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Purification and Characterization of a Cysteine Endopeptidase from *Vasconcellea quercifolia* A. St.-Hil. Latex Displaying High Substrate Specificity

M. José Torres,[†] Sebastián A. Trejo,[‡] M. Inés Martin,[†] Claudia L. Natalucci,*[,†] Francesc X. Avilés,[‡] and Laura M. I. López[†]

[†]Laboratorio de Investigación de Proteínas Vegetales (LIPROVE), Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, C.C. 711, B1900AVW, La Plata, Argentina, and [‡]Institut de Biotecnología i de Biomedicina,Universitat Autònoma de Barcelona, Campus Universitari 08193, Bellaterra, Cerdanyola del Vallès, Barcelona, España

A new proteolytic preparation from *Vasconcellea quercifolia* ("oak leaved papaya") latex containing several cysteine endopeptidases with high proteolytic activity has been obtained. The specific activity of the new enzymatic preparation (VQ) was higher than that of *Carica papaya* latex. VQ was able to coagulate milk and to hydrolyze caseins and then could be used to produce cheeses and/or casein hydrolysates. Ion exchange chromatography of VQ allowed the isolation of a new protease, named quercifoliain I, homogeneous when analyzed by SDS−PAGE, IEF and MALDI-TOF-MS. Molecular mass was 24195 Da, and its isoelectric point was >9.3. The N-terminal sequence was determined (YPESVDWRQ). Insulin B-chain cleavage showed higher specificity than that of papain and was restricted to glycyl and alanyl residues at P1′ position. The tryptic peptide mass fingerprint of quercifoliain I analyzed with the MASCOT search tool did not find a match with papain or any other plant cysteine proteases.

KEYWORDS: ***Caricaceae*; latex; papain; plant cysteine protease; *Vasconcellea quercifolia***

INTRODUCTION

Proteases, which firmly maintain the first place in the world market of enzymes, play an important role in biotechnology, given that proteolysis changes the chemical, physical, biological, and immunological properties of proteins (*1*). Proteolytic enzymes from plant sources have received special attention in pharmaceutical and biotechnology industries by being active over wide ranges of temperature and pH (*2*). Although most proteases used in industrial processes come from microbial sources, some plant cysteine proteases, namely, papain, bromelain, and ficin, are still preferred in a number of cases and are currently used for cheese and beer manufacture, tenderization of meat, production of emulsifiers, and other uses (*1*). At the same time, cysteine proteases of plants play a major role in intra- and extracellular processes such as development and ripening of fruits, nutritional reserve, degradation of storage protein in germinating seeds, activation of proenzymes and degradation of defective proteins (*2*). A number of cysteine endopeptidases have been isolated from plant latices, and there is experimental evidence that latex generally contributes to protecting the plant against predators (*3*−*6*). Caricaceae is a family of flowering plants in the order Brassicales, native to tropical and subtropical regions of Central and South America and Africa. The family comprises five genera (*Carica*, *Cylicomorpha*, *Jacaratia*, *Jarilla* and *Vasconcellea*) and about 50 species. Articulated laticifers, containing milky latex, are present in all organs of members of Caricaceae (*7*−*12*). The most extensively investigated plant cysteine protease is papain (EC 3.4.22.2) from the latex of *Carica papaya* (*13*), which also contains chymopapain (EC 3.4.22.6, the main proteolytic component), caricain (EC 3.4.22.30) and glycyl endopeptidase (EC 3.4.22.25), also named papaya proteinase IV (*14*). Mexicain, the most abundant peptidase from the latex of the fruits of *Jacaratia mexicana*, has been isolated and crystallized (*15*, *16*). Several endopeptidases have been isolated and characterized from the latex of *Vasconcellea cundinamarcensis* (= *Carica candamarcensis*) (*17*−*23*). Recently, cDNA sequences coding for cysteine proteinases in *Vasconcellea x heilbornii* and *Vasconcellea stipulata* have been determined (*24*). In this study, we report the presence of high proteolytic activity in *V. quercifolia* latex, its comparison with the *C. papaya* latex, and its ability to hydrolyze casein and clotting milk. Moreover, a new cysteine protease, with higher specificity than papain, was purified and characterized.

MATERIALS AND METHODS

Plant Material. Ripe and unripe fruits of *Vasconcellea quercifolia* A. St.-Hil., *Caricaceae,* were collected in the beginning of summer in the Guasayan hills, province of Santiago del Estero, Argentina. A voucher specimen was deposited by Dr. Ana María Arambarri in the Herbarium of Área de Botánica, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina, under the accession code LPAG 5647. This species is found in Argentina, Bolivia, Brazil, Paraguay, Peru, and Uruguay (*25*).

*Corresponding author. Fax: 542214226947. Tel: 542214250497 ext 57. E-mail: natalucci@biol.unlp.edu.ar.

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Chemicals. AMPSO [*N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid], azocasein, bovine serum albumin, hemoglobin (H2625), CAPS [3-(cyclohexylamino)-1-propanesulfonic acid], casein (C5890), κ-casein, E-64 [*trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)-butane], iodoacetic acid, MES (2-morpholinoethanesulfonic acid), MOPS [3-(*N*-morpholino) propanesulfonic acid], 1,10-phenanthroline, phenylmethylsulfonyl fluoride, pepstatin A, sodium tetrathionate and TAPS [*N*-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid] were purchased from Sigma-Aldrich Inc. (St. Louis, MO). *N*-α-Benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) was purchased from Bachem AG (Torrance, CA). Coomassie Brillant Blue R-250, acrylamide, and bisacrylamide were obtained from Bio-Rad (Hercules, CA). Peptide Marker kit was purchased from GE Healthcare-Bioscience AB (Uppsala, Sweden). Other reagents used were of the highest grade available.

