Proteolysis in the Subtropical Woody Tree *Anadenanthera colubrina* (Angico) Seeds during and after Germination

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Abstract

Proteolysis of seed storage proteins (SSP) during germination provides a steady supply of amino acids to the embryo development into seedling. This process is coordinated by different peptidases that act sequentially and overlaid mode. These enzymes are an ancient group evolved separately in a wide structural and functional diversity and have many applications in medicine, pharmacy and industry. However, the knowledge about seed peptidases during germination was obtained from studies almost restricted to the cultivated species. This restriction implies caution about generalizations made from these studies, as well limits the biological knowledge about plant kingdom and technological use from plant peptidases. In this work, a scan of the proteolytic activity was held in germinating seeds of a leguminous subtropical woody tree. Eleven proteolytic activities were detected in protein extracts from embryonic axis and cotyledons. The presence and intensity of these activities varied over time and between these tissues. There was indication that aspartyl-endopeptidases (phytepsins) and cysteine-carboxypeptidases (plant cathepsins) were involved in *A. colubrina* SSP hydrolysis. These peptidases differ to that commonly involved in germination of the cultivated leguminous. In addition, one of detected phytepsins showed stability on pH scale, which is important for industrial uses. There was also detected a metallo-carboxypeptidase activity, which has been not described in plants. These peptidases must be isolated to confirm or not these indications. However, these data indicate the biological and technological importance of extending the studies about plant peptidases on a diverse genetic basis.

1. Introduction

The proteolysis of the seed storage proteins (SSP) during germination is crucial to seedling establishment. This process, which is activated at seed hydration and remains active after germination, is coordinated by joint activity of different peptidases. These hydrolases are expressed in temporal- and tissue-specific way. They are accumulated in embryonic axis and reserve tissues during seed maturation, as well synthesized during and after germination. Thus, being activated or synthesized, these enzymes sequentially hydrolyze SSP and provide continuous amino acids supply for resumption of embryonic axis development until seedling establishment [1] [2] [3] [4] [5].

The explanatory models of this proteolytic process were based from studies with some cultivated species. Despite the validity of these models, the restriction of studies to these few and genetically standardized plants limits the biological knowledge about proteolysis during germination and other plant proteolytic processes. Maybe is enough to consider the close genetic base that the model plants represent in front of the angiosperms diversity [250,000 species in the world; 50,000 in Brazil] [6] and of the peptidases diversity (524,000 gene sequences; 2800 characterized enzymes, are deposited in MEROPS database; http://merops.sanger.ac.uk [7]).

Peptidases originate from 60 evolutionary lines that resulted in an extensive multifunctional group of enzymes [8]. With structures ranging from 20 kDa-single catalytic units up to 6000 kDa-agglomerates of dozens of subunits, they are capable to fragment proteins completely for recycle amino acids, as well make specific cleavages to regulate the fate and activity of many proteins, modulate protein-protein interactions, amplify molecular signals and create new bioactive molecules [9].

Indeed, at germination, these enzymes vary even in model plants. The hydrolysis routes of SSP among different plants show more similarities to the SSP cleavage sites than the peptidases that cleave then [10]. This is not expected, because SSP are conserved structures and similar protein structures tend to be cleaved by the same peptidase types [8].

Plant peptidases show direct involvement in essential cell cycles [11] [12] [13] [14]. However, few plant peptidases are well known and still less that ones used technologically, when compared to peptidases from mammalian and microorganisms. These latter are intensively studied and have generated fundamental information about biological knowledge of living beings [8], as well many uses in research, medicine, pharmacy, food and industry [15] [16] [17]. In fact, peptidases account for 60% of all commercially available enzymes [18].

On the other side, from works with peptidases active in germination, the majority focuses isolated enzymes. In spite of the obligatory use of purified enzymes to determine their structures, specific functions, evolutionary relations and technological applications, works that exploit germination in relation to whole proteolytic activity are rare. But it is important to expand the plant peptidases research on a diverse genetic basis to increase knowledge about proteolysis in germination, as well in other plant proteolytic processes; and to explore the technological use that plant peptidases may have [19] [20].

In this way, our objective was to carry out a scan of the proteolyticactivity during and after germination inseeds of a subtropical woody species native to South America. Woody species represent the ecological climax of most terrestrial ecosystems, but there is little biochemistry information about its germination, especially from the tropical and subtropical ones. However, *A. colubrina* (Fabaceae), called Angico in Brazil, has pharmacological [21]-[26], agricultural [27], anthropological [28] [29] and phytogeographic [30] importance, as well to food industry [31]. In addition, *A. colubrina* shows experimental advantages that confer model plant characteristics for studies about woody trees germination: it presents orthodox seeds with high, fast and uniform germination rate, high SSP content and ease of separating embryonic axes from cotyledons.

In this context, we used synthetic peptide substrates and peptidase inhibitors to detect, quantify and partially characterize, via spectrofluorometry and high performance liquid chromatography, the activity of peptidases in protein extracts from *A. colubrina* embryonic axis and cotyledons separated before, during and after germination.

2. Material and Methods

2.1. Sample Preparation

A. colubrina seeds were supplied by LAS/UFLA, from a batch of seeds randomly collected from several local trees (21˚14'42''S 45˚00'00''W). Visibly normal and undamaged seeds were placed to germinate on rolls made from 3 sheets of *germitest* paper soaked in distilled water at volume 2.5 times the dry paper weight. These seed rolls were kept at 30˚C, without photoperiod control, during 0, 6, 12, 18 and 24 hours (h). Fifty seeds were used for each hydration time. Seeds apparently dead or presenting necroses were not collected. The seed coats from the remaining seeds were discarded, embryonic axes (E) and cotyledons (C) were separated and collected for immediate protein extraction. Collected tissues from E and C were macerated using liquid N_2. The powder was homogenized with ice-cold Tris buffer [50 mM/pH 7.5] at 25 mg E or 100 mg C to 1 mL buffer. The homogenate was maintained at 4˚C for 2 h under occasional shaking and then centrifuged twice at 16,000 g for 20 min at 4˚C. The supernatant containing water-soluble proteins (crude extract) was diluted 1:1 with glycerol (v/v) and stored at −40˚C. The protein content of these extracts was estimated by Bradford method [32]. Aliquots of 5 μL were quantified in triplicate and work samples were

diluted to 200 ng·μL⁻¹ with tris buffer + 50% glycerol. New 5 μL-quantifications in triplicate were done with the diluted samples. Uniform working samples in relation to protein concentration were aliquoted, cataloged according to tissue and hydration time of the seeds (E0 to E24/C0 to C24) and stored at −40˚C.

