitor purified from *Poecilanthe parviflora* seeds.

Two of the families of serine proteases, the Kunitz and Bowman-Birk type inhibitors, have been isolated from legume seeds. Such families differ from each other in mass, cysteine content and number of reative sites. The Bowman-Birk type inhibitor has a lower molecular weight (6 to 9 kDa), whereas the Kunitz type are proteins of a molecular weight above 20 kDa (Klomklao *et al.* 2011, Kumar & Gowda 2013).

Two protease inhibitors from seeds of *Cajanus cajan*, an inhibitor of a trypsin-chymotrypsin (CTCI) and only a trypsin inhibitor (CTI), Bowman-Birk type, had a molecular mass of 15 and 10.5 kDa, respectively (Godbole *et al*. 1994). Inhibitors of Kunitz and Bowman-Birk type, isolated from the seeds of *Entada acaciifolia* and *Albizia lebbeck*, both belonging to the subfamily Mimosoideae, have molecular weights of 20 and 12.3 kDa, respectively (Oliveira *et al*. 2012, Sharma *et al*. 2012). Based on these results, the inhibitors partially isolated from different species studied include inhibitors of the Kunitz and Bowman – Birk type.

The variation of the affinity of inhibitors against different proteolytic enzymes is also demonstrated in studies involving the trypsin inhibitor isolated from *Poecilanthe parviflora* seeds (Faboideae) and trypsin-papain inhibitor from *Pithecelobium dumosum*

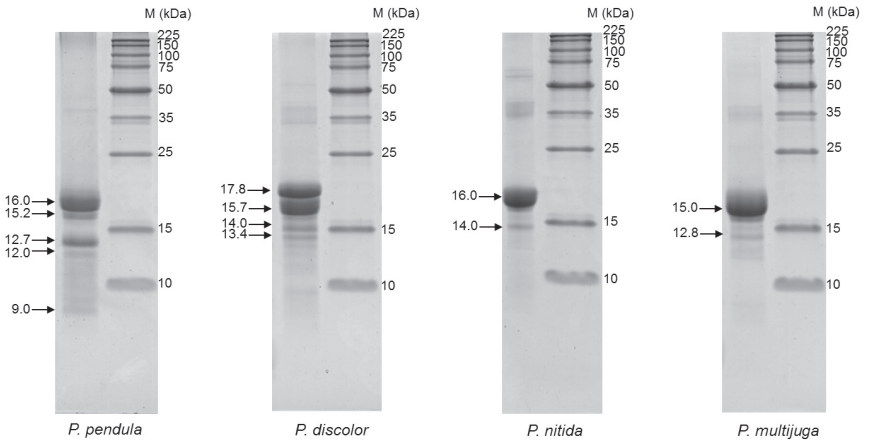

Figure 2. Coomassie stained SDS-PAGE of the affinity chromatography fractions of Amazon *Parkia* species, Brazil.

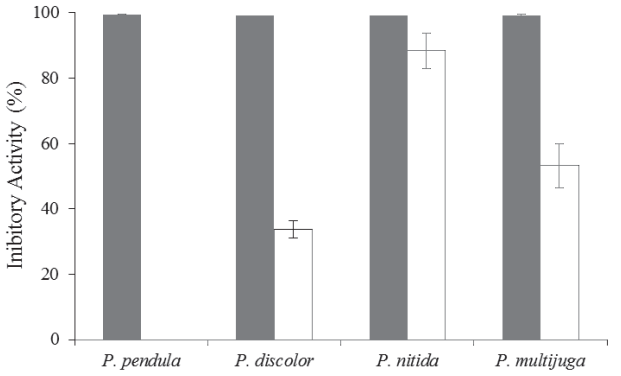

Figure 3. A summary bar graph of the inhibitory activity of the inhibitors partially purified from seeds of Amazon *Parkia* species, Brazil, assayed against trypsin (■) and chymotrypsin (□).

seeds (Mimosoideae), in which the activity of trypsin was completely inhibited, while the inhibition of chymotrypsin and papain by inhibitor from *P. parviflora* was 40% and 10%, respectively and the inhibitor from *P. dumosum* reduced 10% and 49% of chymotryosin and papain activities respectively (Garcia *et al.* 2004, Oliveira *et al.* 2007).

Studies with other leguminous Amazonian species showed the occurrence of trypsin and chymotrypsin inhibitors in extracts from seeds of *Cassia brasselari*, *Cassia occidentalis*, *Dialium guianensis*, *Inga rubiginosa*, *Inga umbratica*, *Inga velutina* and *Mimosa guillandinae*, whereas for *Inga fagifolia* and *Cassia grandis* the inhibition was specific to trypsin (Calderon *et al*. 2001).

Studies have indicated that there is a relationship between the families of proteolytic inhibitors found in legume seeds and the development degree of these plants, where tropical species, for example, Kunitz-type inhibitors are mainly found in basal subfamilies, Caesalpinioideae and Mimosoideae, while the Bowman-Birk inhibitors are more frequent in the derived subfamily, the Faboideae (Norioka *et al*. 1988, Oliveira *et al*. 2007, Bhattacharyya & Babu 2009, Klomklao *et al.* 2011).

Considering its mass characteristics and specificity by trypsin, these inhibitors could be considered as Kunitz and Bowman-Birk type inhibitors. However, the exact classification will only be possible after protein sequencing and further physical-chemical characterization.

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