Isolation and characterization of an Aspartic Protease from *Salpichroa origanifolia* Fruits

Gabriela F. Rocha[1], W. David Obregón[2,4], Fernando Muñoz[3,4], M. Gabriela Guevara[3,4], Graciela Fernández[1], Adriana M. Rosso[1] and Mónica G. Parisi[1*]

[1]*Área de Química Biológica. Departamento de Ciencias Básicas. Universidad Nacional de Luján. Ruta 5 y Avenida Constitución, C.C. 221, B6700AVW, Luján, Buenos Aires. Argentina;* [2]*Laboratorio de Investigación de Proteínas Vegetales, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115 s/N, C.C. 711, B1900AVW, La Plata, Argentina;* [3]*Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata, Funes 3250, B7600AVW, Mar del Plata, Argentina;* [4]*Consejo Nacional de Investigaciones Científicas y Tecnológicas, CONICET*

Mónica G. Parisi

Abstract: This report describes the purification of an aspartic protease (salpichroin) from ripe fruits of *Salpichroa origanifolia* (Solanaceae) starting with precipitation using organic solvents and anion-exchange chromatography with 32.1% recovery and 13.4-fold purification. SDS-PAGE and zymograms of this enzyme showed a single band corresponding to an apparent molecular mass of approximately 32 kDa. The biochemical and kinetic characterization of the pure enzyme showed an acidic behavior with an optimal pH value around 3.0–4.5 with hemoglobin and 5.5–6.0 with casein. Salpichroin activity was inhibited by pepstatin but not by phenylmethylsulfonyl fluoride, E-64, EDTA or 1,10-phenanthroline, thus suggesting an aspartic protease behavior. Salpichroin hydrolyzed natural substrates, such as casein and hemoglobin, with high specific activity. Kinetic studies conducted with the synthetic peptide H-Pro-Thr-Glu-Phe-p-(NO$_2$)-Phe-Arg-Leu-OH showed lower affinity (K_m 494 µM) than other representative aspartic proteases. By investigating the cleavage of oxidized insulin β-chain to establish the hydrolytic specificity of salpichroin, we found six cleavage sites on the substrate of peptide bonds similar to those of chymosin. MALDI-TOF/TOF-MS of the tryptic in-gel digest of salpichroin showed that the isolated protease shared homologous sequences with other plant proteases of the A1 aspartic protease family. This is the first report concerning the isolation and biochemical characterization of an aspartic protease isolated from *Salpichroa origanifolia* fruits.

Keywords: Caseinolytic activity, peptide mass fingerprinting, plant aspartic protease, purification, *Salpichroa origanifolia,* salpichroin.

INTRODUCTION

Proteases are a multifunctional class of enzymes involved in multiple physiological reactions, from simple digestion of food proteins to highly regulated processes, such as activation of zymogens, blood-clotting cascade, and activation of the complement, inflammation, and apoptosis. Proteases are ubiquitous in nature and have commercial importance since they represent half of the enzymes used in the industry [1, 2].

Aspartic proteases (APs) (EC 3.4.23) belong to one of the seven catalytic types of proteolytic enzymes widely distributed in all living organisms [3, 4, 5]. Plant APs have characteristics in common with the aspartic proteinase A1 family: they are active at acidic pH values, specifically inhibited by pepstatin, and two aspartic acid residues are responsible for their catalytic activity [1]. APs have been principally found in seeds but are also present in leaves, tubers, flowers, petals, and fruits of many species [6, 7, 8, 9, 10]. A small number of APs have been isolated and characterized from plant sources including monocotyledons, e.g. barley, rice, maize, and wheat [11, 12, 13, 14], and dicotyledons, e.g. tomato, cacao, and *Arabidopsis* [15, 16, 17]. Only the cDNAs of APs from cardoon, potato, *Arabidopsis*, *Brassica*, rice, barley, tomato, and sweet potato have been studied [18, 19, 20]. A characteristic feature of most typical plant APs is the presence of the plant specific insert (PSI), which is approximately 100 residues long and separates the enzyme sequence into two regions [21]. The PSI sequence shows no homology to mammalian or microbial APs, but is highly similar to that of saposin-like proteins [22, 23]. Plant APs have different physiological roles, including protein turnover, germination, senescence and host pathogen interaction [17, 22, 24]. Besides, the correlation between the different patterns of typical plant APs and their biological functions is still far from being deciphered [4]. In preliminary studies, we have reported that crude extracts from *Salpichroa origanifolia* fruits have proteolytic and milk-clotting activities [25]. In addition, we obtained antimicrobial peptides from milk sources by enzymatic hydrolysis [26].

*Address correspondence to this author at the Área de Química Biológica, Departamento de Ciencias Básicas, Universidad Nacional de Luján, Luján, Buenos Aires, Argentina; Tel: +54 2323 423171; Fax: +54 2323 425795; E-mails: monicaparisiunlu@gmail.com; mparisi@unlu.edu.ar

Salpichroa origanifolia (Lam.) is a perennial herb found in the north and center of Argentina that belongs to the Solanaceae family. The ripe fruits are edible and the leaves are believed to have medical properties [27, 28]. Some compounds present in the leaves have lethal and sub-lethal effects on species of economic importance such as *Musca domestica* and *Ceratitis capitata* [29, 30].

The aim of this work was to identify an AP from ripe fruits of *S. origanifolia* using biochemical and proteomic tools. As the biological role of plant APs is not completely established, the identification of a plant AP is interesting to determine the possible biological function as well as its potential industrial applications.

MATERIALS AND METHODS

Reagents

Hemoglobin, Coomassie Brilliant Blue R-250, E-64, phenylmethylsulfonyl fluoride (PMSF), pepstatin, Tris base, and glycine were either of analytical grade or purchased from Sigma Chemical Company (St. Louis, MO, USA). Acrylamide, bisacrylamide, and low-range molecular weight standards were obtained from Bio-Rad (Hercules, CA, USA). The synthetic peptide H-Pro-Thr-Glu-Phe-p-(NO2)-Phe-Arg-Leu:OH was purchased from Bachem A.G., Bubendorf, Switzerland. All other chemicals were obtained from commercial sources and were of analytical grade.

Plant Material

Ripe fruits from *S. origanifolia* were collected from plants grown near Luján city (Buenos Aires, Argentina). The mature fruits are about 2 cm long and look like small white eggs. After collection, fruits were washed thoroughly with distilled water and then stored at 4°C until they were used.