2.2. Proteolytic Activity Assays

The proteolytic activity (At) in E and C samples was detected by spectrofluorometry using FRET (fluorescence resonance energy transfer) synthetic peptide substrates that emit fluorescence when hydrolyzed[1]. The substrates used were Abz-AIAFFSRQ-EDDnp, Abz-FRAK(Dnp)-OH, F-MCA and Z-FR-MCA. These substrates were synthesized according INFAR protocol [33]. The hydrolysis reactions were performed at pH 3 to 9. Each of these 280 reactions (10 samples × 7 pH points × 4 substrates) was run with 4 μg of proteins and 5 μM of substrate into 1 mL of 50 mM sodium acetate buffer (pH 3; 4; 5), bis-tris (pH 6) or tris (pH 7; 8; 9). Hydrolysis of Abz-peptidyl substrates were monitored at λ_{Ex} = 320 nm and λ_{Em} = 420 nm; and of peptidyl-MCA substrates at λ_{Ex} = 380 nm and λ_{Em} = 460 nm. Both monitoring were done in a spectrofluorometer Hitachi F-2500 with temperature control and magnetic stirring bar in the cuvette cell. Allreactions were monitored for 10 min at 37˚C and the At results were expressed in nM of substrate hydrolyzed per μg protein per min using the Equation 1:

$$A t = \frac{Vh}{Kn \cdot pt} \times 1000$$

where At is proteolytic activity (nM·μg·min⁻¹), Vh is hydrolysis rate (AFU/min), Kn is K constant for each fluorescent group (AFU/μM) and pt is the amount of protein in the sample (μg)[2]. All reactions were performed in duplicate.

2.3. Proteolytic Activity Inhibition Assays

After obtaining At data, were chosen 6 results with Abz-AIAFFSRQ-EDDnp, 3 with Abz-FRAK(Dnp)-OH, 8 with F-MCA and 3 with Z-FR-MCA to repeat the reactions that gave rise to these 20 results, but now using peptidase inhibitors. These results were chosen due At expressivity from individual samples and/or particular variations of At in time profile and/or pH profile. The peptidase inhibitors used were 1 μM Pepstatin, 5 μM E-64, 500 μM PMSF or 5 mMo-phe, which are aspartyl-, cysteine-, serine- and metallo-peptidases inhibitors, respectively. The reactions were performed with protein solutions (4 μg·mL⁻¹) been incubated in absence or presence of inhibitors for 10 min at 37˚C under stirring. Thereafter, substrate was added and its hydrolysis was monitored for 10 min. The results were determined by Equation (1) and converted at

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[1]By resonance to Z, EDDnP and Dnp quenching groups, Abz and MCA fluorescent groups exhibit low fluorescence that increases when the substrate molecule is ruptured.

[2]The spectrofluorometer was adjusted to record the fluorescence every 0.4 s during 10 min. With increasing amount of fluorescence over time, the equipment generates an accurate linear regression (R^2 > 0.99), whose angular coefficient corresponds to hydrolysis speed and represents a direct measure of the proteolytic activity.

percentage of inhibition relative to the control. The inhibitionhydrolysis of Abz-FRAK(Dnp)-OH by o-phe was performed by high performance liquid chromatography (HPLC).

2.4. Proteolytic Activity Inhibition by HPLC Assay

To quantify the inhibition of Abz-FRAK(Dnp)-OH hydrolysis by o-phe, proteins solutions (8 μg·mL^{-1}) containing or not 5 mM o-phe were incubated at 37°C for 10 min under stirring. Thereafter, 30 μM of substrate was added and aliquots (240 μL) were collected at 0, 7.5, 15, 30 and 60 min (serial hydrolysis). At each collect, the reaction was immediately stopped with 10 μL of trifluoroacetic acid (TFA) 1 M and stored at −40°C. From these aliquots, 150 μL were injected into Shimadzu chromatograph with SPD-10AV UV-Vis and RF-535 fluorescence detectors coupled to Ultrasphere C-18 column (5 μm/4.6 mm × 150 mm) equilibrated with solvent [90% acetonitrile + 0.1% TFA (v/v)] at 10%. Samples were solvent-eluted in 10% - 80% gradient at 1 mL·min^{-1} for 28 min and the column eluates were monitoredby their absorbance at 220 nm and by their fluorescence emissionat 420 nm following excitation at 320 nm. As chromatographic standards, were injected 150 μL of: 10 μM hydrolyzed substrate by sample for 15 h at 37°C; 10 μM non-hydrolyzed substrate; 8 μg·mL^{-1} protein + 40 mM TFA; and 8 μg·mL^{-1} proteins + 40 mM TFA + 5 mM o-phe. Linear regression curves were constructed with the data of product amount from serial hydrolysis and the results were expressed as percentage inhibition relative to control without inhibitor.

2.5. Data Analysis

The proteolitic activity (At) is a peptidic substrate function and there is no relation between quantification data and peptidic substrate. Thus, all of the at values from the same substrate were divided by the sample value that displayed maximum At and were rented as Relative Activity ($Ar = At/At_{max}$). Peptidases class was chosen according to the inhibition percentage that each peptidases inhibitor had on tested At. Therefore, the data were also relatively expressed. After the individual analyzes with each substrate we obtained a tissue-temporal map of the proteolytic activity in *A. colubrina* seeds.

