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Characterization, subsite mapping and N-terminal sequence of miliin, a serine-protease isolated from the latex of *Euphorbia milii*

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ABSTRACT

Miliin is a serine protease purified from the latex of *Euphorbia milii*. This work reports the effect of pH and temperature on the catalytic activity of miliin, using fluorescence resonance energy transfer (FRET) substrates. Miliin displayed the highest activity at pH 9 and 35 °C. Subsite mapping shows that subsites S_2 to S_2' prefer uncharged residues. The S_2 subsite prefers hydrophobic aliphatic amino acids (Val, Pro and Ile) and defines the cleavage site. This work is the first one that reports subsite mapping of *Euphorbiacea* proteases. The N-terminal sequence showed higher similarity (40%) with the serine protease LIM9 isolated from *Lilium*. The presence of Tyr, Pro and Lys at positions 2, 5 and 10 respectively, were observed for most of the serine proteases used for comparison. The N-terminal sequence has striking differences with those reported previously for milin and eumiliin, other serine proteases isolated from the latex of *E. milii*.

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1. Introduction

Proteases occur naturally in all organisms and proteolysis is essential for the normal functioning of multicellular organisms. Plant proteases are involved in all aspects of the plant life cycle involving a variety of functions, from mobilization of storage proteins during seed germination to the initiation of apoptosis and senescence programs, and presently they are produced both *in vivo* and *in vitro*, by cell culture and by the expression of recombinant proteases [1,2]. Recently, plant proteases have been receiving special attention from both the pharmaceutical and biotechnology industries, due to their activity over wide ranges of temperature and pH. Possible applications of plant serine proteases include dairy industry, food processing, brewing industry and even in molecular biology, as a protection against nucleases [3–6], since they display high specific activity. Biotechnological developments and particularly protein engineering, predict the advent in the near future of plant proteases with novel and improved industrial properties [7].

The crude latex of Crow-of–Thorns (*Euphorbia milii*) has been reported for medical purposes as potent molluscicidal agent used in schistosomiasis control and a promising alternative to niclosamide (NCL) [8,9]. Other suggested potential applications include antithrombotic drugs and in the food and biotechnology industries [10,11].

Serine endo- and exo-peptidases catalyze peptide bond cleavage via nucleophilic attack of the target carbonyl bond; this involves the formation of an acyl-enzyme intermediate with a reactive serine residue and they are grouped into twelve clans [12,13]; the largest two are the (chymo) trypsin-like and subtilisin-like clans, which possess a highly similar arrangement of catalytic His, Asp, and Ser residues in different β/β (chymotrypsin) and α/β (subtilisin) protein scaffolds [5]. The majority of serine proteases extracted from plants belong to the S8 family (clan SB) which is divided into two subfamilies: S8A and S8. Members of family S8 have a catalytic triad in the order Asp, His and Ser in the sequence and most members are nonspecific peptidases with a preference to cleave after hydrophobic residues, while members of subfamily S8B, such as kexin and furin, cleave after basic amino acids, according to the MEROPS database [14].

Following the scheme proposed by Berger and Schechter [15] the residues on the N-terminal side of the scissile bond of the substrate are labeled P1, P2, P3, and P4, while the residues on the C-terminal side of the scissile bond are labeled P1′, P2′, P3′, and P4′. This nomenclature gives rise to corresponding subsites on the protein, termed S1, S2, S3, S4 and S1′, S2′, S3′, and S4′, that interact with the equivalent residues of the substrate (Fig. 1).

