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ORIGINAL ARTICLE

Biochemical characterization of VQ-VII, a cysteine peptidase with broad specificity, isolated from *Vasconcellea quercifolia* latex

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Abstract The latex from *Vasconcellea quercifolia* ("oak leaved papaya"), a member of the Caricaceae family, contains at least seven cysteine endopeptidases with high proteolytic activity, which helps to protect these plants against injury. In this study, we isolated and characterized the most basic of these cysteine endopeptidases, named VQ-VII. This new purified enzyme was homogeneous by bidimensional electrophoresis and MALDI-TOF mass spectrometry, and exhibited a molecular mass of 23,984 Da and an isoelectric point >11. The enzymatic activity of VQ-VII was completely inhibited by E-64 and iodoacetic acid, confirming that it belongs to the catalytic group of cysteine endopeptidases. By investigating the cleavage of the oxidized insulin B-chain to establish the hydrolytic specificity of VQ-VII, we found 13 cleavage sites on the substrate, revealing that it is a broad-specificity peptidase. The pH profiles toward *p*-Glu-Phe-Leu-*p*-nitroanilide (PFLNA) and casein showed that the optimum pH is about 6.8 for both substrates, and that in casein, it is active over a wide pH range (activity higher than 80 % between pH 6 and 9.5). Kinetic enzymatic assays were performed with the thiol peptidase substrate PFLNA (K_m = 0.454 ± 0.046 mM, k_{cat} = 1.57 ± 0.07 s^{-1}, k_{cat}/K_m = 3.46 × 10^3 ± 14 s^{-1} M^{-1}). The N-terminal sequence (21 amino acids) of VQ-VII showed an identity >70 % with 11 plant cysteine peptidases and the presence of highly conserved residues and motifs shared with the "papain-like" family of peptidases. VQ-VII proved to be a new latex enzyme of broad specificity, which can degrade extensively proteins of different nature in a wide pH range.

Keywords Caricaceae · Latex · Plant cysteine peptidase · Protease · *Vasconcellea*

Abbreviations

AMPSO	3-[(1,1-Dimethyl-2-hydroxyethyl)amino]-2-hydroxy-propanesulfonic acid
BLAST	Basic local alignment search tool
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
2D-PAGE	Two dimensional-polyacrylamide gel electrophoresis
DTT	Dithiothreitol
E-64	*Trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane
HCCA	α-Cyano-4-hydroxy-cinnamic acid
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
MES	2-Morpholinoethanesulfonic acid
MOPS	3-(*N*-morpholino) propanesulfonic acid

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PFLNA	*p*-Glu-Phe-Leu-*p*-nitroanilide
PMF	Peptide mass fingerprinting
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	Sulfopropyl
TAPS	*N*-tris (hydroxymethyl) methyl-3-aminopropanesulfonic acid
TFA	Trifluoroacetic acid

Introduction

Plant cysteine peptidases represent a well-characterized type of proteolytic enzymes that fulfill tightly regulated physiological functions (such as senescence and seed germination) and defense roles. Among the about 800 peptidases encoded by plant genomes, more than 140 correspond to cysteine peptidases that can be grouped in 15 families, which are in turn grouped into five clans (Rawlings et al. 2012). In particular, the papain-like peptidases C1A (family C1, clan CA), subdivided into cathepsin L-, B-, H- and F-like according to their gene structures and phylogenetic relationship, are the most abundant (Martínez and Díaz 2008; Martínez et al. 2012). The enzymes belonging to the C1A subfamily that enter the secretory pathway, intended either for secretion or for the lysosomal (animal) or vacuolar (plant) compartments, are synthesized as precursors, with N-terminal propeptides as well as the signal peptides (Trejo et al. 2009). C1A peptidases play crucial roles in plant pathogen/pest interactions (Shindo and van der Hoorn 2008; van der Hoorn 2008; McLellan et al. 2009). An important source of plant proteases used in traditional medicine and industry is latex. Over 110 latices of different plant families are known to contain at least one proteolytic enzyme. Most of them belong to the cysteine or serine endopeptidases family and only one to the aspartic endopeptidases family (Domsalla and Melzig 2008).