Crude Extract

Fresh and mature fruits were ground in 50 mmol L^{-1} potassium phosphate buffer pH 7.0, filtered through cheese cloth, and centrifuged at 20,000 *g* for 20 min at 4°C (SS-34 Sorvall rotor). The supernatant was diluted with two volumes of cold ethanol at -20°C and kept for 2 h for complete precipitation with gentle stirring, before vacuum filtration. The precipitate was dissolved in the same buffer and then centrifuged at 26,000 *g* for 20 min at 4°C. The supernatant called crude extract was stored at -20°C for further analysis.

Aspartic Protease Purification

The AP was purified by anion exchange chromatography on a Mono Q column (FPLC system, Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 mmol L^{-1} potassium phosphate buffer (pH 7.0). Proteins were eluted with a linear gradient of NaCl (0.15-0.5 mol L^{-1}) at a flow rate of 0.6 mL min^{-1}. The active fraction was desalted by size exclusion chromatography on Sephadex G-10, and the purified protein showing proteolytic activity was stored at -20ºC for further characterization.

Activity Assays

The proteolytic activity of the AP against hemoglobin as a natural substrate was determined according to Anson [31] with slight modifications. Enzyme solution (200 µL) was incubated for 10 min (40ºC) with 200 µL of 1.0% (w/v) acid-denatured bovine hemoglobin in 100 mmol L^{-1} citrate buffer pH 4.0. The reaction was stopped by adding 1 mL of 5% (w/v) trichloroacetic acid (TCA). The reaction mixture was settled for 20 min and then centrifuged at 13,800 *g* for 15 min (Eppendorf Centrifuge 5415 C) to remove the precipitate. The acid soluble material (500 µL) was added to Folin reagent (300 µL) in 0.5 mol L^{-1} sodium hydroxide, and the absorbance recorded at 750 nm. One unit of proteolytic activity with hemoglobin as protein substrate (UHem) was defined as the amount of enzyme required to cause a unit increase in absorbance per minute at 750 nm across a 1-cm path length, under the assay conditions.

The caseinolytic activity of AP was measured with α-casein as protein substrate as performed previously [32]. Enzyme solution (100 µL) in acetate buffer (pH 6.0) was added on 900 µL of 0.5% (w/v) α-casein. After 30 min incubation at 40°C, the reaction was stopped with the addition of 1 mL of 5% (w/v) TCA. Acid soluble products were determined in 1 mL of the supernatant by measuring the absorbance at 280 nm. One unit of caseinolytic activity with α-casein as protein substrate (Ucas) was defined as the amount of enzyme required to cause a unit increase in absorbance per minute at 280 nm across a 1-cm path length, under the assay conditions.

The protease activity against the synthetic peptide H-Pro-Thr-Glu-Phe-p-(NO_2)-Phe-Arg-Leu-OH (PNPE) was also determined. Enzyme solution (100 µl) was incubated at 25°C with 0.2 mmol L^{-1} substrate (1mL) in 50 mmol L^{-1} sodium citrate buffer (pH 3.5) and the hydrolysis rate of the nitrosyl product was monitored at 310 nm in a GeneQuant 1300 UV/visible spectrophotometer (GE, USA). A molar absorption coefficient of 1,800 L mol^{-1} cm^{-1} at 310 nm was used in the calculations [33]. One unit of proteolytic enzyme was defined as the amount of enzyme that hydrolyzed 1 mol min^{-1} of PNPE at 25°C under the assay conditions.

Protein Content

The protein content was quantified in a Bradford assay [34] using bovine serum albumin (BSA) as standard. Along the enzyme purification, the protein content was also estimated by measuring the absorbance at 280 nm.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE under reducing and non-reducing conditions was carried out in 5 and 14% polyacrylamide gels [35]. The gels were stained with Coomassie Brilliant Blue R-250 or silver nitrate [36]. Low Molecular Weight Range Markers (Sigma Aldrich) were used as molecular mass standards.

Zymography was performed on native-PAGE with 0.1% w/v gelatin included in the gel mixture [37]. After electrophoresis, the gel was incubated overnight in sodium citrate

buffer (pH 4.0) with 20 mmol L^{-1} CaCl$_2$ at 40°C overnight. The development of a clear band on the blue background of the gel indicated the presence of protease activity.

Biochemical Characterization

Effect of pH on Proteolytic Activity

The proteolytic activity of the purified enzyme was measured on α-casein and hemoglobin at different pH values. The pH of the reaction mixture was adjusted with the following buffers: 50 mmol L^{-1} glycine–HCl buffer (pH 2.0–2.5), 50 mmol L^{-1} sodium citrate buffer (pH 3.0–6.0) and 50 mmol L^{-1} phosphate buffer (pH 6.5–8.0).

Stability Assays

The thermal stability of the purified enzyme was measured by incubating the enzymatic preparation with 100 mmol L^{-1} sodium citrate buffer (pH 4.0) without a substrate at different temperatures (40-55°C) for 120 min. The standard protease assay with hemoglobin was performed to determine its residual activity. Besides, the self-processing of the activated enzyme at low temperatures (-20°C) was evaluated for a period of 30 days. Native-PAGE was performed to visualize the degradation pattern. The gels were stained with silver and Coomassie Brilliant Blue R-250, as previously described.

Effect of Inhibitors on the Enzymatic Activity

Inhibition assays were performed using pepstatin, PMSF, E-64, and 1,10-phenanthroline to determine class and enzyme specificity [38]. The purified enzyme was incubated for 30 min at 30°C with the inhibitor solutions and the residual activity was measured. The control without inhibitors was taken as 100%. Residual activity was measured with the hemoglobin method [39].

Kinetic Characterization

The effect of substrate concentration on the reaction rate of enzyme hydrolysis was studied using synthetic and natural substrates [25]. Kinetic studies were performed using substrate concentrations between 100 and 1000 μmol L^{-1} for hemoglobin (pH 4.0), 30 and 225 μmol L^{-1} for casein (pH 6.0), and 20 and 700 μmol L^{-1} for PNPE (pH 3.7). Blank samples were carried out simultaneously at the specific substrate concentrations without the enzyme. In each case, Lineweaver-Burk plots were obtained and Michaelis-Menten constants (K_m and V_{max}) were calculated [33, 40]

Enzyme concentration was determined by active site titration with pepstatin, as described by Barrett and Kirschke [41] using PNPE substrate. To express the V_{max} values as k_{cat}, we used a molecular weight of 32 kDa.