3. Results

In this work, we perform an exploratory mapping of the peptidase activity in *A. colubrina* during and after germination seeds. The experiments were performed with crude protein extracts and synthetic substrates not with isolated peptidases and purified seed storage proteins (SSP), which would allow discerning, qualifying and quantifying the activity of specific peptidases. Therefore, no clear distinction was made among peptidases with specific function in SSP hydrolysis from peptidases related to other cellular processes, in spite of the activities were detected at germination. For the same reason, no distinction was made between

possible isoforms. Although many peptidases are common to embryonic axis(E) and cotyledons (C), since cotyledonary tissue is embryonic tissue, there are isoenzyme members differentially present between these tissues [34]. Thus, for results discussion, the same peptidase class, detected in both E and C samples, was considered as represented by enzymes common at both tissues and that the different relative activities (Ar) between these tissues were due to different enzyme concentration in the constant protein concentration of the samples. By this way, our data allowed to discern patterns of tissue-temporal proteolytic activity in the seed submitted to germination and indicate the classes of peptidases correlated with these activities.

In addition, radicle protrusion was used as criterion for germination of the seed batch. By this criterion, 4% of the seeds germinated at 12 h, 58% at 18 h and 93% at 24 h by hydration time (data not shown). No seed germinated in 6 h. Thus, the period up to 12 h refers to the beginning of the seed batch germination, 18 h at its end (>50%) and 24 h to post-germination events. On the other side, the proteolytic activity detected at 6 h was intermediate between 0 and 12 h and that detected at 18 h were intermediate between 12 and 24 h, both for E and C samples. Thus, the proteolytic data of 6 h and 18 h were not shown, being sufficient for analysis the times 0, 12 and 24 h as representative of the proteolytic events occurred respectively before, during and after germination of *A. colubrina* seeds.

3.1. Peptidases Detected with Abz-AIAFFSRQ-EDDnp Hydrolysis

Using Abz-AIAFFSRQ-EDDnp, which is specific for detecting endopeptidases, maximal activities were verified at pH 3 (Figure 1) and were 100% inhibited by pepstatin at all reactions tested (Table 1). The addition of cysteine peptidases (CP) activator DTT (5 mM) or CPs irreversible inhibitor E-64 (5 mM) at pH 5 and pH 7 did not result in activity change (Table 1). These results exclude the presence of CPs [35] and indicate that the activity observed were exclusive of aspartyl-endopeptidases (AP).

Table 1. Peptidases classes identified by Abz-AIAFFSRQ-EDDnp substrate. Peptidases class identificationn on proteic extracts from embryonary axis and cotyledons from *A. colubrina* obtained from Abz-AIAFFSRQ-EDDnp substrate clivage inhibition (%) caused different peptidases inhibitors. Values in parentheses = pH reaction; *null* = activity not altered by E-64 or DTT; (-) not tested.

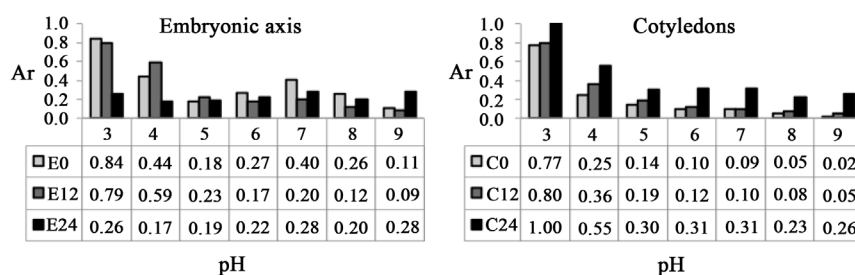| samples	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)
	Pepstatin/Aspartyl	E-64/Cysteine	PMSF/Serine	o-phe/Metallo
E0	100 (3)	*null* (5; 7)	-	-
E12	100 (3; 4)	-	-	-
E24	100 (3)	-	-	-
C0	100 (3)	-	-	-
C24	100 (3)	*null* (5; 7)	-	-

Figure 1. Relative activity (A*r*) at pH 3 to 9, quantified by spectrofluorometry, as a function of Abz-AIAFFSRQ-EDDnp hydrolysis in protein extracts from *A. colubrina* embryonic axis and cotyledons, excised at 0, 12 and 24 h from seed hydration (E0, E12, E24/C0, C12, C24).

Plant aspartyl-peptidases (AP), or phytepsins, are endopeptidases specifically inhibited by pepstatin. They have maximum activity at acidic pH on several organic or synthetic substrates and show preference to hydrolyze peptide bonds formed by hydrophobic residues [36] [37], mainly Leu-Tyr, Phe-Tyr and Phe-Phe bonds at insulin β-chain [38]; as is the case of Phe-Phe (FF) bond present in Abz-AIAFFSRQ-EDDnp.

The temporal activity profile *versus* pH activity profile in Abz-AIAFFSRQ-EDDnp hydrolysis suggests the presence of three APs differentially expressed by the tissues (Figure 1). The first AP (AP1) is stored in both dry seed tissues, as indicated by the activities of E0 and C0 in pH 3. The second AP (AP2) is synthesized during germination, as indicated by the higher E12 or C12 activity on that E0 or C0 at pH 4. AP2 differs from AP1 by being more active at pH 4, although both APs have maximum activity at pH 3. The third AP (AP3) is synthesized in the cotyledons after germination, as indicated by At_{max} at C24/pH3 and by it lower and stable activity at all pH values at C24. An AP with maximum activity at pH 3.5 and stable between pH 2 and pH 10 was characterized in *V. radiata* [37]. Similar pattern was described for a recombinant AP in *Arabidopsis* [38]. In *A. colubrina* there is also indicated a fourth endopeptidase (E0/pH7), whose class was not determined in this study.

These results suggest a sequential events where AP1 initiates the proteolysis in the protein bodies in embryonic axis and cotyledons, which is followed by AP2 mRNAs translation and after by AP3 mRNAs transcription and translation, mainly in cotyledons. The indication of these three APs in *A. colubrina* and the events sequence suggested is in accordance with the general seed pattern to store inactive forms of peptidases and their respective mRNAs during its maturation, as well to transcribe new mRNAs after germination. These suggest that peptidases and mRNAs stored in the embryos are sufficient for germination and that the largest mass of SSP is hydrolyzed from the storage tissues at post-germination events [2] [5].