Papain is a component of papaya latex involved in the defense of the papaya tree against different lepidopteran caterpillars (Konno et al. 2004). The latices from plants of the Caricaceae family are known to produce large amounts of cysteine peptidases. *Vasconcellea quercifolia* belongs to this family and is widespread in South America (southern Peru, Bolivia, Paraguay, Brazil, Uruguay and northern Argentina). *V. quercifolia* latex has a high proteolytic activity and one of its cysteine peptidases has been previously isolated and characterized (Torres et al. 2010). This peptidase showed higher specificity than papain, since the cleavage of the insulin B-chain was restricted to glycyl and alanyl residues at P1′ position. We have recently demonstrated the presence of at least seven different peptidases in this latex when separated by cation exchange chromatography (Torres et al. 2012). These enzymes were named VQ-I, VQ-II, VQ-III, VQ-IV, VQ-V, VQ-VI and VQ-VII, according to their elution order. As a continuation of this work, we report the biochemical characterization of the most basic peptidase present in *V. quercifolia* latex, and compared it with papain and other plant cysteine peptidases.

Materials and methods

Plant material

The latex used as start material was collected from unripe fruits of *V. quercifolia* A. St.-Hil., Caricaceae (voucher specimen accession code: LPAG 5647). The fruits were collected at the beginning of summer in the Guasayán hills, province of Santiago del Estero, Argentina. This species is also found in Bolivia, Brazil, Paraguay, Peru, and Uruguay (Scheldeman et al. 2007).

Purification of VQ-VII peptidase

The VQ-VII peptidase was isolated and purified from the VQ preparation obtained from *V. quercifolia* latex (Torres et al. 2010), following the method described in our previous report (Torres et al. 2012). Briefly, the VQ preparation was purified by cation exchange chromatography (SP-Sepharose High Performance) at pH 7.5, using a sodium chloride two-step gradient (0.15–0.45 and 0.45–1.0 M).

Protein concentration

Protein content was determined by the Bradford method (Bradford 1976), using bovine serum albumin as standard. The protein profile of the chromatogram was obtained by measuring absorbance at 280 nm.

Proteolytic activity on casein

The reaction mixture contained 1.1 ml of 1 % casein solution and 0.1 ml of enzyme solution, both in 0.1 M Tris–HCl buffer (pH 8.0), containing 10 mM cysteine. The reaction was carried out at 37 °C and stopped by the addition of 5 % trichloroacetic acid (1.8 ml). Each test tube was then centrifuged at 4,000*g* for 20 min and the absorbance of the supernatant read at 280 nm. An arbitrary enzyme unit ("caseinolytic unit", Ucas) was defined as the amount of enzyme that produces an increase of one absorbance unit (1 cm light path) per minute under the assay conditions (Natalucci et al. 1996).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970) with slight modifications (Hames 1990). The polyacrylamide (12 %) gel electrophoresis was performed in a Miniprotean III Cell (Bio-Rad) and electrophoresis was performed for 45 min at 60 mA. Previously, protein samples were boiled for 5 min at 100 °C in sample buffer. Gels were stained with colloidal Coomassie [17 % (w/v) ammonium sulfate, 34 % methanol, 0.5 % acetic acid, and 0.1 % (w/v) Coomassie Brilliant Blue G-250] and then destained using distilled water.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The first dimension was performed with an Ettan IPGphor 3 IEF System (GE Healthcare) using Immobiline DryStrip (GE Healthcare), pH 7–11 NL (7 cm). Immobiline DryStrip gel was rehydrated (rehydration solution: 8 M urea, 2 % CHAPS, 0.5 % IPG buffer pH 6–11) overnight. Then, 50 µl of sample (30 µg of protein in rehydration solution containing 0.2 M DTT) was loaded. Before its application into the second dimension (12.5 % SDS-PAGE), the strip was treated with 6.5 mM DTT and 13.5 mM iodoacetamide in SDS equilibration buffer (50 mM Trizma, 6 M urea, 30 % glycerol and 2 % SDS, pH 8). The gel was dyed by the method of colloidal Coomassie.