Specific Cleavage on Oxidized Insulin β-Chain

Oxidized insulin β-chain (1 g/L) was incubated with the purified enzyme (substrate:enzyme mass ratio of 100:1) in 0.1 mol L^{-1} sodium acetate buffer (pH 4.0). After 12 h incubation at 40°C, the reaction mixture was centrifuged at 16,000 g for 6 min (Eppendorf Centrifuge 5415 C) and the insulin-digested fragments were separated by reversed phase high-pressure liquid chromatography (RP-HPLC) using a Brownlee C$_{18}$-column. The chromatography was carried out at room temperature and the column was equilibrated with 0.1 % (v/v) aqueous trifluoroacetic acid (TFA). The peptides were eluted with a linear gradient of acetonitrile (0-80 %) at a flow rate of 0.2 mL/min.

Protein Sequencing

The N-terminal sequence of the protein was determined on a 477a protein sequencer (Applied Biosystems; LANAIS PRO, Universidad de Buenos Aires, Buenos Aires, Argentina).

MALDI-TOF/TOF Mass Spectrometry

In-gel protein digestion was carried out using the In-Gel Digest$_{ZP}$ Kit (Millipore, Billerica, MA, USA). Protein bands of SDS-PAGE corresponding to the active fraction eluted from the chromatographic column were excised with a scalpel and stored in Eppendorff tubes at -20°C until processed. The protein samples were reduced and carbamidomethylated with 10 mmol L^{-1} dithiothreitol (DTT) and 50 mmol L^{-1} iodoacetamide in 25 mmol L^{-1} NH$_4$HCO$_3$ for 1 h at 37°C and then digested with 0.3 μg of trypsin on 100 mmol L^{-1} NH$_4$HCO$_3$ pH 8.5 for 12 h at 37°C. The resulting peptides were recovered by extraction with 50% (v/v) aqueous acetonitrile, dried in a SpeedVac vacuum centrifuge and dissolved in 0.1% (v/v) aqueous TFA. Tryptic digestion products were placed in an MP 384 ground steel plate using α-cyano-4-hydroxycinnamic acid as matrix, and then analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) using a *m/z*-range from 800 to 4,000. External calibration was performed with peptide calibration standards. Comprehensive peak assignments were accomplished using the BioTools software package (Bruker Daltonics). The MASCOT search tool URL http://www.matrixscience.com) was used to identify the related tryptic maps. Peptides of the tryptic digest were sequence "*de novo*" on a MALDI-TOF/TOF-MS (Ultraflex Extreme, Bruker Daltonics, Germany) as reported [42, 43].

Phylogenetic Tree Construction

The evolutionary history was inferred using the Maximum Parsimony (MP) method. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1, in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 20 amino acid sequences. The final data set had a total of 229 positions. Evolutionary analyses were conducted in MEGA5 [44].

Statistical Analysis

Results are means of at least two independent experiments carried out in triplicate.

RESULTS AND DISCUSSION

Purification and Characterization of the Aspartic Protease (AP) from *Salpichroa origanifolia* Fruits

Partially purified crude extracts derived from ripe fruits of *S. origanifolia* were obtained by precipitation with cold

ethanol (-20ºC). This procedure was used to eliminate soluble carbohydrates and phenolic compounds which could oxidize and react irreversibly with the proteins present in the fruits. The ethanolic precipitate was then homogenized in phosphate buffer and centrifuged. The enzymatic preparation (called *crude extract*) had a protein content of 2.5 mg and a caseinolytic activity of 598.33 Units min^{-1}.

It was not possible to purify the crude extract by affinity chromatography on a pepstatin-agarose column, because we could not elute the enzyme from the column. Therefore, an anion exchange chromatography on DEAE-Sepharose Fast Flow was performed. In SDS-PAGE a band at an apparent molecular mass of around 29 kDa and two faster migrating bands corresponding to about 17 and 9 kDa were stained, suggesting a typical self-degradation process [25]. In recent experiments, a simple chromatographic scheme consisting of anion-exchange chromatography on a Mono Q column (FPLC) was used to purify the AP present in the crude extract. The eluate was collected directly in tubes containing pepstatin (1 μmol L^{-1} in fraction) to prevent autolysis. The elution profile indicated several protein peaks of which one contained most of the caseinolytic activity (Fig. **1**).
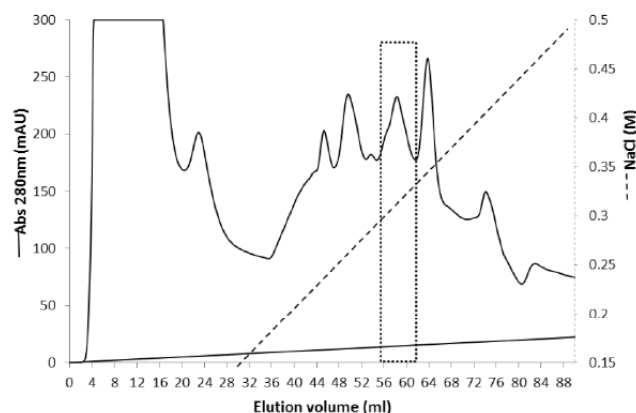

Figure 1. Anion-exchange chromatography on a Mono Q column equilibrated in 50 mmol L^{-1} phosphate buffer (pH 7.0) and eluted in the same buffer using a linear gradient from 0.15 to 0.5 mol L^{-1} NaCl. Fractions of 2 mL per tube were collected.

The active fraction was desalted by size-exclusion chromatography on a Sephadex G-10 column. After the purification step, the AP was purified 13.4-fold with a recovery of 32.1% and a specific activity of 3203.3 Ucas mg^{-1} from the starting material using casein as protein substrate (Table **1**). This protease previously named SoAP was renamed salpichroin according to Kervinen [45].

SDS-PAGE of the purified enzyme showed a single protein band of ca. 32 kDa (Fig. **2a**) under denaturing and reducing conditions. The purity of salpichroin was also evaluated by native PAGE and only one band was observed (Fig. **2b**, lane 1). The proteolytic activity of the enzyme was also investigated in 0.1% gelatin copolymerized gels (zymogram), where lane 2 indicated substrate digestion in the gel with a mobility similar to that of the native gel confirming the homogeneity of the purified enzyme (Fig. **2b**). The native molecular mass of salpichroin estimated by the size exclusion method was consistent with the SDS-PAGE (data not shown).