Partially Purified Preparation. The latex (1 g) was obtained by superficial incisions of fruits, collected in 15 mL of 0.1 M citric acid−sodium citrate buffer (pH 5.6) containing 1 mM sodium tetrathionate and 5 mM EDTA (ethylendiaminetetraacetic acid), in an ice−water bath. The preparation was clarified by centrifugation at 11500*g* during 20 min at 4 °C, and the clear supernatant was named VQ preparation, aliquoted and conserved at −20 °C for further assays.

Activity Assays with Synthetic Substrates. *Amidase Activity*. The sample (100 μL of a 1:30 dilution of VQ) was preincubated for 2 min at 37 °C with 850 μL of reaction buffer (0.1 M Tris/HCl pH 7.0, containing 20 mM cysteine) for its activation. The enzymatic hydrolysis of substrate at 37 °C was started by addition of 50 μL of 10 mM BAPNA (α-*N*-benzoyl-L-arginine *p*-nitroanilide) in 1% DMSO (dimethyl sulfoxide), and the release of 4-nitroaniline was measured spectrophotometrically at 410 nm ($\varepsilon_{410} = 8800\ mol^{-1}\ cm^{-1}$) for 3 min. An Agilent 8453E UV−visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA) was used in all spectrophotometric measurements.

One nkat of enzyme is the amount that hydrolyzes 1 nmol of substrate per second under the above-cited conditions. The specific activity is expressed as the nanomolar concentration of product per second per milligram of protein.

Activity Assays with Protein Substrates. *Casein*. The reaction mixture contained 0.1 mL of the enzyme solution (dilution 1:40 of the VQ) and 1.1 mL of 1% casein in buffer of appropriate pH containing 20 mM cysteine. The reaction was carried out at 37 °C and stopped at different intervals by the addition of 1.8 mL of 5% TCA (trichloroacetic acid). Then each test tube was centrifuged at 4000*g* for 20 min, and the absorbance of the supernatant was read at 280 nm. An arbitrary enzyme unit ("caseinolytic unit", U_{cas}) was defined as the amount of enzyme that produced an increase of one absorbance unit (1 cm light-path) per minute in the assay conditions (*26*).

Hemoglobin. The reaction mixture contained 50 μL of 2.5% hemoglobin in formic acid−NaOH buffer (pH 3.3) and 20 μL of enzyme solution (dilution 1:40 of the VQ), 730 μL of 0.1 M formic acid−NaOH buffer (pH 3.3) containing 20 mM cysteine. The reaction was carried out at 37 °C and stopped at different intervals by the addition of 10% TCA (500 μL); each test tube was centrifuged at 11500*g* for 30 min, and the absorbance of the supernatant was read at 280 nm. One unit of activity was defined as the amount of enzyme that produces an increase of one absorbance unit at 280 nm (1 cm light-path) per minute in the assay conditions (*27*).

Azocasein. Azocasein was the substrate used in inhibition assays with 1,10-phenanthroline. The reaction was carried out at 37 °C in a microfuge tube containing 0.25 mL of 2% azocasein and 0.15 mL of enzyme solution (dilution 1:100 of the VQ), both in 0.1 M Tris-HCl buffer, pH 8.5, containing 20 mM cysteine. The reaction was stopped at different intervals by the addition of 1.2 mL of 10% TCA, and then centrifuged at 4000*g* for 20 min, the supernatant (1.2 mL) was transferred to a test tube containing 1.4 mL of 1 M NaOH, and the absorbance was read at 440 nm. One unit of protease activity was defined as the amount of enzyme required to produce an absorbance change of one unit (1 cm light-path) per minute under the assay conditions (*28*).

Protein Content. Protein concentration was determined according to the Coomassie Blue dye binding method (*29*), using bovine serum albumin as standard. In chromatographic fractions, proteins were detected by measuring absorbance of eluates at 280 nm.

Inhibition Assays. The effects of specific inhibitors (*30*) on proteolytic activities were determined by preincubating VQ (dilution 1:40 for casein substrate or 1:100 for azocasein substrate) with each inhibitor at 37 °C for 30 min, and the residual activity was estimated at pH 8.5 (0.1 M Tris-HCl buffer). E-64 (1−100 μM, aqueous solution), iodoacetic acid (0.1−1 mM, aqueous solution), pepstatin A (1−10 mM, methanol solution), 1,10-phenanthroline (1−10 mM, methanol solution), and phenylmethylsulfonyl fluoride (1−10 mM, methanol solution) were the chemicals assayed. Controls were prepared by preincubating VQ with the appropriate solvent used to dissolve the inhibitors.