In fact, APs are accumulated in protein bodies [39], hydrolyze SSP *in vitro* and *in vivo* [40] [41] [42], showing that APs should participate in SSP hydrolysis at initial germination stages, according to the criteria established by Shutov and

Vaintraub [1], followed to date to restrict peptidases and their functions to the specific processes of SSP hydrolysis: 1) the peptidase must be localized (or transported) next to its native substrate; 2) the peptidase must hydrolyze its native substrate *in vitro* and; (3) the temporal patterns between peptidase activity and substrate degradation *in vivo* must coincide. In addition, two or more cDNAs for different APs were obtained from dry seeds and during germination in buckwheat [43], wheat [41], soybean [44] and *Arabidopsis* [45], as well as aspartyl activity was observed during seed germination in *Araucaria* megagametophyte [46].

3.2. Peptidases Detected with Abz-FRAK(Dnp)-OH Hydrolysis

The hydrolysis of Abz-FRAK(Dnp)-OH, which was designed for detect carboxypeptidase activities, shown that the activity increased with time in both tissues, but in different pH profiles (Figure 2). This suggests the presence of different peptidases stored and synthesized during and after germination in each tissue. According the results, these peptidases belongs to the classes of AP, CP and metallo-peptidases (MP), at embryonic axis; and to CP and MP classes at cotyledon, as indicated by the inhibitors effectiveness (Table 2 and Table 3).

The indication of AP and CP presences were respectively based on the relative spectrofluorometric quantification of Abz released during Abz-FRAK(Dnp)-OH hydrolysis from samples pre-incubated with pepstatin or E-64 (Table 2). By the other side, the indication of MP presence was based on the relative chromatographic
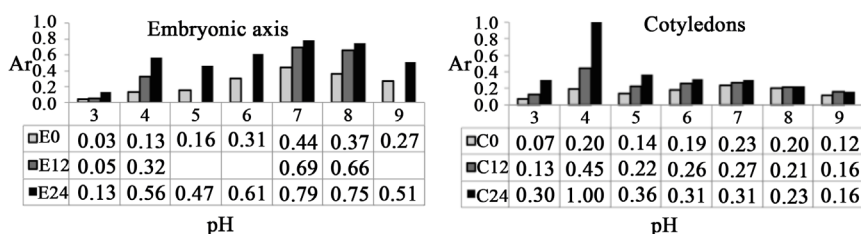

Figure 2. Relative activity (Ar) at pH 3 to 9, quantified by spectrofluorometry, as a function of Abz-FRAK(Dnp)-OH hydrolysis, in protein extracts from *A. colubrina* embryonic axis and cotyledons, excised at 0, 12 and 24 h from seed hydration (E0, E12, E24/C0, C12, C24).

Table 2. Peptidases classes identified by Abz-FRAK(Dnp)-OH substrate. Peptidases class identification on proteic extracts from embryonary axis and cotyledons from *A. colubrina* obtained from Abz-FRAK(Dnp)-OH substrate clivage inhibition (%) caused different peptidases inhibitors. Values in parentheses = pH reaction; null = activity not altered by E-64 or DTT; (-) not tested.

Samples	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)
	Pepstatin/Aspartyl	E-64/Cysteine	PMSF/Serine	o-phe/Metallo
E24	36 (4)	57 (4); 3^{nr} (7)	1^{nr} (7)	HPLC (4; 7)
C24	14^{nr} (4)	87 (4)	-	HPLC (4)

Table 3. Abz-FRAK(Dnp)-OH hydrolyzed by samples E24/pH 4, C24/pH 4 and E24/pH 7 generated 3 fragments [Rt = 11.8; 12.3 and 12.7 (Figure 3)] obtained in proportional amounts to hydrolysis times (ht = 7.5, 15, 30, 60 min) with or without o-phe [inhibited (i) and control (c), respectively]. Linear regression curves were constructed with the peak area values of these quantities [angular coefficient (α) and R^2 indicated]. The substrate hydrolysis inhibition per sample is given by i/c ratio of the total quantities (Qt) [or relative quantities (Qr)] obtained by the sum of i and c of all ht peak values of the 3 fragments.

	ht	E24/pH4		C24/pH4		E24/pH7	
		i	c	i	c	i	c
Rt 11.8	7.5	100,188	274,256	568,197	964,183	166,120	407,029
	15.0	126,810	326,520	951,756	1,467,040	325,765	556,018
	30.0	239,538	528,838	1,075,611	1,969,896	530,966	852,088
	60.0	387,110	905,879	1,136,122	2,389,160	993,327	1,485,255
	a/R^2	5601/0.991	12,320/0.996	8669/0.623	25,171/0.896	15,432/0.997	20,559/0.999
Rt 12.3	7.5	223,359	256,982	201,983	272,680	39,393	62,147
	15.0	316,272	325,827	423,603	388,173	0	63,848
	30.0	536,795	519,355	451,907	503,666	0	76,122
	60.0	925,441	911,347	644,766	659,826	0	99,579
	a/R^2	13,447/0.999	12,643/0.998	7218/0.854	6961/0.955		740/0.990
Rt 12.7	7.5					0	51,812
	15.0					0	68,870
	30.0					0	114,616
	60.0					0	200,842
	a/R^2						2872/0.999
	Qt	2,855,513	4,049,004	5,453,945	8,614,624	2,055,571	4,038,226
	Qr	0.33	0.47	0.63	1	0.24	0.47
Inhibition (%)		29		37		49	

quantification of three fragments released from this hydrolyzed substrate by samples pre-incubated with o-phe (Table 3). These fragments presented different retention times (Rt): a prominent peak (Rt = 11.8 min) was observed in the three samples; a second equally prominent peak (Rt = 12.3 min) in E24/pH 4 sample, which is greatly reduced at C24/pH 4 and insignificant at E24/pH 7; and a third little expressive peak (Rt = 12.7 min), observed only at E24/pH 7 (Figure 3).