Miliin, a new thiol-dependent serine protease purified from the latex of *Euphorbia milii* possesses a molecular weight of 79 kDa, an isoelectric point of 4.3 and activity at temperatures up 60 °C in the

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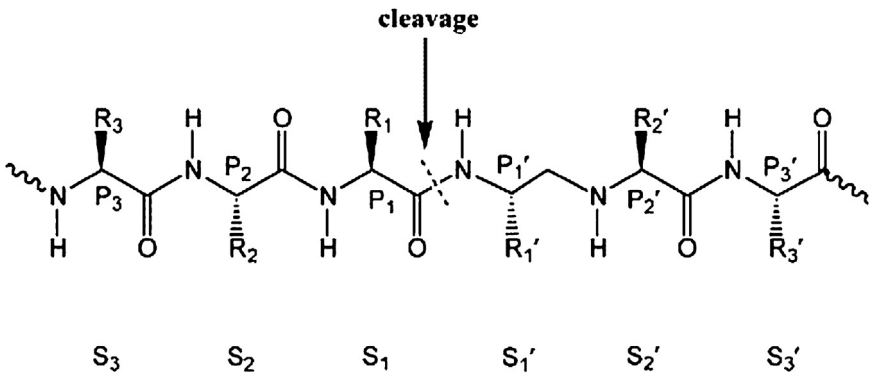

Fig. 1. Scheme of protease specificity [49]. It depends upon the amino acid side chains of the peptidic substrate (P3 through P3′) and the surface contacts with the enzyme's subsites (S3 through S3′), according to the scheme of Berger and Schecter [15].

pH range from 7.5 to 11.0 [11]. The present paper reports miliin's characterization, investigating the specificity of the enzyme subsites S_2 to S_2' using fluorescence resonance energy transfer (FRET) peptides derived from the peptide Abz- KLRSSKQ-EDDnp (Abz, *ortho*-aminobenzoic acid; EDDnp, ethylenediaminedinitrophenyl; Abz/EDDnp: donor/acceptor fluorescent pair). Miliin was also characterized using FRET peptides with peptide families derived from the lead sequence Abz-KLLFSKQ–EDDnp. This is the first work that reports subsite mapping of *Euphorbiacea* proteases.

2. Experimental procedures

2.1. Enzyme

Miliin from the latex of *Euphorbia milii* was purified as described [11]. The molar concentration of the enzyme solutions was determined by active-site titration with phenylmethylsulphonyl fluoride (PMSF) [16,17].

2.2. Peptides

All FRET peptides were obtained by the solid-phase peptide synthesis strategy as described previously [18] and using the *N*-(9-fluorenyl)methoxycarbonyl (Fmoc) (Bachem, Torrance, CA, USA) procedure in an automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu, Tokyo, Japan). The peptides were synthesized in NovaSyn_TGRresin (Novabiochem-Merck) loading 0.2 mmol/g and using 2-(1-H-benzotriazole-1-yl) 1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole as coupling reagent, and the cleavage of peptide-resin was accomplished with trifluoroacetic acid:anisol:1,2-ethanedithiol:water (85:5:3:7) (Fluka Buchs). All peptides were purified by semi-preparative HPLC on an Econosil C-18 column. The molecular weight and purity of synthesized peptides (94% or higher) were checked by amino acid analysis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry, using a TofSpec-E (Micromass, Manchester, UK). Stock solutions of the peptides were prepared in *N*,*N*-dimethylformamide, and the concentrations were measured spectrophotometrically using the molar extinction coefficient of 17,300 M^{-1} cm^{-1} at 365 nm [18].

2.3. Determination of the cleavage sites

The cleavage sites were determined by LC/MS–2010 from Shimadzu with an ESI probe. HPLC was performed using a binary system from Shimadzu with a SPD-20AV UV–vis detector and a RF fluorescence detector, coupled to an Ultrasphere C18 column (5 μM, 4.6 mm × 250 mm) which was eluted with solvent systems A1 (H_2O/TFA 1:1000) and B1 (ACN/H_2O/TFA 900:100:1) at a flow rate of 1.0 mL min^{-1} and a 10–80% gradient of B1 in 20 min. The HPLC column elutes were monitored by their absorbance at 220–365 nm and by fluorescence emission at 420 nm, upon excitation at 320 nm.