Optimum Concentration of Tetrathionate for Enzyme Inactivation. In order to establish the most suitable conditions of reversible inactivation of the enzyme preparation, the assays were performed by incubating VQ (final dilution 1:40) during 30 min with different concentrations (0, 1, 5, and 10 mM) of sodium tetrathionate. The residual caseinolytic activity was measured in 0.1 M Tris-HCl buffer (pH 8.5) with different concentrations of cysteine (0, 10, 20, and 50 mM, aqueous solution) to reverse the inhibition.

pH Profile. Proteolytic activity of VQ (dilution 1:40) was measured on casein (range: pH 6.0 to 11.0) using 0.025 M sodium salts of the following "good" buffers (*31*): MES, MOPS, TAPS, AMPSO and CAPS, adjusting the corresponding pH value with 0.025 M HCl.

Thermal Stability. In order to study the effect of incubation time and temperature heating on the activity of VQ at the optimum pH, VQ (dilution 1:40) was incubated at pH 8.5 (0.1 M Tris-HCl buffer, containing 20 mM cysteine) during different times (0, 5, 10, 30, 60, 90, and 120 min) at different temperatures (20 °C, 37 °C, 45 °C, 55 °C, 65 °C, and 75 °C), and the residual caseinolytic activity was measured as described before.

Effect of the Ionic Strength on Enzyme Activity. The caseinolytic activity was determined, as was above-described, using a dilution 1:40 of VQ, containing increasing quantities of NaCl (0.0, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0 M). Casein (1%) in 0.1 M Tris-HCl buffer (pH 8.5) containing 20 mM cysteine was added to the reaction mixture.

SDS−PAGE. SDS−polyacrylamide (12%) gel electrophoresis was performed in a Miniprotean III Cell (Bio-Rad) based on the method of Laemmli (*32*) with slight modifications (*33*).

Protein samples were boiled for 5 min at 100 °C in sample buffer. Electrophoresis was performed for 45 min at 60 mA. 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 were destaining using destilled water.

Isoelectrofocusing. A Mini IEF Cell (model 111, Bio-Rad) was employed in isoelectrofocusing experiments. Five percent polyacrylamide gels were used, containing broad pH range ampholytes (Pharmalyte 3-10, Pharmacia) following recommendations of Bio-Rad. Cold acetone (5 volumes) was added to the sample, and the precipitate was redissolved with redistilled water. Focusing was carried out under constant voltage conditions in a stepwise mode: 100 V for 30 min, 200 V for 15 min and 450 V for 60 min. Gels were fixed, stained by Coomassie Brilliant Blue R-250 and then destained following Bio-Rad's protocols.

Zymogram. Unstained IEF gels were contacted for 10 min in an oven at 45 °C with an agarose gel imbibed with 1% casein in 50 mM Tris-HCl buffer (pH 8.5) containing 20 mM cysteine. After incubation, the agarose gel was dehydrated and stained by Coomassie Brilliant Blue R-250. Unstained zones evidence proteolytic activity (*34*).

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 rehydratation solution containing 0.2 M DTT) was loaded. Before its application into the second dimension (SDS−PAGE 12.5%) the strip was treated with 6.5 mM dithiothreitol 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.

Clotting Assay. Clotting activity was measured at 33 °C by mixing 1 mL of dry skim milk (100 g/L, 10 mM $CaCl_2$, pH 6.5) with 0.1 mL of VQ solution. Blanks were prepared by replacing the enzyme preparation by distilled water. One unit of clotting activity (CU) was defined as the reciprocal of the time (min) necessary to start milk clotting (*35*).

Casein Hydrolysates. Bovine milk casein suspensions were prepared by dissolving casein (12.5 g/L) in 0.1 M Tris-HCl buffer (pH 8.5) containing 20 mM cysteine. The enzyme/substrate ratio was 1.5 U_{cas}/g

of protein substrate. Each reaction tube contained 450 μL of casein solution and 50 μL of VQ (dilution 1:100). The mixture was incubated at 37 °C and the reaction stopped at different times (2 min to 60 min) by the addition of 500 μL of TCA (5%). Blanks were prepared by adding TCA to the enzyme prior to the addition of the substrate solution.

Hydrolysis assay samples (500 μL) were neutralized and mixed with 500 μL of sample buffer (125 mM Tris-HCl, pH 8.5, containing 4% SDS, 10% 2-mercaptoethanol, 0.4% bromophenol blue, and 20% glycerol), boiled for 5 min and centrifuged at 11500*g* for 15 min. Molecular weight standards were prepared in sample buffer.

Samples were subjected to denaturing electrophoresis in Tricine gels composed of a stacking gel (4% T, 3% C), a spacer gel (10% T, 3% C) and a separating gel (16.5% T, 3% C), which is especially suitable to resolve the mixture of peptides produced (*36*). The electrophoresis was performed in a Mini-Protean III dual slab cell (Bio-Rad, Hercules, CA 94547). Electrophoresis was carried out in duplicate and performed at room temperature using a voltage stepwise procedure: voltage was kept constant (40 V) until the samples completely left the stacking gel, then voltage was increased to 90 V for the spacer gel and finally voltage was maintained constant (150 V) until the tracking dye reached the bottom of the gel. Gels were fixed and stained as was indicated above.

Gels were scanned and the images processed by means of a specific software (Scion Image Beta 4.03 for Windows, Scion Corporation, Frederick, MD 21701) to obtain the corresponding densitograms.