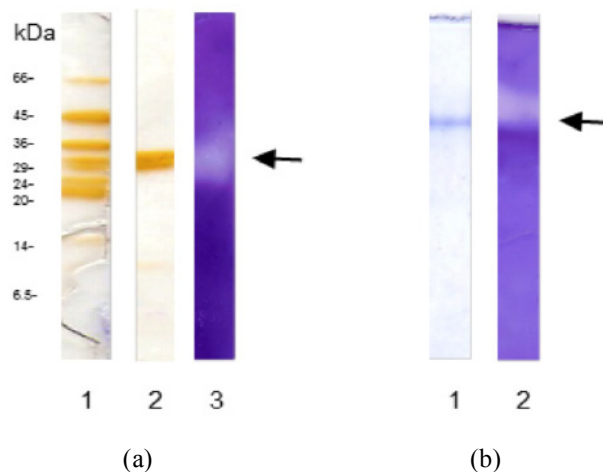(a)   (b)

Figure 2. (a) SDS-PAGE using a 15% gel. Lane 1: low molecular weight markers, lane 2: fraction eluted from the Mono Q column, lane 3: zymogram of salpichroin. **(b)** 10% Native-PAGE. Lane 1: purified fraction and lane 2: zymogram of the purified fraction. Left scale shows the molecular masses of marker proteins. The position of the AP is indicated by the arrow on the right.

The biochemical characterization of the pure enzyme showed an acidic behavior with an optimal pH value around 3.0 -4.5 with hemoglobin and 5.5-6.0 with casein (Fig. **3**). These results are consistent with those reported for other typical plant APs [8, 46, 47, 48, 49].

The thermal stability of the enzyme was examined by measuring the residual activity at different temperatures. The enzyme showed low stability at moderate temperatures (45°C) compared with the crude extract and after 30 min it retained less than 20% of the initial activity (Fig. **4a**). Immobilization techniques are now under development to increase the stability of the pure enzyme.

Table 1.   Purification chart of the aspartic protease from *Salpichroa origanifolia* fruits.

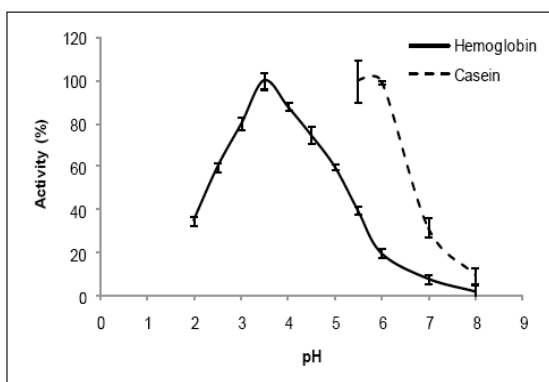| Purification step	Total activity (Ucas.min^{-1})	Total protein (mg)	Specific activity (Ucas .mg^{-1})	Recovery (%)	Purification fold
Crude extract	598.33	2.50	239.33	100	1
Mono Q	192.21	0.06	3203.33	32.1	13.4

Figure 3. Effect of pH on activity of the purified protease.

Auto-digestion of the enzyme at low temperature was also evaluated because proteases are generally prone to undergo autolysis. Fig. (**4b**) shows the native PAGE of the enzyme stored for 30 days at -20°C. As expected, the proteolytic cleavage increased with storage time, and salpichroin was highly processed after 30 days. We next studied the behavior of class-specific inhibitors on salpichroin activity. The enzymatic activity was completely inhibited by pepstatin, a well-known inhibitor that binds the active site cleft of the enzyme, indicating that the protease belongs to the catalytic group of aspartic proteases [50]. E-64 as well as PMSF and 1,10-phenantroline did not affect protease activity (Table **2**).

Pepstatin, a tight binding inhibitor specific for APs [51], inhibited salpichroin at relatively low concentrations. Titration of the active sites of salpichroin using pepstatin showed an effective concentration for the active enzyme of 0.88 μmol L^{-1} (Fig. **5**).

Table 2.   Effect of protease inhibitors on enzyme activity.

Modulator	Concentration (mM)	Activity (%)
None	-	100 ± 3
1,10-phenanthroline	10.000	90 ± 3
PMSF	1.000	84 ± 2
E64	0.010	95 ± 1
Pepstatin	0.001	7 ± 2

A complete steady-state kinetic analysis revealed that the tight binding inhibition constant (K_i) of salpichroin for pepstatin was 500 nmol L^{-1}. This value was higher than that of pepsin and chymosin, under the same assay conditions (Table **3**).