Mass spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used to determine purity and molecular mass of VQ-VII by time-of-flight (TOF) analysis. Samples were mixed with sinapinic acid (matrix) dissolved in 0.1 % trifluoroacetic acid (TFA) and processed in a MALDI-TOF MS, Bruker equipment, model Biflex, using carboxypeptidase B as standard.

Activity assays with synthetic substrates (kinetic studies)

The initial rates of VQ-VII hydrolysis toward *p*-Glu-Phe-Leu-*p*-nitroanilide (PFLNA) were measured spectrophotometrically at 410 nm (Filippova et al. 1984) at pH 6.5 and 45 °C for substrate concentration between 0.1 and 1 mM in the reaction mixture. K_m and V_{max} were calculated by regression analysis using the non-linearized form of the Michaelis–Menten equation (Sigma Plot v9.0).

Inhibitory profile

The effect of specific inhibitors (Salvesen and Nagase 1989) on proteolytic activity was determined by preincubating the protease preparation with each inhibitor at 37 °C for 30 min and the residual activity estimated on PFLNA. E-64 (1.0 µM), iodoacetic acid (10.0 µM), pepstatin A (1.0–10.0 µM), 1,10-phenanthroline (1.0–10.0 mM), and phenylmethylsulfonyl fluoride (0.1–1.0 mM) were the specific inhibitors assayed. Controls were prepared by preincubating the protease preparation with the appropriate solvent used to dissolve the inhibitors.

Titration of active site with E-64

The active site was titrated as described by Barrett and Kirschke (1981), with some modifications. The enzyme (10 µM) was preincubated with activator buffer (50 mM Tris–HCl pH 8.2, containing 20 mM cysteine). Fractions (40 µl) were incubated with 80 µl E-64 at various concentrations between 0 and 10 µM for 30 min at 30 °C. The residual activity was measured on 2 mM PFLNA at pH 6.5, as previously described. The enzyme concentration was established by determining both protein content (Bradford 1976) and molecular mass (mass spectrometry).

Profile of pH

The proteolytic activity of VQ-VII was measured on casein (pH 6.0–11.0) or PFLNA (pH 5.0–9.0) using 0.025 M sodium salts of the following “Good” buffers: MES, MOPS, TAPS, AMPSO and CAPS, adjusting the corresponding pH value with 0.025 M HCl (Good and Izawa 1972).

N-terminal sequence

The N-terminal sequence was determined by Edman’s automated degradation using an Applied Biosystems (Procise 492) peptide sequencer. Protein homology searches were performed using the BLAST network service (Altschul et al. 1997).

Digestion of the insulin B-chain

The reaction mixture contained 150 µl of 0.2 mM insulin B-chain solution and 40 µl of enzyme solution (25 µg/µl), both in 0.1 M Tris–HCl buffer (pH 8.0) containing 20 mM cysteine. The reaction was carried out at 37 °C and stopped at different times by adding 0.1 % aqueous TFA. The peptides obtained by oxidized insulin B-chain degradation were analyzed by MALDI-TOF MS using α-cyano-4-hydroxy-cinnamic acid (HCCA) as matrix and spotted on

MTP 384 target plate ground steel. The identity of the peaks present in the mass spectrum was established using the GPMAW v6.0 program.

Results

Table 1 shows the purification scheme of VQ-VII peptidase, which represents the 8.61 % of the proteolytic activity present in the VQ preparation and containing 6.41 Ucas/mg of protein. The SDS-PAGE analysis of VQ-VII showed a single band of ca. 26 kDa, characteristic of cysteine peptidases. This enzyme was homogeneous by 2D-PAGE and MALDI-TOF MS. The molecular mass of VQ-VII estimated by SDS-PAGE (Fig. 1a) was 26.8 kDa, whereas that obtained by MALDI-TOF MS (Fig. 2) was 23.984 Da. The 2D-PAGE (Fig. 1b) showed an isoelectric point ≥11 for VQ-VII.

The newly isolated enzyme was able to hydrolyze PFLNA (synthetic peptide substrate) and casein (natural protein substrate). Figure 3 shows the pH profile of VQ-VII on both substrates. The enzymatic activity was completely inhibited by E-64 and iodoacetic acid, but it was not affected by 1–10 phenanthroline, pepstatin, neither PMFS. Titration of the active sites of VQ-VII using E-64 showed 93 % of active molecules (Fig. 4). The kinetic parameters V_{max}, K_m and k_{cat} of VQ-VII (77.3 nM in the reaction medium) were 0.113 μmol s^{-1}, 0.454 mM, and 1.57 s^{-1}, respectively, when PFLNA was used as substrate (Table 2).