With hydrolysis inhibition of Abz-FRAK(Dnp)-OH was observed carboxypeptidase activity of APs, CPs and MPs at acidic and neutral pH. The detected acidic CPs activity could be related with plant cathepsin-like. Although cathepsins are usually endo-CPs from papain family, several human cathepsins have preference to Lys (K) and Arg (R), as indicated by assays with a large substrates set [47] and human cathepsin-B has carboxypeptidase activity at acidic pH on Abz-FRAK(Dnp)-OH [33] [48]. In plants, homologous genes to cathepsin-B were described in wheat (*T. aestivum*), barley (*H. vulgare*) and *Arabidopsistha-liana*. In wheat [49] and barley [50], plant cathepsin-B is regulated by abscisic
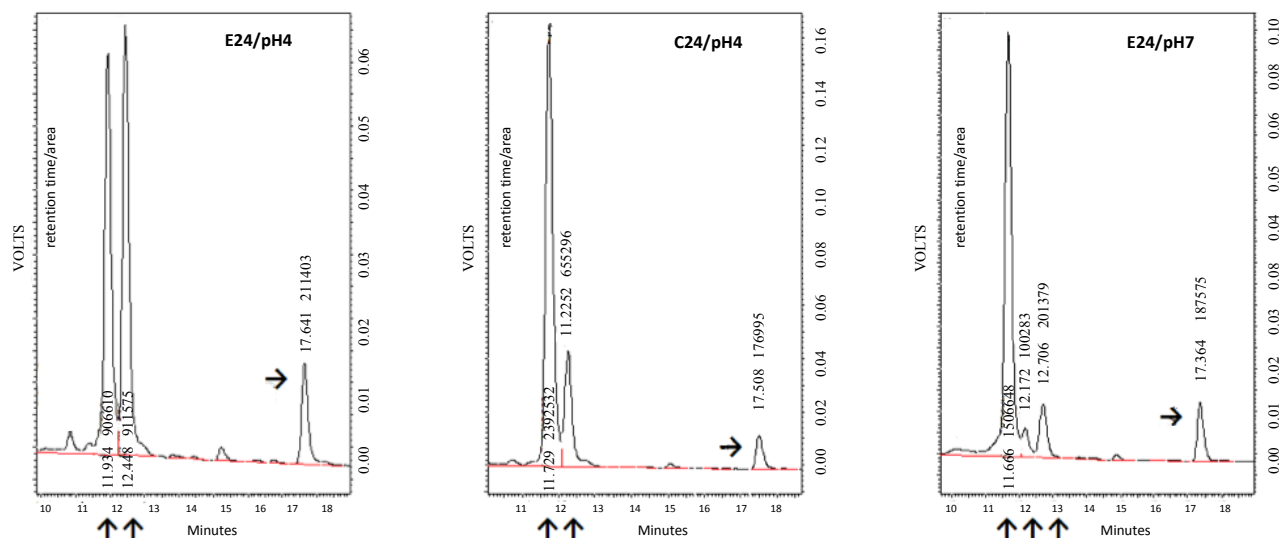
Figure 3. HPLC analysis of Abz-FRAK(Dnp)-OHhydrolyzed almost entirely by E24/pH 4, C24/pH 4 and E24/pH 7 samples showing 3 fragments that generated peaks at retention times (R*t*) 11.8; 12.3 and 12.7 min approximately (vertical arrows below the graphs). Peak values = R*t* and peak area. Horizontal arrow indicates peak of non-hydrolyzed substrate.

acid (ABA) and gibberellin (GA) in aleurone layers and its expression increases with germination, as well in *Arabidopsis*, were a gene for cathepsin B-like, *At-CathB*3, is expressed at germination and post-germination events in embryonic axis and cotyledons, respectively [51]. Several plant cathepsins were discovered, among them the HvPap-1, a barley cathepsin-F [52]. Recombinant HvPap-1 degrades *in vitro* synthetic substrates and the native hordeins, albumins and globulins. The enzyme is immunodetected in the protein bodies (acidic environment) and the gene is expressed in isolated aleurone layers under treatment of GA. These information support the direct participation of HvPap-1 in SSP hydrolysis during germination. As it was indicated that the carboxypeptidase activity detected in E24/pH4 and C24/pH4 (**Figure 2**) can be due to CPs (**Table 2**), it seems possible that plant cathepsin-like participate in post-germination events in *A. colubrina*. However, carboxypeptidase activities of APs and MPs at acidic pH, detected in the assays with Abz-FRAK(Dnp)-OH, are conflicting data.

According to MEROPS database [7], none carboxypeptidase belongs to APs. All known APs are restricted endopeptidases (endo-APs). However, the amino acids sequence from plant APs has 45% of homology with animal cathepsin-D (EC 3.4.23.5) [44]. Indeed, cathepsin-D is an endo-AP classified as member of A1 family (pepsin, phytepsin; MEROPS classification), which are active at acid pH and inhibited by pepstatin. *Arabidopsis* has three genes described for cathepsin D-like, one of them is expressed only in seeds [53]. APs from the pepsin family, as well as CPs from the papain family, cleave a wide variety of substrates. Abz-FRAK(Dnp)-OH was used to characterize carboxypeptidase activity at acidic pH of cathepsin-B of the papain family [33] [48] and *AtCathB3* (cathepsin B-like) is related to germination [51]. Thus, it seems possible that this substrate is also cleaved by some plant cathepsin-D-like from pepsin family. However,

based on more than 800 assays deposited at the MEROPS database, it appears that human cathepsin-D has little preference to hydrolyze Abz-FRAK(Dnp)-OH, although this substrate is not included in these assays. Therefore, given the available information and our data limits, it is unreasonable to indicate the presence of a carboxy-AP in *A. colubrina*, but a carboxy-CP from A1 family seems to be possible.