Chromatographic Purification. VQ was chromatographed on an FPLC system (Pharmacia) using a Sephacryl S-100 column (60 mL) previously equilibrated with 50 mM citrate buffer (pH 4.5) containing 0.5 M NaCl and 5 mM sodium tetrathionate. The column (i.d., 1.5 cm; length, 45 cm) was eluted (flow rate: 0.5 $mL \cdot min^{-1}$) using the same buffer. Fractions (1 mL) were collected, and the caseinolytic activity of each fraction was measured. The whole process was carried out at 18 °C.

The main active fraction from Sephacryl S-100 was concentrated and desalted using Millipore centrifugal filter devices MW 10000. The resulting solution was chromatographed onto a column (Pharmacia XK 16/40, with AK16 adaptors) packed with 20 mL of SP-Sepharose HP (Pharmacia), equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM sodium tetrathionate. The unbound proteins were washed with the starting buffer, and the retained proteins were eluted with a NaCl linear gradient (0.15−0.45 M). Fractions (1.2 mL) were collected, and the caseinolytic activity of all fractions was measured.

N-Terminal Amino Acid 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 (*37*).

Mass Spectrometry. Homogeneity and molecular mass of quercifoliain I were determined by MALDI-TOF/MS (matrix assisted laser desorption ionization time-of-flight mass spectrometry). Mass spectra were acquired on a Bruker Ultraflex spectrometer equipped with a pulsed nitrogen laser (337 nm), in linear positive ion mode, using a 19 kV acceleration voltage. Samples were prepared by mixing equal volumes of a saturated solution of the matrix (2,6-dihydroxyacetophenone) in 0.1% TFA (trifluoroacetic acid) in water/acetonitrile 2:1, and a 1−10 μM protein solution. From this mixture, 1 μL was spotted on MTP 384 target plate polished steel (Bruker Daltonik GmbH) and allowed to evaporate to dryness. A protein of known molecular mass (trypsinogen) was used as standard for mass calibration.

Peptide Mass Fingerprinting (PMF). All assays were carried out in a ZipPlate micro-SPE Plate with a MultiScreen vacuum manifold, 96 wells (MILLIPORE, Billerica, MA). The protein band (purified enzyme) from SDS−PAGE was excised using a fresh scalpel and diced into 1 mm^3 pieces. Gel pieces were further destained in 3 × 100 μL of 25 mM NH_4HCO_3 and then washed with 100 μL of 25 mM NH_4HCO_3/ACN (acetonitrile) 50% and finally with 100 μL of ACN. Cysteine groups from proteins were then reduced and alkylated with 10 mM DTT (dithiothreitol) and 50 mM iodoacetamide, respectively. The tryptic digestion was carried by addition of 30 ng of trypsin (Promega Trypsin Gold, TPCK treated) per band from gel. At the end of the reaction, the C18 resin was washed and activated with 5 μL of ACN in each well. Peptides were extracted from the gel pieces 3× with 100 μL of 0.2% TFA and finally 1× with 15 μL of ACN:H_2O−TFA 0.1% (1:1). Supernatants were then dried in a SpeedVac, and then the peptides were suspended in 5 μL of extraction buffer.

MALDI-TOF-MS analyses were used to determine protein identities by PMF. Analysis was performed using an UltraFlex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). The sample was spotted on a GroundSteel target (Bruker Daltonics) mixing 0.5 μL with 1 μL of freshly prepared matrix solution of HCCA (α-cyano-4-hydroxycinnamic acid) 10 mg/mL in aqueous solution 30% ACN and 0.1% TFA. External calibration was performed using peptide calibrants (Bruker Daltonics). Peptide masses were acquired within a range of ca. 800 *m*/*z* to 4000 *m*/*z*. MASCOT search tool (URL http://www.matrixscience.com) was used for identification of tryptic maps.

Digestion of the B-Insulin Chain. The reaction mixture contained 150 μL of 0.2 mM B-insulin 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 the addition of 0.1% aqueous TFA. The peptides obtained by oxidized insulin B-chain degradation were analyzed by MALDI-TOF MS using 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 (http://www.gpmaw.com/GPMAW/gpmaw.html) v6.0 program.

RESULTS AND DISCUSSION

Vasconcellea quercifolia (Caricaceae) is a wild native species of South America currently not used in agriculture. Ripe and unripe fruits were studied. Latex recovered and the specific proteolytic activity were higher in unripe fruits: 18 μL latex/g fruit and 5 U_{cas}/mg protein for unripe fruits versus 15 μL latex/g fruit and 3.4 U_{cas}/mg protein for ripe fruits, respectively. On the basis of these results, unripe fruits were selected for further assays.

A partially purified preparation, named VQ, was obtained from the latex of *V. quercifolia* unripe fruits. The latex was received on buffer containing sodium tetrathionate to avoid autodigestion and EDTA for phenoloxidase inactivation. The suspension was clarified by centrifugation, to remove gums and other insoluble materials. Fifteen milliliters of VQ came from 1 mL of latex.

Characterization of VQ. An initial characterization of VQ was carried out in view of its potential industrial application (*38*).

The total protein content of the VQ solution was 4.2 mg/mL, while latex protein content was 56 mg/g. This value was higher than those reported by Kyndt et al. (*24*) for the latex of other species of *Caricaceae* with proteolytic activity (*C. papaya* 26.69 mg/g; *Vasconcellea monoica* 36.31 mg/g; *V. stipulata* 40.57 mg/g; *V. heilbornii* 31.75 mg/g).