2.4. Enzyme assays and kinetic measurements

Hydrolysis of the FRET peptides was assayed in a RF-5301PC (Shimadzu) spectrofluorimeter at 37 °C. Control experiments were carried out to check whether the substrates presented hydrolysis in the absence of the enzyme, giving negative results. The assays were performed in 50 mmol L^{-1} glycine buffer, pH 9.0. Fluorescence changes were monitored continuously at 320 nm (excitation) and 420 nm (emission). The enzyme concentration for initial rate determinations was chosen at a level intended to hydrolyze less than 5% of the added substrate over the time course of data collection. The slope was converted into micromoles of substrate hydrolyzed per minute based on a calibration curve obtained from the complete hydrolysis of each peptide. The inner-filter effect was corrected using empirical equations as previously described [19]. The apparent second-order rate constant (k_{cat}/K_m) was determined at low substrate concentrations where the reactions follow first-order conditions ([S] « K_m). The experiments were performed in duplicate and the errors were less than 5% for any of the obtained kinetic parameters. For peptides hydrolyzed at more than one site, the apparent k_{cat}/K_m values correspond to the sum of the individual values of k_{cat}/K_m for each cleavage site [20].

2.5. Optimum pH and temperature determination

The pH dependence was studied using the substrate Abz-KLLFSKQ-EDDnp over the pH range from 4.5 to 11.0, performing the experiments in duplicate; the standard deviation was lower than 5%. Buffers were used at 50 mmol L^{-1} concentration to maintain a constant pH: acetate (pH 4.5–5.5); *N*-(2-acetamido)iminodiacetic acid (ADA) (pH 6.0–7.0); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) (pH 7.5); *N*-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid (Taps) (pH 8.0–8.5); glycine (pH 9.0–9.5) and 3-(Cyclohexylamino)-1-propanesulfonic acid (Caps) (pH 10.0–10.5–11.0). Enzymatic activity upon the FRET peptide Abz-KLLFSKQ-EDDnp was followed using the continuous fluorimetric assay, at 37 °C. Similarly, the effect of temperature on the activity was also studied to determine the optimum temperature for the enzyme. Miliin was incubated at the desired temperature, in the range from 25 to 65 °C.

2.6. Effect of monovalent and divalent ions on the hydrolysis of Abz-KLLFSKQ-EDDnp

The protease was assayed in the presence of chloride salts of different metal ions including Ca^{2+}, Mg^{2+}, Zn^{2+}, Hg^{2+}, Na^{+} and Li^{+} at 0.5 mM (final concentration). The reactions were performed under pseudo first-order conditions of substrate hydrolysis [21] in 50 mmol L^{-1} glycine buffer pH 9.0. The experiments were carried out in duplicate and the standard deviation was lower than 5%. The activity of the enzyme assayed in the absence of added salts was taken as control and considered as 100%.

2.7. N-terminal sequencing analysis

Automated Edman degradation (HPLC system) of *E. milii* protease was performed with a Shimadzu Corporation (Tokyo, Japan) PPSQ/23 protein sequencer.

3. Results and discussion

3.1. Effect of pH and temperature on miliin activity

The profile of activity vs. pH (Fig. 2) displayed high levels of hydrolyzing activity above optimal pH 9.0; this characteristic suggests its suitability in a variety of food and biotechnology industries that demand enzymes with high activity at alkaline pH.

According to the literature serine proteases show maximal activity at pH values between 8 and 10.5, like cucumisin from *Cucumis melo* [22], and the enzymes from *Cucurbita ficifolia* [23] and *Benincasa cerifera* [24], which display an optimum pH of 9.2. Other serine proteases such as those extracted from *Taraxacum officinale* (taraxilin) [25] and *Euphorbia milii* (milin and eumiliin) [10,26] present an optimum pH of 8.0.