MEROPS database [7] also revealed that plant MPs are generally endo- or amino-peptidases. No carboxy-MP has been characterized in plants. However, gene sequences relating to M14 family, which contains carboxy-MPs, has been deposited for many plant species. But MPs are characteristically neutral enzymes. Therefore, it is plausible that o-phe inhibited the activity of a possible plant carboxy-MP at E24/pH 7, but the same occurrence at E24/pH 4 and C24/pH 4 is not consistent (Table 3). The substrate Abz-FRAK(Dnp)-OH was used to characterize carboxy-CPs activity at acidic pH; neverthelesscarboxy-MPs has affinity to this substrate (Prof. LuizJuliano, personal communication). Thus, it seems possible that this substrate is cleaved by carboxy-CPs (at acidic pH) and by carboxy-MPs (at neutral pH). In fact, the cleavage profile of this substrate was more similar among different tissues at the same pH, than among different pH in the same tissue. In Figure 3, it may be noted that R*t*11.8 and R*t*12.3 fragments are released by E24 and C24 in pH4, while the R*t*11.8 and R*t*12.7 fragments are released only by E24 in pH7. Furthermore, E-64 was more effective in the inhibition of C24/pH 4 activity than E24/pH 4 activity, but did not inhibited E24/pH 7 (Table 2), whereas o-phe inhibited E24 activity more significantly at pH 7 than in pH 4 (Table 3).

By the data set from Abz-FRAK(Dnp)-OH hydrolysis, it seems more plausible to suppose that, at acid pH, o-phe inhibited not specifically the activity of some carboxy-CP (possibly a cathepsin-like) and that, at neutral pH, specifically inhibited the activity of some carboxy-MP, not yet described in plants.

3.3. Peptidases Detected with F-MCA Hydrolysis

The substrate F-MCA is generally used to detect aminopeptidases and the results shown that the major activities occurred at pH 7 (Figure 4) due to serine-peptidases (SP) and MPs (Table 4).

The temporal activity profile in E samples describes an increase in activity during germination, followed by decay after that, mainly at pH 7. By the other side, the temporal activity of C samples profile was constant (Figure 4). These results suggest activation of stored enzymes in E followed by synthesis *de novo* and enzyme degradation after germination, possibly due SSP stocks depletion. The uniformity of activity in C does not show whether a similar process has occurred.

Plant metallo-aminopeptidases (amino-MPs) are distributed into M1, M17 and M24 families [7]. This distribution is mainly based on the *Arabidopsis* genome sequencing. Among the amino-MPs of these families, the most likely to
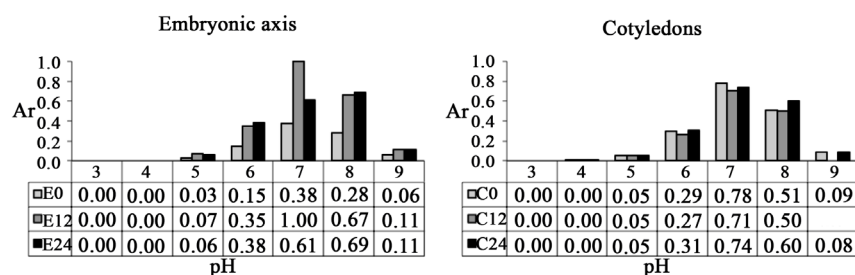
Figure 4. Relative activity (A*r*) at pH 3 to 9, quantified by spectrofluorometry, as a function of F-MCA hydrolysis, in protein extracts from *A. colubrina* embryonic axis and cotyledons, excised at 0, 12 and 24 h from seed hydration (E0, E12, E24/C0, C12, C24).

Table 4. Peptidases classes identified by F-MCA substrate. Peptidases class identification on proteic extracts from embryonary axis and cotyledons from *A. colubrine* obtained from F-MCA substrate clivage inhibition (%) caused different peptidases inhibitors. Values in parentheses = pH reaction; *null* = activity not altered by E-64 or DTT; (-) not tested.

Samples	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)
	Pepstatin/Aspartyl	E-64/Cysteine	PMSF/Serine	o-phe/Metallo
E0	-	-	38 (7)	76 (7)
E12	-	-	35 (7)	77 (7)
E24	-	19^{nr} (7); 5^{nr} (8)	47 (7); 13^{nr} (8)	74 (7); 73 (8)
C0	-	-	32 (7)	77 (7)
C12	-	-	32 (7)	80 (7)
C24	-	0 (7); 13^{nr} (8)	39 (7); 14^{nr} (8)	77 (7); 75 (8)

hydrolyze F-MCA at neutral pH are: leucilaminopeptidase (M17), which is related to wounds and pathogens responses and it was detected in barley [54]; and membrane alanylaminopeptidase, or aminopeptidase M1 (APM1), which hydrolyzes an inhibitor of auxin transport. Loss-of-function mutants (*apm*1) in *Arabidopsis* result in uncoordinated cell divisions during embryogenesis and lethality after germination [55]. Studies with serine-aminopeptidases (amino-SP) in plants are still rarer. There were relates of a tripeptidyl-peptidase II (TPPII) purification from *Arabidopsis*, which hydrolyzes AAF-MCA at pH 7 and is inhibited by PMSF [56]. TPPII is an intermediate cytoplasmic exopeptidase in amino acids recycling centralized on proteasome complex [57]. It is not known if TPPII acts on the cytoplasmic phase of SSP degradation, since no direct function is attributed to proteasome on germination. However, proteasome degrades proteins that promote dormancy in *Arabidopsis*, allowing germination to proceed [58].