Endopeptidase activities of VQ and a preparation obtained in our laboratory by the same procedure from unripe fruits of *Carica papaya* (papain) were analyzed for comparative purposes. The proteolytic activity of VQ on casein was 20.8 U_{cas}/mL, and the specific activity 5 U_{cas}/mg protein, which was higher than that of the papain preparation (3.5 U_{cas}/mg protein). Moreover, the specific activity of the VQ using D,L-BAPNA as substrate was 1.01 nkat/mg protein, higher than the papain preparation (0.56 nkat/mg protein). Additionally, amidase activity per milligram of dried latex using BAPNA as substrate was evaluated by Sheldeman et al. (*39*) for several *Vasconcellea* species. These authors reported that *V. cundinamarcensis, V. stipulata* and *V. x heilbornii* latices showed proteolytic activities 4−13 times higher than the papaya latex. More recently, Kyndt et al. (*24*) using the same substrate and latex from three *Vasconcellea* species reported higher specific activities than that of *C. papaya* (*V. monoica,* 1.2 times, *V. stipulata*, 2.0 times, and *V.* x *heilbornii*, 1.3 times). The study confirms a higher degree of proteolytic activity in the latex of three *Vasconcellea* spp. in comparison with *C. papaya*, due to the higher protein content and the presence of other, more active,

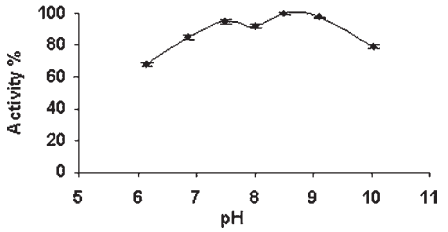

Figure 1. Effect of pH on hydrolysis of casein by VQ. Activity on casein at pH 8.5 was taken as 100%. Data points represent the mean value of five determinations.

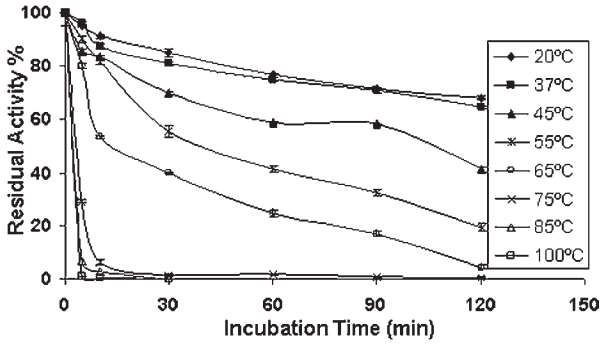

Figure 2. Thermal stability of VQ. Activity on casein at pH 8.5 after 8 min at 37 °C was taken as 100%. Data points represent the mean value of five determinations.

cysteine proteinases in the latex (*24*). The chromatographic analysis of VQ revealed the presence of several proteolytic fractions (not shown); this fact could justify its high specific activity.

According to the pH profile of activity using casein (**Figure 1**) VQ shows a wide pH range of high activity (more than 80% between pH 6.70 and 10.00), an interesting characteristic for industrial applications. Additionally, the proteolytic activity was assayed at pH 3.3 using hemoglobin as substrate, and the activity was 1.7 units/mL, demonstrating that VQ is also active at acid pH values.

VQ showed good thermal stability (**Figure 2**), retaining about 70% of activity when incubated at 20 and 37 °C for 90 min, and even 30% of the initial activity was retained when exposed at 75 °C for 5 min. The complete enzyme inactivation was achieved by heating at 75 °C for 90 min, at 85 °C for 30 min or at 100 °C for 5 min.

Moderate sodium chloride concentrations (0.2 M) did not affect caseinolytic activity, but higher salt concentrations (1.0 and 2.0 M NaCl) decreased the optimal activity to 50% and 37%, respectively, a frequent behavior seen in most enzymes (*40*).

The isoelectrofocusing of VQ showed several polypeptide fractions, some of them with acid p*I* value while most of them had basic p*I*. The corresponding zymogram revealed proteolytic activity only for the most basic proteins (**Figure 3 A**). The use of immobiline 7-11 in the 2D-PAGE revealed the complexity of the sample that contained multiple proteins with the same MW (*ca.* 24 kDa) and p*I* values in the range 8.5−11.

When VQ was treated with protease group-specific inhibitors, the enzymatic activity was inhibited by E-64 (100 μM) and iodoacetic acid (1 mM), but was not affected by 10 mM 1,10-phenanthroline, pepstatin, nor PMFS, revealing that VQ contained cysteine-type peptidases. In C1 cysteine proteinases the free thiol function on C25 (mature papain numbering) is essential for catalytic activity (*41*) and must be protected from air

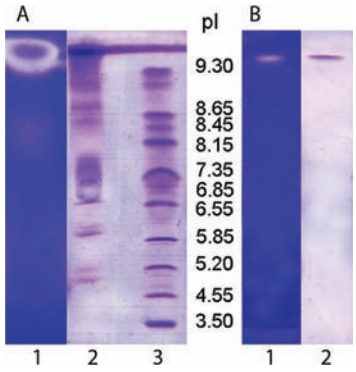

Figure 3. (**A**) Isoelectric focusing and zymogram of VQ preparation. Lane 1, zymogram of VQ preparation; lane 2, IEF of VQ preparation; lane 3, p*I* markers: amyloglucosidase (p*I* 3.50), trypsin inhibitor (p*I* 4.55), β-lactoglobulin a (p*I* 5.20), carbonic anhydrase II (p*I* 5.85), carbonic anhydrase I (p*I* 6.55), myoglobin (p*I* 6.85 and 7.35), lectins from *Lens culinaris* (p*I* 8.15, 8.45 and 8.65) and trypsinogen (p*I* 9.30). (**B**) Isoelectric focusing and zymogram of quercifoliain I. Lane 1, zimogram of quercifoliain I; lane 2, IEF of quercifoliain I.