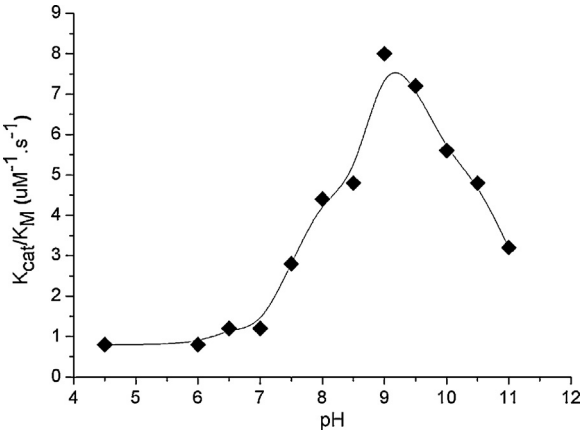

Fig. 2. Influence of pH on hydrolysis of Abz-KLLFSKQ-EDDnp by purified miliin. The assays were performed at 37 °C in 50 mmol L^{-1} glycine buffer, pH 9.0.

Concerning the effect of temperature, the catalytic activity displayed a maximum value around ca. 35 °C using a FRET substrate (Fig. 3). The hydrolytic activity of Taraxalisin [25] a subtilisin-like proteinase with synthetic substrate presented similar maximum temperature at 40 and 35 °C respectively. Another plant subtilisin-like proteinase from *Heliantus annus* was examined using synthetic substrates and presented maximum activity at 55 °C [27]. Optimum temperatures for serine proteases have been reported in the range from 40 to 70 °C using natural substrates. *Trichosantus kirrilowi A* [27] displayed an optimum temperature of 70 °C using casein as substrate. These high values could be a result of simultaneous thermal denaturation of the substrate, which would ease its hydrolysis, masking the denaturation of the enzyme.

3.2. Effect of cations on the proteolytic activity

Concerning the effect of mono and divalent cations, all the tested metals decreased the proteolytic activity (Table 1), and EDTA did not affect miliin activity. Remarkably, in the presence of mercury the enzyme maintained approximated 70% of its activity; usually this metal promotes inhibition of cysteine and serine proteases [28]. This behavior is also exhibited by taraxalin and other subtilisin-like serine proteases that are inhibited by PMSF, but not by mercury [25]. Kumar et al. [29] reported inhibition by several cations on the activity of a protease isolated from *Euphorbia cotinifolia*, with the only exception of Ni, able to increase substantially the activity.

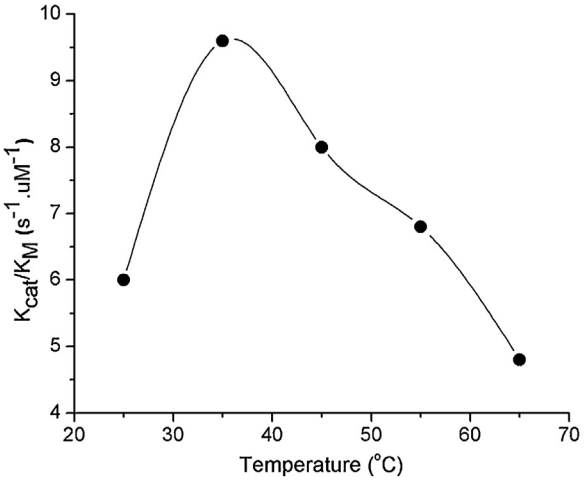

Fig. 3. Hydrolysis of the peptide Abz-KLLFSKQ-EDDnp by miliin at different temperatures. The reaction was performed in 50 mmol L^{-1} glycine, pH 9.0.

Table 1
Effect of different cations (0.5 mmol L^{-1} final concentration) on the hydrolysis of Abz-KLLFSKQ-EDDnp by miliin.

Compound	Relative activity (%)
Control	100.0
EDTA	100.0
$CaCl_2$	94.5
NaCl	91.7
$ZnCl_2$	86.1
LiCl	85.0
$AlCl_3$	77.8
$HgCl_2$	69.5
$MgCl_2$	47.2

Table 2
Kinetic parameters for the hydrolysis by *Euphorbia* miliin in the series of FRET peptides Abz-KL**X**SSKQ-EDDnp. The underlined residues occupied position "X", and the arrow indicates the cleaved peptide bonds. The assays were performed at 37 °C in 50 mmol L^{-1} glycine buffer, pH 9.0.