To determine the cleavage specificity of VQ-VII, we studied the digestion pattern of the insulin B-chain by this peptidase. The GPMAW v6.0 program was used to identify the peptides showed in the mass spectra at different digestion times. Table 3 shows the masses of the peaks appearing in the spectra (mass detected) together with the masses corresponding to the ions identified within the sequence of the insulin B-chain (masses predicted) and the corresponding peptide sequences. The analysis of peptides revealed a total of 13 cleavage sites on the substrate for VQ-VII during the 30-min interval. The P1′ residue of the observed cleavages for VQ-VII are compared (highlighted) with the cleavage sites for quercifoliain I, papain, CMS1MS2 and CMS2MS2 (Table 3).

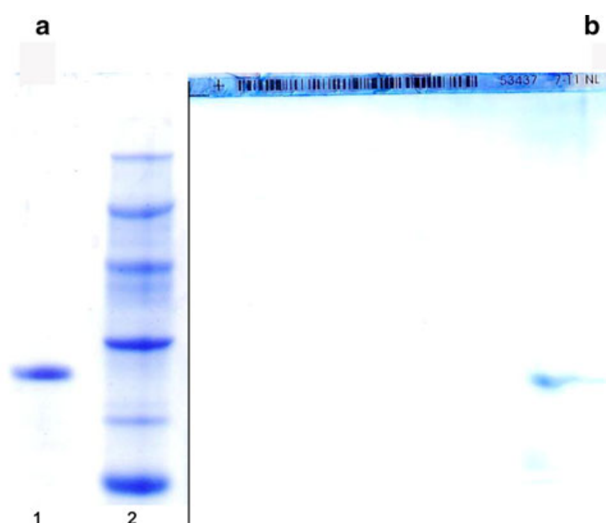

Fig. 1 Electrophoretic analysis of VQ-VII. **a** SDS-PAGE. *Lane 1* purified VQ-VII; *lane 2* molecular weight markers (94.0, 67.0, 43.0, 30.0, 20.1 and 14.4 kDa). **b** 2D-PAGE, immobilines pH 7–11 NL were used for the first dimension and 12.5 % polyacrylamide gel in the second dimension

Discussion

We have recently reported that the VQ preparation obtained from the latex from fruits of *V. quercifolia* can be purified in a single step using ion exchange chromatography (SP-Sepharose HP). In that work, analysis by SDS-PAGE of the seven fractions with proteolytic activity obtained revealed the presence of an intense band of ca. 26 kDa, characteristic of cysteine peptidases, in all fractions. In most cases, additional less intense bands of smaller size and two fractions with a 31-kDa band were found. The most basic fraction, which we named VQ-VII, was the only homogeneous protein detected by SDS-PAGE (Torres et al. 2012). We purified VQ-VII according to these results; Table 1 shows the purification scheme corresponding to this enzyme. The yield was 8.61 %, which is not very low taking into account that the starting enzymatic extract has at least seven proteolytically active fractions. Furthermore, the purification factor (1.28-folds) is consistent with that expected for an extract from plant lattices with high proteolytic activity, where peptidases represent the bulk of protein (López et al. 2000).

Table 1 Purification scheme of VQ-VII

Purification step	Protein (mg/ml)	Activity (Ucas/ml)	Specific activity (Ucas/mg)	Purification (*n*-fold)	Recovery (%)
VQ preparation	4.16	20.76	4.99	1.00	100
VQ-VII	0.47	2.98	6.41	1.28	8.61