Natural substrates, such as α-casein and hemoglobin, and the synthetic peptide PNPE were used for kinetic analysis. Salpichroin followed a Michaelis-Menten kinetics. The K_m values, obtained from the Lineweaver-Burk plots (Table **3**), were 164 μmol L^{-1} for α-casein and 378 μmol L^{-1} for hemoglobin. The K_m for α-casein was in the same order as that determined for bovine chymosin (370 μmol L^{-1}). Furthermore, the K_m for hemoglobin was similar to that of an AP from the insect *Plutella xylostella* (538 μmol L^{-1}) and higher than that of an AP from S*ardinella aurita* (73 μmol L^{-1}). The K_m value for PNPE was 494 μM, which is similar to earlier reports for other milk-clotting enzymes [33], such as bovine chymosin (460 μmol L^{-1}) and human pepsin (170 μmol L^{-1}), but higher than that of Cynarase A, an AP from *Cynara cardunculus* (58 μmol L^{-1}).
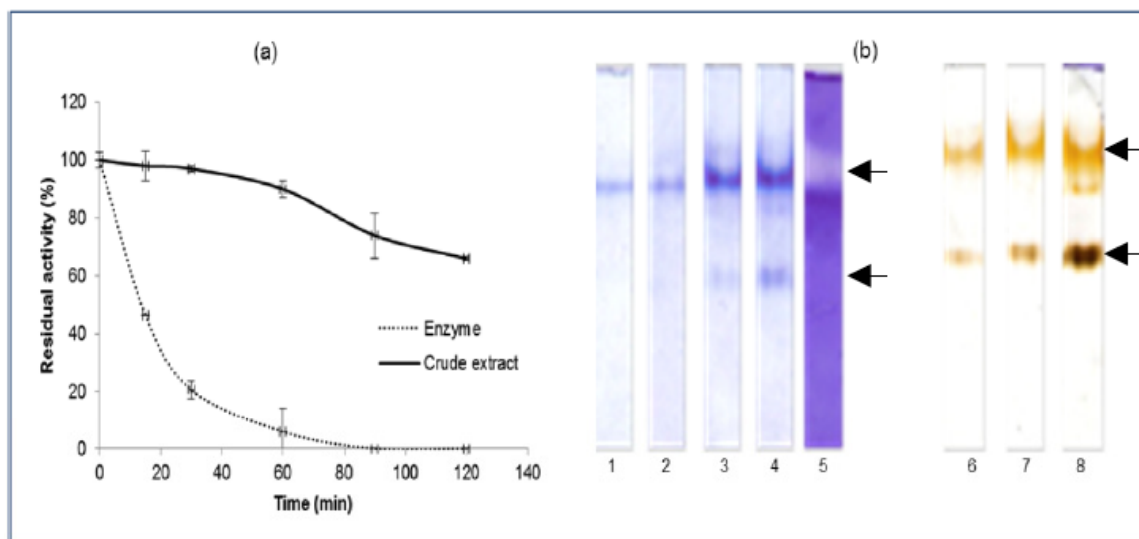
Figure 4. (a) Thermal stability at 45°C of the crude extract and the purified protease. Activity was assayed using hemoglobin as a substrate. **(b)** Native-PAGE (10%) of the self-processed salpichroin stored at -20°C. Lane 1: purified fraction, lane 2: day 7, lane 3: day 14, lane 4: day 30 and lane 5: zymogram of the purified fraction. Silver staining of the Native gels. Lanes 6, 7 and 8: days 7, 14 and 30 respectively. The position of the AP and the corresponding fragment are indicated by arrows.

F**igure 5.** Active site titration of the aspartic protease from *Salpichroa origanifolia* fruits with pepstatin. The residual enzymatic activity was measured with the synthetic substrate PNPE.
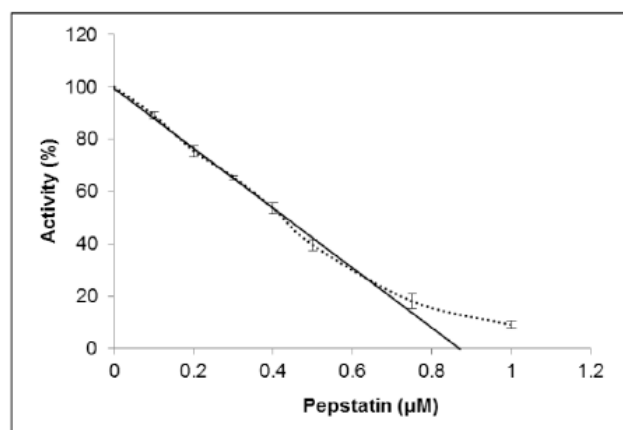
The k_{cat} values calculated from the effective concentration of the active enzyme (0.88 μmol L^{-1}) and the k_{cat}/K_m ratio of salpichroin were similar to APs of Cynarase A (Table **3**). On the other hand, salpichroin showed lower affinity for the synthetic substrate than the other proteases evaluated.

Cleavage specificity of oxidized insulin β-chain

The proteolytic specificity of salpichroin was investigated for oxidized insulin β-chain, which was cleaved at six sites between residues Phe_1-Phe_2, Leu_{11}-Val_{12}, Leu_{15}-Tyr_{16}, Tyr_{16}-Leu_{17}, Phe_{24}-Phe_{25}, and Phe_{25}-Tyr_{26} (Table **4**). These cleave sites are similar to those of chymosin including one common cleavage site between Leu_{15} and Tyr_{16}. This peptide bond is also cleaved by other plant APs, such as cardosins A, B, E, F, G, and H [55, 56], procirsin [4], phytepsin-Barley [57], StAP1, and StAP3 [58]. The fact that salpichroin cleaves the peptide bond Phe_1-Phe_2 suggests that this enzyme does not only have endoproteolytic activity but also exoproteolytic activity [58].

Sequence Analysis of Salpichroin

The 32 kDa band was excised from SDS-PAGE, digested with trypsin, and analyzed by MALDI-TOF-MS. The peptide mass fingerprint (PMF) was submitted to MASCOT (http://www.matrixscience.com) using the National Center for Biotechnology Information database (Fig. **6**). Considering at least a score of 54 and four peptide masses matched, it was not possible to identify a protease in the database.

Tandem mass spectra were recorded for the five most prominent peaks at *m/z* 1294.6 (P1), 1743.7 (P2), 1970.9 (P3), 2317.1 (P4), and 2611.2 (P5) (Fig. **6**). The tandem mass spectra recorded for P1 and P2 matched well (significant threshold P < 0.05) with the theoretical ion values of tryptic peptides from an AP of *Nepenthes alata* (called NaAP3) and from a predicted A1-like AP of *Solanum lycopersicum* (called *Sl*AP1-like; GenBank acc. number: XP_004231616.1).

Peptides P3 and P4 did not provide reliable MASCOT score. However, "*de novo*" sequencing provided the partial sequences FTV**V**FDTGSSNLWVPSSK and NTEEEQGG EI**I**FGGVDPNHFK, respectively. These sequences correspond to two tryptic peptides of *Sl*AP1-like, i.e. FTV**I**FDT GSSNLWVPSSK and NTEEEQGGEI**V**FGGVDPNHFK, respectively, showing substitutions V→I and I→V, respectively (Table **5**). The tandem mass spectrum of P5 indicated the sequence FSEDNVKVGDLVVTDQEFIEATR, which contains one missed lysine cleavage site.

The deduced sequences clearly show that the enzyme under consideration belongs to the A1 AP family, with high similarity to *Sl*AP1-like. This uncharacterized enzyme is predicted from a genomic sequence (NW_004194303.1, BioProject: PRJNA66163). The open reading frame of the enzyme consists of a 65-amino acid preprosequence and a 441-residue-long mature protein, about the same size as that revealed for rice and cardoon enzymes [61].