Substrate	k_{cat}/K_m (mM s)$^{-1}$
Abz-KL**H**↓SSKQ-EDDnp	122.3
Abz-KL**M**↓SSKQ-EDDnp	106.2
Abz-KL**F**↓SSKQ-EDDnp	75.0
Abz-KL**T**↓SSKQ-EDDnp	39.9
Abz-KL**N**↓SSKQ-EDDnp	38.6
Abz-KL**A**↓SSKQ-EDDnp	15.7
Abz-KL**K**↓SSKQ-EDDnp	15.5
Abz-KL**S**↓SSKQ-EDDnp	10.0
Abz-KL**C**↓SSKQ-EDDnp	7.0
Abz-KL**Y**↓SSKQ-EDDnp	5.2
Abz-KLR↓SSKQ-EDDnp	**5.0**
Abz-KL**W**↓SSKQ-EDDnp	2.2
Abz-KL**D**↓SSKQ-EDDnp	0.7
Abz-KL**I**S↓SKQ-EDDnp	6.7
Abz-KL**P**S↓SKQ-EDDnp	6.6
Abz-KL**V**S↓SKQ-EDDnp	5.4
Abz-KL**G**SSKQ-EDDnp	No hydrolysis

3.3. Subsite mapping

Tables 2–5 show the catalytic efficiency and the cleavage site by miliin for the FRET peptides series derived from the leader sequence Abz-KLRSSKQ-EDDnp with systematic modifications in residues P2 till P2′. The site of cleavage appears to be mostly defined by S_2.

Tables 2 and 3 display the cleavage parameters for the peptide series Abz-KL**X**SSKQ-EDDnp and Abz-K**X**RSSKQ-EDDnp respectively, where X stands for the varying residues. For most of the

Table 3
Kinetic parameters for the hydrolysis by *Euphorbia* miliin in the series of FRET peptides Abz-K**X**RSSKQ-EDDnp. The underlined residues occupied position "X", and the arrow indicates the cleaved peptide bonds. The assays were performed at 37 °C in 50 mmol L^{-1} glycine buffer, pH 9.0.

Substrate	k_{cat}/K_m (mM s)$^{-1}$
Abz-K**Q**RS↓SKQ-EDDnp	7.2
Abz-K**Y**RS↓SKQ-EDDnp	5.3
Abz-K**F**RS↓SKQ-EDDnp	4.4
Abz-K**T**RS↓SKQ-EDDnp	4.1
Abz-K**N**RS↓SKQ-EDDnp	3.9
Abz-K**A**RS↓SKQ-EDDnp	3.2
Abz-K**C**RS↓SKQ-EDDnp	2.7
Abz-K**E**RS↓SKQ-EDDnp	2.0
Abz-K**W**RS↓SKQ-EDDnp	0.4
Abz-K**R**RS↓SKQ-EDDnp	No hydrolysis
Abz-KLR↓SSKQ-EDDnp	**5.0**
Abz-K**P**R↓SSKQ-EDDnp	4.0
Abz-K**I**R↓S↓SSKQ-EDDnp	4.1
Abz-K**M**↓RS↓SKQ-EDDnp	1.8

Table 4
Kinetic parameters for the hydrolysis by *Euphorbia* miliin in the series of FRET peptides Abz-KLR**X**SKQ-EDDnp. The underlined residues occupied position "X", and the arrow indicates the cleaved peptide bonds. The assays were performed at 37 °C in 50 mM glycine buffer, pH 9.0.