oxidation; moreover, reversible inhibition of the enzyme prevents its autodigestion. The reaction between tetrathionate ions and the free thiol residues results in a reversible inhibition of proteases, while the addition of low molecular mass thiols (e.g., dithiothreitol, cysteine) allows the regeneration of the free thiol function when required (*42*, *43*). In the case of VQ complete inhibition was achieved by addition of 1 mM sodium tetrathionate, which was fully reversed with 20 mM cysteine.

Milk Clotting and Casein Hydrolysates by VQ. Clotting activity of VQ was determined and compared with papain obtained by us. Both enzymes clotted bovine skim milk, but the coagulant capacity of VQ was higher than that of papain (1.50 CU and 1.07 CU, respectively).

VQ preparation was used to obtain casein hydrolysates, and the produced peptides were analyzed by Tricine-SDS−PAGE (**Figure 4 A**), showing that the breakdown kinetics of α_{S2}-, α_{S1}-, β- and κ-casein varied to different degrees, as incubation progressed, while new bands with higher mobilities appeared, as early as 2 min. Densitograms from the corresponding electrophoretic lanes were performed to analyze the degradation kinetics of each fraction, and the results were plotted (**Figure 4 B**). Briefly, the degradation profile was as follows: the κ-casein fraction, involved in milk clotting, was quickly degraded in the beginning of the process (63% at 10 min of reaction) but then, the rate of the hydrolysis decreased considerably (67% of degradation at 60 min); the α_{S2}- and α_{S1}-casein fractions, associated with cheese texture, showed similar degradation patterns but at 60 min of hydrolysis α_{S1}-casein was more degraded than the α_{S2} fraction (α_{S1}-casein disappeared almost completely whereas 23% of α_{S2}-casein remained without degradation). Finally, the β-casein fraction, related to bitterness (*44*), was 94% degraded after 60 min of hydrolysis. As a consequence of hydrolytic action new peptides (15.6, 14.2, 13.0, and 10.9 kDa) appeared 10 min after the reaction started, which were fully hydrolyzed by 60 min, and a band of *ca.* 8.6 kDa (**Figure 4 A**) was generated.

Purification of Quercifoliain I. As a first purification step, VQ was submitted to molecular exclusion chromatography (Sephacryl S-100) (not shown). The main active fraction was concentrated, desalted and submitted to cation exchange chromatography (SP-Sepharose HP), which afforded two active fractions (**Figure 5**). The most basic fraction, named quercifoliain I, was homogeneous, as determined by SDS−PAGE, IEF and MALDI-TOF-MS.

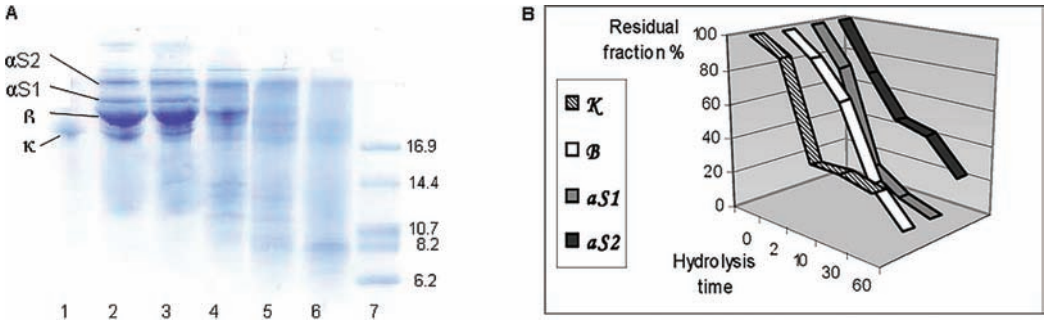

Figure 4. (**A**) Tricine-SDS−PAGE of hydrolysis products of caseins. Lane 1, κ-casein enriched standard; lane 2, 0 min of hydrolysis; lane 3, 2 min of hydrolysis; lane 4, 10 min of hydrolysis; lane 5, 30 min of hydrolysis; lane 6, 60 min of hydrolysis; lane 7, Peptide Marker (GE Healthcare): Horse myoglobin peptides. (**B**) Kinetics of degradation of casein fractions produced by the VQ preparation.