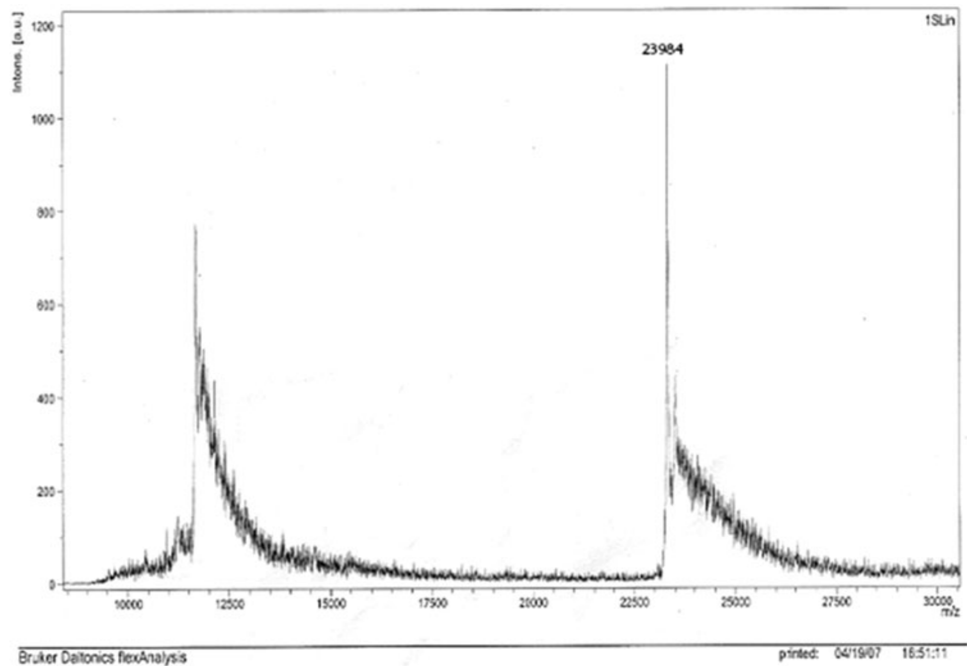

Fig. 2 Mass spectrometry analysis of VQ-VII

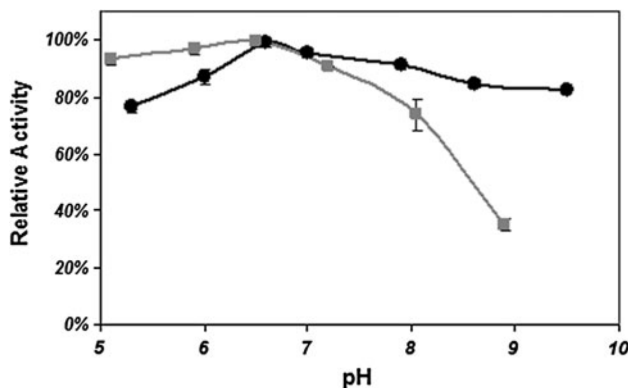

Fig. 3 pH profile of VQ-VII. Proteolytic activity measured toward casein as substrate (*filled circle*) and amidolytic activity toward PFLNA (*filled square*) using "Good" buffers

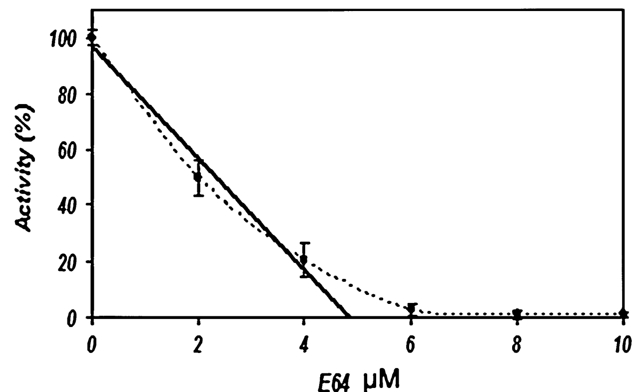

Fig. 4 Titration of active site with E-64

Characterization of VQ-VII

The molecular mass of VQ-VII estimated by SDS-PAGE (Fig. 1a) was 26.8 kDa, whereas that obtained by MALDI-TOF MS (Fig. 2) was 23,984 Da. MALDI-TOF MS analysis confirmed the homogeneity of the new enzyme. To determine its isoelectric point and confirm its homogeneity, VQ-VII was analyzed by two-dimensional electrophoresis (Fig. 1b). The use of immobiline pH 7–11 in the 2D-PAGE revealed that the plant peptidase has an isoelectric point $\geq$11 and showed its full homogeneity. The cysteine proteases from plant latex are almost exclusively basic proteins. Extremely alkaline isoelectric points have been previously reported for caricain (11.7), chymopapain (10.3–10.7), glycyl endopeptidase (>10), endopeptidases from *Carica candamarcensis* CCI, CCII, CCIII, CCIV, and CC28 (10.5–11.5), philibertain gI (>10.25) and heynein (10.8) (Domsalla and Melzig 2008).