Substrate	k_{cat}/K_m (mM s)$^{-1}$
Abz-KLR↓SSKQ-EDDnp	**5.0**
Abz-KLR**P**S↓KQ-EDDnp	207.8
Abz-KLR**V**S↓KQ-EDDnp	132.0
Abz-KLR**I**S↓KQ-EDDnp	93.9
Abz-KLR**L**S↓KQ-EDDnp	10.3
Abz-KLR**H**S↓KQ-EDDnp	7.7
Abz-KLR**N**S↓KQ-EDDnp	4.3
Abz-KLR**G**↓SKQ-EDDnp	0.6
Abz-KLR**A**↓S↓KQ-EDDnp	15.3
Abz-KLR**F**↓S↓KQ-EDDnp	1.0
Abz-KLR**E**↓S↓KQ-EDDnp	0.4
Abz-KLR**D**↓S↓KQ-EDDnp	0.1
Abz-KLR**R**SKQ-EDDnp	No hydrolysis

tested peptides a single cleavage occurred at an X-S or an S-S bond. Histidine, a basic residue, showed the highest catalytic efficiency at P_1 (Table 2) followed by two hydrophobic residues with large side chains: Met and Phe, and accordingly, S_1 has poor selectivity. However, at pH 9.0 the imidazole group predominates in an unprotonated form and does not carry a positive charge, behaving as a polar side chain. Under those circumstances histidine side chain would have a hydrophilic character due to the presence of two electronegative nitrogens [30]. Arg and Lys, that have a positive charge at pH 9.0 and would characterize a good substrate for a trypsin-like protease, exhibited poor hydrolysis.

The peptide Abz KLXSSKQ-EDDnp with aromatics residues in X would resemble a chymotrypsin-like substrate [14], as observed with Phe, but for Tyr and Trp, for which miliin displayed low efficiency.

Although the efficiency values are low, when X = Ile, Pro, or Val it can be observed the cleavage between Ser-Ser, so residue X is dislocated to subsite S_2. The series Abz KXRSSKQ-EDDnp and Abz KLXSSKQ-EDDnp, (Tables 2 and 3) and Abz KLRXSKQ-EDDnp (Table 4), where X is one of the hydrophobic residues Pro, Val and Ile display a displacement that shifts these variations into the P_2 position which confirms that the presence of cyclic or medium size aliphatic hydrophobic side chains determines their binding to P_2 and influences the site of cleavage.

Analyzing the behavior for Abz KLRXSKQ-EDDnp (Table 4), the peptides showing the highest values of catalytic efficiency are the hydrophobic residues Pro, Val and Ile, and the cleavage site showed that they were dislocated to P_2 position. Other amino acids also induced a displacement of the cleavage site and, in the case of Ala, which has a small hydrophobic side chain, two cleavage sites were observed: after X and Ser, a behavior also observed for substrates

Table 5
Kinetic parameters for the hydrolysis by *Euphorbia* miliin in the series of FRET peptides Abz-KLRS**X**KQ-EDDnp. The underlined residues occupied position "X", and the arrow indicates the cleaved peptide bonds. The assays were performed at 37 °C in 50 mM glycine buffer, pH 9.0.

Substrate	k_{cat}/K_m (mM s)$^{-1}$
Abz-KLR↓SSKQ-EDDnp	**5.0**
Abz-KLRS**H**↓KQ-EDDnp	57.0
Abz-KLRS**L**↓KQ-EDDnp	25.2
Abz-KLRS**F**↓KQ-EDDnp	14.5
Abz-KLRS**Q**↓KQ-EDDnp	8.0
Abz-KLRS**N**↓KQ-EDDnp	7.8
Abz-KLRS**I**↓KQ-EDDnp	2.4
Abz-KLRS↓A↓KQ-EDDnp	6.2
Abz-KLRS↓V↓KQ-EDDnp	1.8
Abz-KLRS↓E↓KQ-EDDnp	1.1
Abz-KLRS↓G↓KQ-EDDnp	0.8

Table 6
N-terminal sequence of Miliin as compared to other plant serine proteases (first 10 residues).