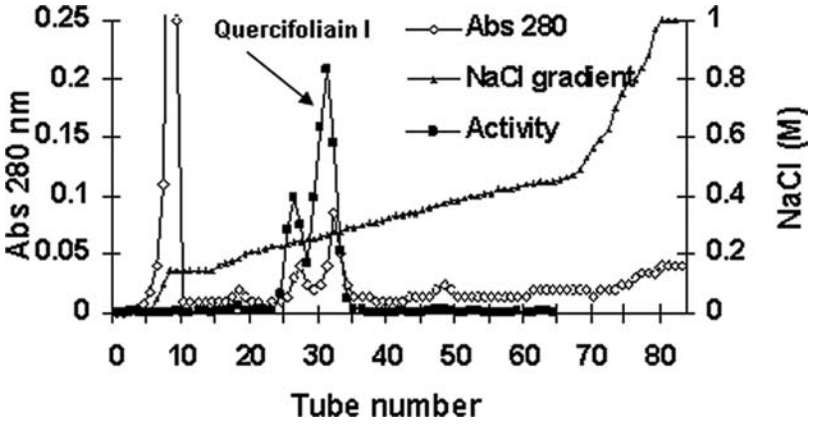

Figure 5. Cation exchange chromatography after size-selection pool. Column, SP-Sepharose HP; buffer, 50 mM Tris-HCl, pH 7.5; flow rate, 1 mL·min^{-1}; gradient, 0.15−0.45 M NaCl. Fractions of 1.2 mL were collected.

The molecular mass obtained by MS was 24195 Da (**Figure 6**) while the value obtained from SDS−PAGE was *ca.* 26.9 kDa (not shown). The IEF of quercifoliain I (**Figure 3 B**) showed a single band at p*I* higher than 9.3, coincident with that observed in the corresponding zymogram. So, the IEF-zymogram technique confirmed that quercifoliain I corresponds to one of the basic proteins present in VQ (**Figure 3 A**).

Quercifoliain I was irreversibly inhibited by E-64 (100 μM) and iodoacetate (1 mM), and activated by 20 mM cysteine, suggesting that the enzyme belongs to the cysteine-type proteases, as all other endopeptidases isolated from other species belonging to family *Caricaceae* (*45*).

The cleavage of oxidized B-chain insulin was investigated to establish the hydrolytic specificity of quercifoliain I. The identity of produced peptides was established by MALDI-TOF-MS, which yields information about the substrate specificity in a sensitive and quick way (*46*). Analysis of peptides (**Table 1**) revealed that quercifoliain I is more specific than papain (*47*). After 18 h incubation with quercifoliain I, only three excisions were observed and the specificity of the enzyme was practically restricted to glycyl and alanyl residues at position P1′. This behavior is similar to that of glycyl endopeptidase from *C. papaya*, in marked contrast to the broad specificity of papain, caricain and chymopapain; however, quercifoliain I preferred hydrophobic amino acid residues in the P2 subsite, like papain (*48*−*50*).

The N-terminal sequence (YPESVDWRQ) of quercifoliain I was compared with those of other plant proteases. Despite that 9 of the amino acid residues could be identified, little differences were detected with the N-terminus of other proteases from species belonging to the family Caricaceae (**Table 2**). No difference was found with VxH-I, a protease isolated from *V. x heilbornii* latex, but one different amino acid residue was detected between the N-terminal of quercifoliain I and those of 12 proteases isolated from the genus *Vasconcellea*. Pro2 and DWR motif are present in almost all cases with the exception of CMS1MS1-B N-terminal. The Pro2 residue is often conserved in the mature peptidases of family C1, and it is suggested that this prevents attack by aminopeptidases, since the Xaa−Pro bond is resistant to many such enzymes (*54*), while the DWR motif is characteristic of most cysteine plant proteases. When the N-terminal of quercifoliain I is compared with that of papain, the archetype protease of the subfamily C1A, only 67% of identity was shown.

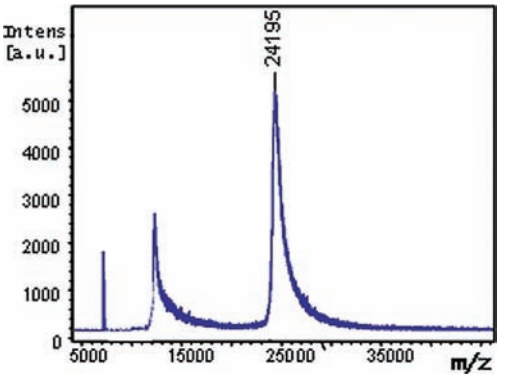

Figure 6. Mass spectrometry analysis of quercifoliain I.

Peptide Mass Fingerprint. Trypsin digested of quercifoliain I was analyzed by MALDI-TOF-MS. MASCOT search tool (URL http://www.matrixscience.com) was used for identification

Table 1. Determination of the B-Chain Insulin Cleavage Sites by Quercifoliain I[a]

Incubation Time	Ion Mass (predicted)	Ion Mass (detected)	Sequence of insulin B-chain
0 min	3515.00	3515.00	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	1273.40	1273.52	---------- GERGFFYTPKA
2 min	3515.00	3515.00	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	2260.80	2260.71	FVNQHLÇGSHLVEALYLVÇ ----------
	1272.10	1273.64	---------- GERGFFYTPKA
	1993.30	1993.30	---------- ALYLVÇGERGFFYTPKA
10 min	3514.00	3514.00	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	2260.80	2259.64	FVNQHLÇGSHLVEALYLVÇ ----------
	1273.40	1273.52	---------- GERGFFYTPKA
	1539.50	1540.76	FVNQHLÇGSHLVE ----------
	1993.30	1993.30	---------- ALYLVÇGERGFFYTPKA
60 min	3514.00	3514.00	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	2260.80	2259.64	FVNQHLÇGSHLVEALYLVÇ ----------
	1273.40	1273.52	---------- GERGFFYTPKA
	1539.50	1540.76	FVNQHLÇGSHLVE ----------
	1993.30	1993.30	---------- ALYLVÇGERGFFYTPKA
	1418.60	1418.62	------ GSHLVEALYLVÇ ----------
180 min	3514.00	3514.00	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	2260.80	2259.64	FVNQHLÇGSHLVEALYLVÇ ----------
	1273.40	1273.52	---------- GERGFFYTPKA
	1539.50	1540.76	FVNQHLÇGSHLVE ----------
	1418.60	1418.62	------ GSHLVEALYLVÇ ----------
18 hs	3514.00	3514.00	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA
	1273.40	1273.52	---------- GERGFFYTPKA
	1539.50	1540.76	FVNQHLÇGSHLVE ----------
	1418.60	1418.62	------ GSHLVEALYLVÇ ----------
VQ 1	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA		
Papain	FVNQHLÇGSHLVEALYLVÇGERGFFYTPKA (47)		