3.4. Peptidases Detected with Z-FR-MCA Hydrolysis

The hydrolysis of Z-FR-MCA, generally substrate for CP and SP, was more expressive in the E samples, in which the activity increased with time and pH scale (Figure 5). At all times in E samples, the activity was inhibited by o-phe,
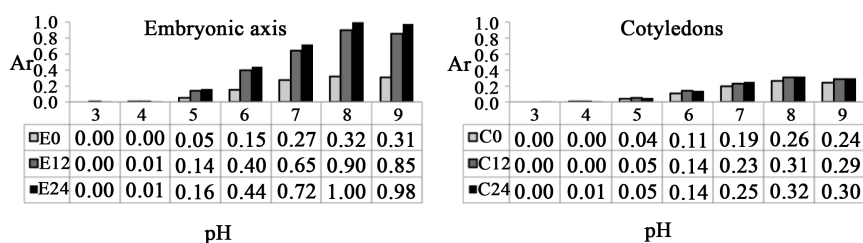
Figure 5. Relative activity (A*r*) at pH 3 to 9, quantified by spectrofluorometry, as a function of Z-FR-MCA hydrolysis, in protein extracts from *A. colubrina* embryonic axis and cotyledons, excised at 0, 12 and 24 h from seed hydration (E0, E12, E24/C0, C12, C24).

indicating presence of MP, whereas in E24, it was also inhibited by E-64 and PMSF, indicating presence of CP and SP, respectively (Table 5). The low activity of C samples did not increase in the presence of 5 mM DTT (data not shown), indicating that CP activity had already reached its maximum.

Legumains are a group of endo-CP related to storage and hydrolysis of globulins in some model plants. One of the major evidences for this is the fact that they are stored together with globulins in the acidic environment of the protein bodies [34] [59]. In fact, the maximum activity of these purified CPs occurred at acid pH [59] [60], including Z-FR-MCA as substrate [61]. Therefore, our results indicate that the cysteine activity detected with Z-FR-MCA in *A. colubrina* embryonic axis is related to another cellular process, since the maximum activity occurred in neutral-alkaline pH (Figure 5). The only peptidase found for this condition was the cytoplasmic phytocalpain, encoded by corn DEK-1 (Defective Kernel) and its orthologues in 11 species. This CP, associated to cell polarization, is a key enzyme in cell proliferation and differentiation during plant organogenesis, including leaf primordia [62]. According to phytocalpain function, the temporal profile of neutral-alkaline CP activity in the embryonic axis, as well as the possible presence of these enzymes in lower concentration in cotyledons, suggests a process of seedling development.

Due to the low proteolytic activities in C samples (Figure 5), the cysteine, serine and metallo activities were not tested. However, it is assumed that this low activity is due to lower concentrations of the same peptidases detected at high concentration in E after germination – which makes it strange that neutral-alkaline activity of CP, as well as SP and MP, is low in *A. colubrina* cotyledons. Although there are CPs, such as bean LLP [63] and soybean C2 protease [64], which are detected around 5 days after hydration, there are also SPs, such as soybean protease C1 [65] and several CP of *Vicia sativa* [34], that are stored in protein bodies from embryonic axis and cotyledon, which are activated by hydration. On the other hand, neutral-alkaline amino-MP with preference for Phe or Leuare associated with the final globulin hydrolysis. Acidic endo- and carboxy-peptidases release oligopeptides that are diffused from protein bodies into cytoplasm, where they are finally cleaved by neutral-alkaline SP and MP [1]. Considering that this final phase of SSP hydrolysis must be intense in protein storage tissues, it is possible that metallo activity would increase after 24 h in

Table 5. Peptidases classes identified by Z-FR-MCA substrate. Peptidases class identification on proteic extracts from embryonary axis and cotyledons from *A. colubrine* obtained from Z-FR-MCA substrate clivage inhibition (%) caused different peptidases inhibitors. Values in parentheses = pH reaction; *null* = activity not altered by E-64 or DTT; (-) not tested.

Samples	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)	Inhibitor/Peptidase (class)
	Pepstatin/Aspartyl	E-64/Cysteine	PMSF/Serine	o-phe/Metallo
E0	-	23^{nr} (8)	8^{nr} (8)	52 (8)
E12	-	21^{nr} (8)	14^{nr} (8)	56 (8)
E24	-	52 (8)	56 (8)	52 (8)

A. colubrina cotyledons. Another possibility to explain this tissue-temporal profile of neutral-alkaline MP in *A. colubrina* takes place by the action of plant matrix metallo-peptidases (MMP). MMP are cytoplasmic endo-MP associated with cell membrane remodeling in developmental and/or wound processes [66]. The temporal stability of the metallo activity in the Z-FR-MCA hydrolysis by E samples (Table 5) suggests proteolytic activity associated with constant remodeling of membranes in developing tissues and not activity associated with SSP hydrolysis, which is expected to increase with the germination, especially in cotyledons. However, it seems that MMP has no preference for Phe or Arg [8].

Among plant SP, a reasonable assumption is the trypsin-like plant oligopeptidase-B (SC clan, S9 family, MEROPS classification). This enzyme is expressed in quiescent and germinating embryos of wheat and cleaved Z-FR-MCA with maximum activity at pH 8.5 [67]. Their role in plants and their native substrate is not known, but due to their cleavage specificities and inefficiency against some protein substrates, Tsuji *et al*. [67] discuss their role in the cytoplasmic protein turn-over and not in the SSP final hydrolysis. A similar result was observed in *Canavalia ensiformis*. A SP purified from seeds showed high preference for Arg (Z-RR-MCA) at pH 8 and poor efficiency against the native concavaline [68]. Although the final stages of SSP degradation occur in the cytoplasm by neutral peptidases, which do not recognize the unmodified native protein present in the acidic environment of the protein bodies [1], the indication of the plant oligopeptidase-B does not participate in SSP hydrolysis [67] is consistent with the low proteolytic activity in *A. colubrina* cotyledons on Z-FR-MCA hydrolysis (Figure 5), considering that the SP detected in the embryonic axis is also present in cotyledons, although at low concentrations.

4. Discussion

Using fluorescent peptides and peptidase inhibitors, we detected several proteolytic activities associated with aspartyl-, cysteine-, metallo- and serine-peptidases (AP, CP, MP and SP, respectively) in protein extracts from *A. colubrina* embryonic axis (E) and cotyledons (C) excised before, during and after germination. The experimental conditions do not allow to directly relating the observed

activities in E and C samples with specific germination events, since they do not allow discerning isolated peptidases, specific functions or native substrates. However, it was possible to make inferences about the possible peptidase identity related to the activities detected. These inferences were made from the determination of the catalytic class (based on pH of enzymatic activity, substrates used and peptidase inhibitors effectiveness) and from the tissue-temporal profile of seed proteolytic activity.