Enzyme or source	N-terminal	References
Miliin	DTGPPDYAPL	This work
Lily LIM 9	T**T**HT**PDY**LGI	[42]
Alnus ag12	T**T**HT**P**RFLS**L**	[43]
Bamboo protease	T**T**RT**P**SFLR**L**	[44]
Euphorbia supine protease B	T**T**RT**P**NFLG**L**	[45]
Euphorbia pseuchamaesyce	T**T**RT**P**NFLG**L**	[45]
Carnein	T**T**HS**P**EFLG**L**	[46]
Tomato P69 B	T**T**RS**P**TFLG**L**	[47]
Arabidopsis ARA12	T**T**RT**P**LFLG**L**	[47]
Tomato P69 A	T**T**HTSSFLG**L**	[48]
Milin	DVSYVGLILE	[26]
Hirtin	YAVYIGLILE	[41]
Eumiliin	AFLLQIIVTP	[10]

that did not display significant activity. Charged residues Asp/Glu or Arg did not show significant hydrolysis.

In Abz-KLRSXKQ-EDDnp (Table 5) the profile resembles the preferences at P_1, with Leu substituted in the second place instead of Met. For some residues (Ala, Glu, Val and Gly) occurred two cleavages, on their N and C sides. Miliin did not display significant activity in the presence of Gly, Arg and Pro.

For Abz XLRSSKQ-EDDnp and Abz KLRSSXQ-EDDnp the activities were low (under 10 s^{-1} mM^{-1}) and for most of the substrates two cleavages were observed (results not shown).

In summary, subsites S_1', S_1 and S_2' presented, respectively, the highest values of catalytic specificity. Miliin was shown to be a non-specific protease, with poor selectivity at S_1, but is influenced by some hydrophobic side chains, able to induce apparently a binding to S_2 and influence the site of enzyme hydrolysis. Since miliin was able to cleave at several peptide bonds, it could be used to effectively hydrolyze substrates with low specificity. These properties are probably fundamental due to the role of latex proteases, as a defense against pathogens and insects, and miliin would be useful for applications where proteins need to be cleaved efficiently. These properties are probably fundamental due to the role of latex proteases, as a defense against pathogens and insects, and miliin would be useful for applications where proteins need to be cleaved efficiently.

If milin, a different protease also isolated from the latex of *E. milii* shares the poor selectivity exhibited by miliin, it could explain the findings reported by Yaday and Jagannadham [31], showing its efficiency against proteinase K, trypsin and chymotrypsin. For the other peptides the data for P_2, P_3 and P_3' show lower rates of hydrolysis.

Usually, subtilisins exhibit a broad specificity, preferring to cleave substrates containing neutral residues at the S_1 subsite. Subtilisins also cleave substrates containing hydrophobic residues, such as Phe, at the S_1 subsite and small hydrophobic residues, such as Val or Pro, at the S_2 subsite [32,33].

Plant proteases show broad substrate specificities and activities over a wide range pH and temperature values [24]; it is supposed that they might protect ripening fruits against plant protease pathogens, especially fungi and insects [34,35]. The presence of bacteriolytic activity in lattices of *Carica papaya* [36], *Ficus glabarata* [37], and *Ervatamia coronaria* [38,39] support that assumption [40].

3.4. N-terminal sequence

The sequence of 10 residues at the N-terminus of purified miliin was determined, and compared to other serine proteases (Table 6); it displays higher identity and conserved residues when compared to several proteases, but no similarity at all with those reported by Patel et al. [41] and Yadav et al. [26], allegedly isolated from latex

from the genus *Euphorbia* and from the same species, respectively. Remarkably, eumiliin, another serine protease obtained from the same species *E. milii* [10] does not show any similarity for the first ten residues.

Most serine proteases have Thr (T) residues as the first two residues, and Leu at position 10 is common among most serine proteases [40]. The N-terminal sequence also showed similarity with those from other plant serine proteases such as Tomato P69B, *Euphorbia supine* protease B and *Euphorbia pseuchamaesyce*, published by Asif-Ullah et al. [4].

In conclusion, the preliminary data gathered from subsite mapping show a preference for hydrophobic residues, poor selectivity, and the N-terminal sequence confirms that miliin is different from the protease reported by Yadav et al. [26] and Fonseca et al. [10] from the same source.

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