The N-terminal sequence of VQ-VII peptidase (IPASIDWRQKGAVTPIRLQGQ) was determined by Edman's automated degradation (Torres et al. 2012). This sequence was subjected in this work to basic local alignment search tool (BLAST) analysis for its comparison with plant peptidases. Table 4 shows the results for peptidases with identity >70 %. Notably, cysteine peptidases isolated from *Lotus japonicus* (Fabaceae), *Philibertia gilliesii* (Apocynaceae), *Phalaenopsis* sp. (Orchidaceae), *Daucus carota* (Apiaceae) had higher percentages of identity than some proteases belonging to Caricaceae family (Torres et al. 2012).

Table 2 Kinetic parameters of VQ-VII toward PFLNA, and its comparison with the parameters of cysteine proteases from *Carica candamarcensis* (CMS1MS2, CMS2MS2), *Asclepias fruticosa* (asclepain f), *Bromelia pinguin* (pinguinain A1), *Araujia angustifolia* (araujiain aII), *Solanum granuloso-leprosum* (granulosain I) and *Hohenbergia penduliflora* (penduliflorain I)

Protease	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	References
VQ-VII	0.454 ± 0.046	1.57 ± 0.07	$3.46 \times 10^3 \pm 14$	
CMS1MS2	0.27 ± 0.05	15.5 ± 2.10	$57.4 \times 10^3 \pm 5.33$	Gomes et al. (2010)
CMS2MS2	0.32 ± 0.08	0.12 ± 0.03	$0.38 \times 10^3 \pm 68.1$	Gomes et al. (2010)
Asclepain f	0.055 ± 0.007	0.24	4.4×10^3	Trejo (2005)
Pinguinain A1	0.2937	0.431	1.47×10^3	Abreu Payrol et al. (2008)
Araujiain aII	0.18 ± 0.03	1.078 ± 0.06	$5.99 \times 10^3 \pm 0.57$	Obregón et al. (2011)
Granulosain I	0.6158	9.79	15.4×10^3	Vallés et al. (2008)
Penduliflorain I	0.33	4.27	12.9×10^3	Pérez et al. (2010)

The N-terminal sequence of VQ-VII showed the presence of highly conserved residues and motifs of ''papain-like'' cysteine peptidases: the Pro2 and Ser4 residues and the DWR and GAV motifs. In addition, it showed a Gln residue in the 19 position that participates in the catalysis of the hydrolysis of the peptide-bond, whose amide side-chain stabilizes the oxyanion of the tetrahedral intermediate (Barrett and Rawlings 2004).

The peptide mass fingerprinting (PMF) of VQ-VII was reported (Torres et al. 2012). The trypsin digested was analyzed by MALDI-TOF MS, and the MASCOT search tool (http://www.matrixscience.com) was used to identify tryptic maps. No matches with other plant cysteine peptidases were found, confirming that VQ-VII is a new cysteine peptidase.

The pH profile for VQ-VII (Fig. 3) was obtained using two different substrates: a natural protein (casein) and a synthetic peptide (PFLNA). The activity profile of VQ-VII with casein showed a wide pH range displaying high activity >80 % between pH 6.0 and 9.5; this is a remarkable characteristic for industrial applications. This pH behaviour is similar to that found for the VQ preparation (Torres et al. 2010); however, the VQ preparation has an optimum pH of 8.5, while VQ-VII showed optimum pH near neutrality (around 6.8). On the other hand, when PFLNA was used as substrate, VQ-VII showed maximum activity at a pH range between 6.2 and 6.8. This result is similar to those reported for other cysteine peptidases such as papain, bromelain, funastrain cII and philibertain gI (Filippova et al. 1984; Morcelle et al. 2004; Sequeiros et al. 2005).