[a] 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 and carbamidomethylated cysteine. For comparison papain cleavage sites previously established are shown at the lower end.

of tryptic maps. No matches were found with papain or with other plant cysteine protease. When the Peptide Mass Fingerprint (PMF) of quercifoliain I was compared with that obtained by in silico digestion of papain, only two peptides matched the search, even when using a high peptide mass tolerance (1 Da) indicating the presence of significant differences in the primary sequences. Protein identification and differentiation by PMF has been adopted in our group as an excellent tool to differentiate, in a fast and unequivocal way, proteases with very similar physicochemical and functional properties (*55*). The robustness of the method even allowed the differentiation of the homologue isoenzymes of the latex of *Asclepias curassavica* (*55*), as well as the identification of rAfCP, the recombinant enzyme of *A. fruticosa* (*56*).

In sum, a new proteolytic preparation (VQ) obtained from the latex of *Vasconcellea quercifolia* ("oak leaved papaya") is reported. VQ specific activity is higher than that of crude papain, and the preparation was able to coagulate milk and could be used

Table 2. N-Terminal Amino Acid Sequences[a]

Plant Protease	N-terminal Sequence	Reference	Identity (%)
Quercifoliain I [*V.quercifolia*]	YPESVDWRQ		
VXH-I [*V. x heilbornii*]	YPESVDWRQ	24	100
VXH-III [*V. x heilbornii*]	YPESVDWRG	24	89
VXH-IVa [*V. x heilbornii*]	YPESIDWRQ	24	89
VS-B [*V. stipulata*]	YPESIDWRQ	24	89
CMS2MS2, CMS2MS3, CMS3MS1, CMS3MS2, CMS3MS3, CMS3MS6 [*V. cundinamarcensis*]	YPESIDWRQ	21	89
CMS2MS1, CMS2MS4 [*V. cundinamarcensis*]	YPGSVDWRQ	21	89
CC-II [*V. cundinamarcensis*]	YPGSVDWRQ	17	89
VXH-IVb [*V. x heilbornii*]	YPESIDWRK	24	78
CC-III [*V. cundinamarcensis*]	YPESIDWRK	17	78
CC-IV [*V. cundinamarcensis*]	YPESIDWRK	17	78
CC28 [*V. cundinamarcensis*]	YPESIDWRK	21	78
CMS2MS5 [*V. cundinamarcensis*]	IPESIDWRQ	21	78
Mexicain [*Jacaratia mexicana*]	YPESIDWRE	16	78
CCIb [*V. cundinamarcensis*]	IPTSIDWRQ	19	67
CCIa [*V. cundinamarcensis*]	IPASIDWRQ	19	67
Glycylendopeptidase [*C. papaya*]	LPESVDWRA	51	67
Papain [*C. papaya*]	IPEYVDWRQ	52	67
Chymopapain [*C. papaya*]	YPQSIDWRA	53	67
VXH-A [*V. x heilbornii*]	IPASIDWRQ	24	67
VXH-B [*V. x heilbornii*]	IPASIDWRQ	24	67
VXH-D [*V. x heilbornii*]	IPTSIDWRQ	24	67
VS-A [*V. stipulata*]	IPASIDWRQ	24	67
CMS1MS1-A [*V. cundinamarcensis*]	IPASIDWRQ	21	67
Caricain [*C. papaya*]	LPENVDWRK	42	56
CMS1MS1-B [*V. cundinamarcensis*]	IPTSIDQRQ	21	56
CC23 [*V. cundinamarcensis*]	-PQRMDWRK	21	44

[a] N-Terminal sequence of quercifoliain I compared with the sequences from proteases *of Caricaceae.* Coincident amino acids are on a gray background.

for the production of cheeses and/or casein hydrolysates, taking into account that crude extracts or partially purified enzymes are normally used in food industry.

From VQ a new cysteine protease, named quercifoliain I, was isolated and characterized. The new enzyme showed a molecular mass of 24195 Da (mass spectrometry) and an isoelectric point >9.3. Its behavior on the insulin B-chain is highly specific, as it only cleaved glycyl and alanyl residues at P1 position.

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Supporting Information Available: Figures depicting cation exchange chromatography on CM-Sepharose FF of VQ, effect of ionic strength on proteolytic activity of VQ, effect of group specific inhibitors on proteolytic activity of VQ, and bidimensional electrophoresis of VQ. This material is available free of charge via the Internet at http://pubs.acs.org.

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