In general, plant APs are expressed in their zymogen form, undergo N-terminal processing during activation, and then some of them undergo further internal processing until they reach the mature form. The presence of a 100-residue

Table 3. **Kinetic parameters of salpichroin with α-casein, hemoglobin, and PNPE as substrates**

Substrate	Enzyme	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})	K_i (mM)	References
α-casein	Salpichroin	164	11.9	72.0	-	-
	Bovine chymosin	370	0.7	1.9	-	[52]
Hemoglobin	Salpichroin	378	1.4	3.6	-	-
	AP *Sardinella aurita*	73	-	-	-	[53]
	AP *Plutella xylostella*	538	-	-	-	[54]
PNPE	Salpichroin	494	10.3	20.9	500.0	-
	Human pepsin	170	72.0	425.0	57.2	[33]
	Bovine chymosin	460	51.0	110.0	10.5	[47]
	Cynarase A	58	2.6	45.3	30.0	[47]

Table 4. Comparison of proteolytic cleavages of salpichroin a related APs for the β chain of insulin. Shown is the sequence of insulin β chain with cleavage sites of each protease highlighted in gray .

Salpichroin	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	
Chymosin	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[59]
Pepsin	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[60]
Procirsin	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[4]
Cardosin E, F, G, H	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[56]
Cardosin B	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[56]
Phytepsin (barley)	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[57]
StAP1	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[58]
StAP3	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G	F	F	Y	T	P	K	A	[58]
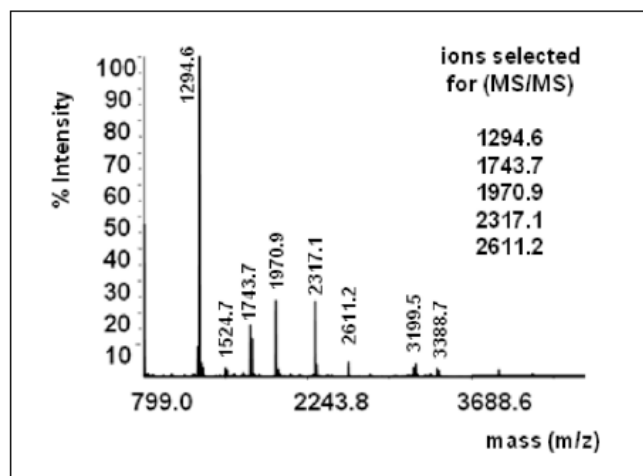
Figure 6. Mass spectrum of the purified protease digested with trypsin. Dominant signals assumed to represent tryptic peptides were selected to record tandem mass spectra (insert in the graph).

long sequence known as PSI is a unique feature of plant APs [62]. The PSI is an independent subunit of five amphi-pathic α-helices linked by three disulfide bridges inserted into the C-terminal domain. The PSI shares a high sequence homology with mammalian saposins, which are lysosomal, sphingolipid-activating proteins involved in membrane degradation [19], and can exhibit antimicrobial activity by inducing membrane leakage [63, 64]. It has also been suggested that the PSI is involved in vacuolar targeting of APs and excised during activation, which results in the formation of a two-chain enzyme [65, 21]. However, the reason why some plant APs exist as monomers and others as dimers is not known. Both autocatalytic and heterocatalytic steps are needed to activate the enzyme. This mechanism presumably depends both on the prevailing pH and on the presence of processing proteases within the particular intracellular compartment traversed by the enzyme precursor [45]. Thus, the activation process *in vitro* is not always complete under this condition [65].

Additionally, HPLC analysis of the pure enzyme showed two peaks corresponding to salpichroin and another peptide of 9 kDa, probably a autolytic product of the enzyme. The aminoterminal sequence of the protein was not available since the N-terminus was blocked. The N-terminal sequence of the 9-kDa fragment, SAVDCGKLSSMPTCXF, matched with the C-terminal domain of *Sl*AP1-like and may be inserted into the PSI present in the monomeric APs. The peptide fragments with 95% of sequence identity with *Sl*AP1-like are shown in Fig. **7**.

The relationship between the salpichroin fragment and other plant APs containing the PSI domain was evaluated by phylogenetic analyses using the Neighbor-Joining method of MEGA 5 program [44]. The resulting tree splits into two major groups (I and II; Fig. **8**). Group I splits further into two sub-groups, where group Ia includes the salpichroin fragments and contains the proteases from *Arabidopsis thaliana*