The enzymatic activity of VQ-VII was completely inhibited by E-64 and iodoacetic acid, confirming that this peptidase belongs to the catalytic group of cysteine endopeptidases, as all the other endopeptidases purified from the latex of species belonging to the Caricaceae family. By titrating the active site of VQ-VII with E-64, the proportion of active enzyme molecules (93 %) was determined, a very good proportion compared with those reported for other cysteine peptidases (Bruno et al. 2003, 2006, 2008; Vallés et al. 2008).

Kinetic studies

The amidase activity of the enzyme was assayed toward PFLNA, a specific substrate for peptidases belonging to the papain family. VQ-VII was able to hydrolyze PFLNA; the K_m value (0.454 ± 0.046 mM) was in the same order as those determined for papain (0.34 mM), ficin (0.43 mM), and bromelain (0.30 mM), the most known plant cysteine peptidases (Filippova et al. 1984). To determine the k_{cat} value, we calculated the effective concentration of active enzyme (71.9 nM). We then compared the k_{cat}/K_m ratio of VQ-VII, the kinetic parameter that expresses the catalytic efficiency, with those from other plant cysteine peptidases (Table 2), and found that it was similar to those of asclepain f and araujiain aII, obtained from latex of *Asclepias fruticosa* and *Araujia angustifolia*, respectively, and to that of pinguinain A1, isolated from fruits of *Bromelia pinguin.* Although VQ-VII had a lower affinity from PFLNA than the compared peptidases, VQ-VII showed the highest catalytic constant (k_{cat}).

Cleavage specificity of VQ-VII

The cleavage of oxidized B-chain insulin was investigated to establish the hydrolytic specificity of VQ-VII. The identity of the peptides produced was established by MALDI-TOF MS, which yields information about the substrate specificity in a sensitive and quick way (Siigur et al. 2002). The analysis of identified peptides (Table 3) revealed that there are a total of 13 cleavage sites on the substrate for VQ-VII along the reaction. Six of them were the same as those generated by papain, for which seven cleavage sites were identified on the insulin B-chain (Kaneda et al. 1995). Based on the peptides identified and

Table 3 Determination of the B-chain insulin cleavage sites by VQ-VII

Incubation Time	Ion Mass (predicted)	Ion Mass (detected)	Sequence of insulin B-chain
0 min	3512.800	3512.924	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
2 min	3512.904	3512.924	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	1992.033	1992.005	-------------ALYLVÇGERGFFYTPKA
	1539.736	1539.737	FVNQHLÇGSHLVE------------------
	1807.902	1807. 884	----------------YLVÇGERGFFYTPKA
	1644.837	1644.820	-----------------LVÇGERGFFYTPKA
	1531.740	1531.736	------------------VÇGERGFFYTPKA
	1272.653	1272.637	--------------------GERGFFYTPKA
	1431.715	1431.709	--------------ALYLVÇGERGFF-----
5 min	3512.934	3512.724	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	3152.527	3152.544	----QHLÇGSHLVEALYLVÇGERGFFYTPKA
	2557.379	2557.291	---------SHLVEALYLVÇGERGFFYTPKA
	1539.774	1539.737	FVNQHLÇGSHLVE------------------
	1992.087	1992.005	--------------ALYLVÇGERGFFYTPKA
	1610.810	1610.774	FVNQHLÇGSHLVEA------------------
	1644.895	1644.820	-----------------LVÇGERGFFYTPKA
	1531.784	1531.736	------------------VÇGERGFFYTPKA
	1272.688	1272.637	--------------------GERGFFYTPKA
	2952.538	2952.428	FVNQHLÇGSHLVEALYLVÇGERGFF------
	1431.744	1431.709	--------------ALYLVÇGERGFF------
	1649.827	1649.766	-------ÇGSHLVEALYLVÇGE----------
	2477.262	2477.199	------LÇGSHLVEALYLVÇGERGFFYTP--
10 min			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	1272.649	1272.637	--------------------GERGFFYTPKA
	1307.609	1307.616	--NQHLÇGSHLVEA-----------------
	1649.784	1649.766	------ÇGSHLVEALYLVÇGE----------
30 min			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	1649.898	1649.766	-------ÇGSHLVEALYLVÇGE---------
	1551.823	1551.730	----------------YLVÇGERGFFYTP-
VQ-VII			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
Quercifoliain I			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA (Torres et al. 2010)
CMS1MS2			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA (Gomes et al. 2010)
CMS2MS2			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA (Gomes et al. 2010)
Papain			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA (Kaneda et al. 1995)
Phytolacain R			FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA (Kaneda et al. 1995)