Considering experimental conditions, we speculate that phytepsins (AP group) initiate the SSP hydrolysis in *A. colubrina*. In cultivated dicots, the most common enzymes in SSP hydrolysis are CPs. Legumains and other CPs are involved in reserve globulin processing during seed maturation and its hydrolysis within the acidic protein bodies during germination [59] [63]. The substrate Abz-AIAFFSRQ-EDDnp was designed to specifically detect endo-CPs (Dr. LuizJuliano, personal communication), but acidic endo-CPs were not detected. Only AP activity at acidic pH was observed in *A. colubrina* using this substrate. By the other side, legumains are not able to cleave Abz-AIAFFSRQ-EDDnp, since they has restricted specificity *in vitro* to cleave substrates with Asn (N) at P1 position[3] [69]. Nevertheless, no activity at acid pH was detected using Abz-KLNSSKQ-EDDnp as substrate (data not shown).

Although non-detection of CPsusing these substrates does not exclude the possibility of CPs presence in *A. colubrina* seeds, phytepsins have been directly associated with SSP hydrolysis, by their presence in protein bodies [39], and ability to hydrolyze *in vitro* native substrates from peanut (*Arachis hypogaea* L.) [42] and wheat (Dunaevsky *et al.* 1989; [41] [70]). Phytepsins were also indirectly associated with germination in soy [44], buckwheat [43], *Arabidopsis* [45], wheat [41] and *Araucaria* [46].

However, Shutov and Vaintraub [1] consider that protein bodies has pH 4 - 5 and globulins, at pH 3, undergo conformational changes that would expose cleavage sites toAPs, which explain their participation in the SSP hydrolysis, but not to trigger the process, likewise CPs. In fact, the APs temporal activity profile in *A. colubrina* embryonic axis is different between pH 3 and pH 4. However, a MP initiate the legumin hydrolysis in buckwheat (*Fagopyrum esculentum*) [71], which indicates that native globulins can be cleaved by more than one class of peptidases. Anyway, the temporal activity of endo-APs in *A. colubrina* it is in agreement with the fact that embryonic axis readily consume its reserves by the activity of peptidases stored during seed maturation and synthesized during germination, while cotyledons increase peptidases synthesis mainly after germination, in order to increase the amino acids supply to the growing seedling. Thus, regardless of phase or possible presence of CPs, phytepsins may participate in *A. colubrina* SSP hydrolysis.

In the case of carboxy-MP activity detected by the hydrolysis of Abz-FRAK(Dnp)-OH, it was not possible to suppose some specific enzyme,

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[3]P1 refers to the 1st residue of sessile bond in the substrate accommodated by the 1st residue of enzyme catalytic site at N-terminal orientation.

since carboxy-MP is not described in plants, although gene sequences for several plant species are deposited in MEROPS databank. Carboxypeptidases that act with endo-CP in the globulin hydrolysis are SP (carboxy-SP) [1]. In fact, carboxy-SP were confirmed in the germination of wheat [70] and triticale [72] (Drzymała and Bielawski 2009). However, we detected expressive carboxy-CP activity at acidic pH by Abz-FRAK(Dnp)-OH hydrolysis, mainly in cotyledons; and carboxy-CP (plant cathepsin-like) are described in wheat aleurone [49] (Cejudo *et al.* 1992), barley [50] (Martinez *et al.* 2003) and germinative and post-germinative events in *Arabidopsis* [51] (Iglesias-Fernández *et al.* 2014). Therefore, it is possible that some plant cathepsin-like is the carboxypeptidase that acts together with phytepsins in *A. colubrina* SSP hydrolysis. Considering the acidic activities of endo-AP and carboxy-CP, detected after germination at higher levels in cotyledons than the axis, both enzymes should act sequentially in the protein bodies and would be responsible for the SSP hydrolysis in *A. colubrina*.

In general, these information agrees with the observations of Tan-Wilson and Wilson [10] where the pathway of SSP hydrolysis in different plants present more similarities about cleavage sites than peptidases that cleaves them. Due evolutionary implications, it considers that there should be caution with generalizations about proteolytic mechanism of SSP made from models formulated on basis in few long-cultivated species and, therefore, studies should be expanded to wild species [10]. Similar considerations were made by Bewley and Black [73], which consider the regulatory mechanisms of germination a reflex from the biological diversity.

On the other hand, none of neutral-alkaline peptidases detected appear to be involved in cytoplasmic phase of SSP final degradation in *A. colubrina*. Cytoplasmic amino- and di-peptidases from metallo and serine classes are involved in final degradation of the oligopeptides generated by acidic endo- and carboxy-peptidases activities within protein bodies [1]. Although amino-MP and amino-SP activities were detected with F-MCA, the tissue-temporal profile of these activities and literature informations, indicated as supposed enzymes those related with meiotic prophase (metallo APM1 [55]) or with protein turn-over (serine TPPII [56]). Likewise, the alkaline CPs, MPs and SPs activities detected with Z-FR-MCA are indicative of the presence of phytocalpain, MMP and oligopeptidase-B, which are related with developmental processes, in general.

5. Considerations

We generated initial information about the proteolytic activity in *A. colubrina* germinating seeds. Our results showed that *A. colubrina* germination presents different peptidases from that commonly found in cultivated leguminous. In addition, we possibly detected a peptidase with useful characteristics for industrial purposes and another one not yet described in plants. This study reinforces the need to amplify the detection of plant peptidases on a diverse genetic basis, since

the research focused on commercial species is a limiting factor for the knowledge of proteolysis in germination, as in other proteolytic processes in the plant kingdom, and the potential technological use of plant peptidases. In this context, the scanning method chosen for this study can be applied in comparative studies of germination between different species and in other biological processes aiming wide detection of peptidases.

Acknowledgements

Conflict of Interest

The authors declare that they have no conflict of interest.

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