Table 5. **Identification of salpichroin by mass spectrometry.**

Observed Mass (kDa)	Predicted Mass (kDa)	Mascot Ion Score	Peptide Secuence	Match to
1294.6417	1293.6506	126	K.EPVFSFWLNR.K	AP 3 *Nepenthes alata* AP A1-like *Solanum lycopersicum*
1743.7896	1742.7974		K.GYWQFDMGDVLIDGK.A	
1743.7896	1742.7974	94	K.GYWQFDMGDVIIDGK.S	AP-like isoform X1 de *Cicer arietinum*
1294.6417	1293.6506	85	K.EPVFSFWLNR.N	AP *Brassica olearacea* AP *Brassica napus* AP *Arabidopsis thaliana*
1970.9624	1969.9786		K.FTVVFDTGSSNLWVPSSK.C	
1743.7896	1742.7974	82	K.GYWQFDMGDVLIGDK.T	AP *Helianthus annuus*

```
                                                             50
Salpichroin : ------------------------------------------------------------- :   -
SlAP1-like  : MGAKIFLVALFLSALLIPLASSSNDGFVRIGLKKMKFDQNNRLAARLESKERDLMRPSIRKYNFR :  65

                                         100
Salpichroin : ----------------------------KFTVVFDTGSSNLWVPSSKC------------ :  20
SlAP1-like  : GKLGDSEDTDIVALKNYMDAQYFGEIGVGTPPQKFTVIFDTGSSNLWVPSSKCYFSVPCFFHAKY : 130

                                    150
Salpichroin : ----------------------FSEDNVKVGDLVVTDQEFIEATR--------------- :  43
SlAP1-like  : KSSLSSTYKKNGESAAIQYGSGAISGFFSEDNVKVGDLVVTDQEFIEATREPSVTFLVAKFDGIL : 195

                      200                                250
Salpichroin : ----------------------KEPVFSFWLNRNTEEEQGGEIVFGGVDPNHFK-------- :  75
SlAP1-like  : GLGFQEISVGNAVPVWYNMVKQGLIKEPVFSFWLNRNTEEEQGGEIVFGGVDPNHFKGEITYVPV : 260

                                  300
Salpichroin : -KKGYWQFDMGDVLIDGKA--------------------------------------- :  93
SlAP1-like  : TRKGYWQFDMGDVLIDGKATGYCKSGCSAIADSGTSLLAGPTTVITMINQAIGASGVASQQCKAV : 325

                                350
Salpichroin : ------------------------------------------------------------- :   -
SlAP1-like  : IQQYGQTIMDLLLAEAHPKKVCSQVGVCTFDGTHGVSMGIESVVNEKAGRSAGLHDGMCSACEMA : 390
                                      PSI domain

                 400                                 450
Salpichroin : ------------------------------SAVDCGKLSSMPTQ-F--------------- : 108
SlAP1-like  : VVWMENQLRQNQTQDRILDYLNELCERLPSPMGESAVDCGKLSSMPTVSFTIGGKVFDLSPNEYI : 455

                                 500
Salpichroin : ------------------------------------------------------ :   -
SlAP1-like  : LKVGEGDQAQCISGFIGLDVPPPRGPLWILGDIFMGRYHTVFDYGKLRVGFAEAA : 510
```
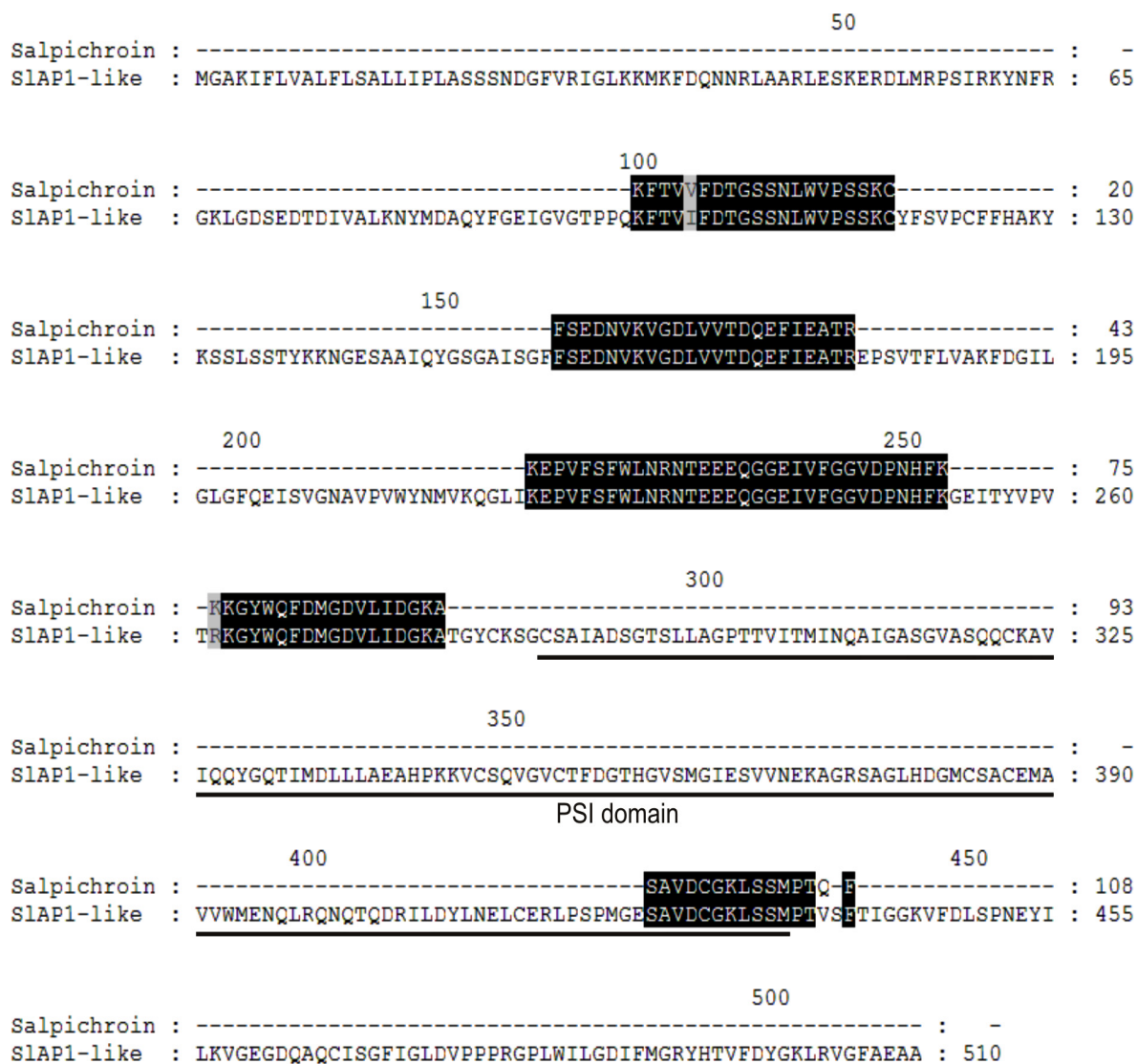
Figure 7. Sequence alignment of salpichroin and *Sl*AP1-like. Sequences were automatically aligned using the CLUSTAL W algorithm. Identical residues are highlighted in black, similar residues are highlighted in gray and gaps are indicated by dashes.
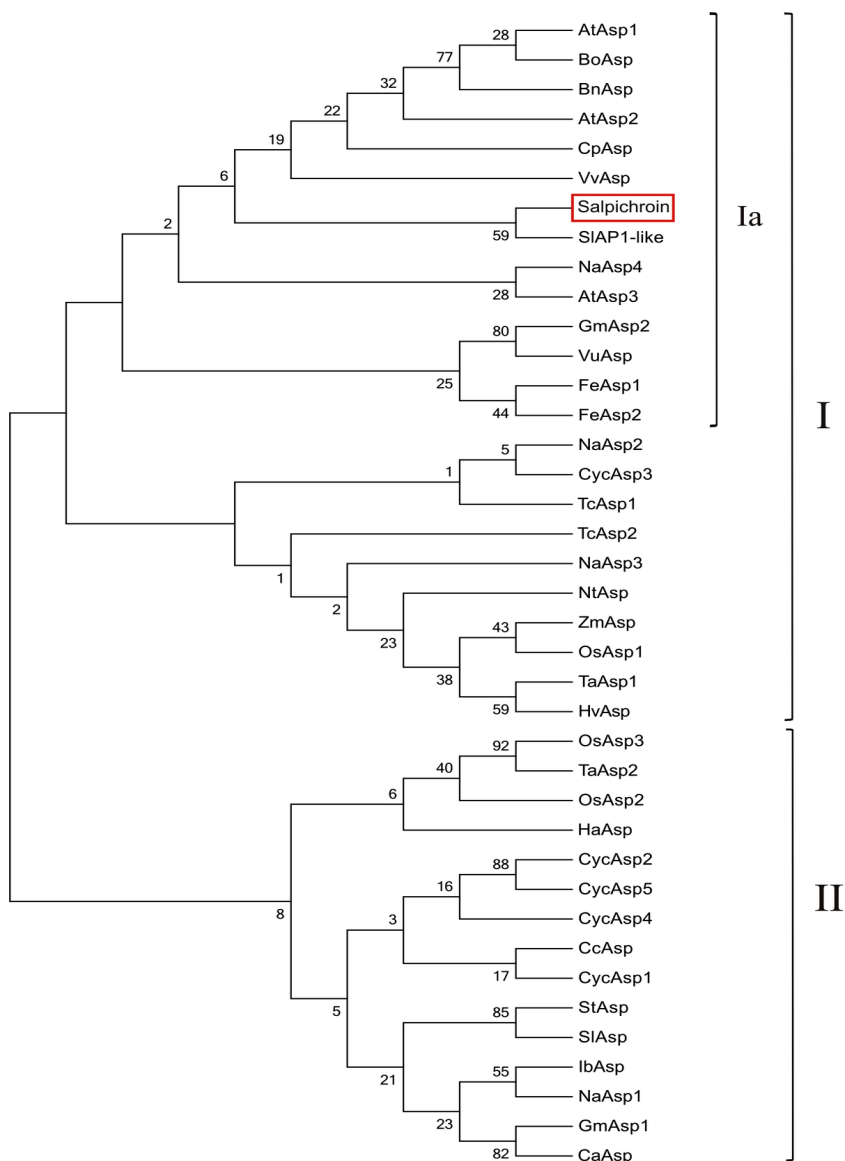
Figure 8. Unrooted Neighbor-Joining tree of plant aspartic protease sequences containing PSI domains. Sequences retrieved from databases were aligned using the CLUSTAL W algorithm [72] and protein sequence similarity searches were performed by using GeneDoc alignment editor version 2.6.002 [73]. Phylogenetic analysis was conducted using MEGA version 5 [44]. The phylogenetic tree was inferred using the Neighbor-Joining method [74] and the robustness of each node was assessed by bootstrap resampling (1000 replicates) [75]. The AP amino acid sequences were either obtained from previously reported sequences or translated from the reported cDNA sequences at the NCBI: *St*Asp (*S. tuberosum*, AY672651); *Sl*Asp (*S. lycopersicum*, AAB18280); *Ib*Asp (*I. batatas*, DQ903691); *Na*Asp1 to *Na*Asp4 (*N. alata*, AB045894, AB045891, AB045892, AB045893); *Gm*Asp1 and *Gm*Asp2 (*G. max*, AB070857, AB069959); *Zm*Asp (*Z. mays*, EU960771); *Ca*Asp (*C. arietinum*, AB024999); *Os*Asp1 to *Os*Asp3 (*O. sativa*, D32144, NP_001042785, AAS98423); *Ta*Asp1 and *Ta*Asp2 (*T. aestivum*, AB219968, AB219969); *Hv*Asp (*H. vulgare*, X56136); *Fe*Asp1 and *Fe*Asp2 (*F. esculentum*, AY826351, AAV84086); *Tc*Asp1 and *Tc*Asp2 (*T. cacao*, AJ313384, AJ313385); *Vv*Asp (*V. vinifera*, EF123256); *At*Asp1 to *At*Asp3 (*A. thaliana*, U51036, AAL49856, NP_192355); *Cc*Asp (*C. calcitrapa*, Y09123); *Bo*Asp (*B. oleracea*, X77260); *Bn*Asp (*B. napus*, AAB03108); *Ha*Asp (*H. annuus*, AB025359); *Nt*Asp (*N. tabacum*, DQ648018); *Cp*Asp (*C. pepo*, AB002695); *Vu*Asp (*V. unguiculata*, U61396); and *Cys*Asp1 to *Cys*Asp5 (*C. cardunculus*, CAA57510, CAL07969, CAA48939, AJ132884, AJ237674).