The data on each column (*left* to *right*) refers to the incubation intervals for the reaction, the predicted mass and detected mass for insulin produced peptides; the right column shows each peptide sequence with a gray square indicating insulin cleavage site. The Ç symbol corresponds to reduced carbamidomethylated cysteine. For comparison quercifoliain I, CMS1MS2, CMS2MS2, papain and phytolacain R cleavage sites previously established are shown at the lower end

their comparison with those obtained for other cysteine peptidases (Table 3), we can conclude that this new enzyme (VQ-VII) has lower cleavage specificity than the other peptidases.

A comparison between different cysteine peptidases in their ability to cleave the B-chain of insulin is presented in Table 3. The results show that all enzymes cleave Ala14 at P1 position, whereas VQ-VII was able to cleave five positions (N3, L6, C7, Y16, and K29) that are not cleaved by the other peptidases. The cleavage pattern suggests a preference for a hydrophobic residue at P2 and P3 and no preference at the P1 subsite, similar to other papain-like enzymes (Turk et al. 1998). An exception to this relatively broad specificity is the glycyl endopeptidase from *Carica papaya*, characterized by its specificity for C-terminal glycyl residues (Thomas et al. 1995) and the quercifoliain I (Torres et al. 2010), characterized by its specificity for N-terminal glycyl and alanine residues.

In sum, a new plant cysteine peptidase named VQ-VII was obtained from latex of *V. quercifolia*. The results of 2D-PAGE, mass spectrometry, inhibition assays, kinetic parameters and the N-terminal amino acid sequence show that the purified VQ-VII is a “papain-like” peptidase. VQ-VII proved to be an enzyme of broad specificity, which extensively degrades proteins of different nature within a wide pH range. This feature can bear a physiological implication probably related to the kind of substrate targeted by this enzyme.

Table 4 Comparison of N-terminal sequence of the VQ-VII protease with other plant cysteine proteases

Protease	N-terminal sequence	Reference	Identity (%)
VQ-VII [*V. quercifolia*]	IPASIDWRQKGAVTPIRLQGQ	Torres et al. 2012	
VXH-A [*V. X heilbornii*]	IPASIDWRQKGAVTPVRNQGS	Kyndt et al. 2007	86
VXH-B [*V. x heilbornii*]	IPASIDWRQKGAVTPVRHQGS	Kyndt et al. 2007	86
VS-A [*V. stipulata*]	IPASIDWRQKGAVTPVRNQGS	Kyndt et al. 2007	86
CMS1MS2 [*V. candamarsencis*]	IPTSIDWRQKGAVTPVRNQGS	Gomes et al. 2008	81
VXH-D [*V. x heilbornii*]	IPTSIDWRQKGAVTPVRNQGS	Kyndt et al. 2007	81
CC-I [*V. candamarsencis*]	IVASIDWRQKGAVTPVRNQGS	Walreavens et al. 1993	81
Cysteine protease [*Lotus japonicus*]	VPASLDWRQKGAVTPIKDQGQ	Deguchi et al. 2007	81
Cysteine protease [*Phalaenopsis sp.*]	LPASIDWRQKGAVTAVKDQGQ	Nadeau et al. 1996	81
Mexicain-like CP [*Jacaratia mexicana*]	YPESIDWRQKGAVTPVKNQNP	Ramos-Martinez et al. 2012	71
Philibertain gI [*Philibertia gilliesii*]	LPASVDWRKEGAVLPIRHQGQ	Sequeiros et al. 2005	71
Cysteine protease [*Daucus carota*]	VPATMDWRKKGAVTPIKNQGQ	Mitsuhashi et al. 2004	71

Coincident amino acids are on a gray background

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