(*At*Asp1, *At*Asp2, *At*Asp3)*, Brassica oleracea* (*Bo*Asp) and *Brassica napus* (*Bn*Asp), for which no specific function has yet been attributed, the protease from cucumber (*Cp*Asp), which is related to protein maturation [67], *Fe*Asp1 and *Fe*Asp2 from buckwheat, which are involved in seed germination and protein maturation [68], *Na*Asp4, which has been identified in the flower pit of the carnivorous plant *Nepen-*

thes alata [69], soy *Gm*Asp2, which has been suggested to be involved in programmed cell death [70], cowpea *Vu*Asp, which is up-regulated in senescing leaves [7], and *Vitis vinifera Vv*Asp, which is related to abiotic stress responses [71]. Overall, the phylogenetic analysis did not allow us to assess the biological function of the protein that corresponds with the fragment identified from salpichroin.

CONCLUSIONS

In this study, we carried out a biochemical and proteomic characterization of the aspartic protease isolated from ripe fruits of *Salpichroa origanifolia*. Salpichroin hydrolyzes natural substrates such as hemoglobin and casein with high specificity, is active in acid pH range, but shows a low thermal stability at moderate temperatures. However, immobilization techniques are now in process to improve an increase in the stability of salpichroin.

SDS-PAGE, MALDI-TOF/TOF-MS, inhibition assays, kinetic parameters, and substrate specificity demonstrate that salpichroin is an enzyme belonging to the A1 family of APs. This enzyme showed high similarity to *SlAP1-like*, although the phylogenetic analysis did not allow us to assess its biological function. Additional studies are needed to evaluate the physiological role of the enzyme in the plant and its potential application in biotechnological processes.

ABBREVIATIONS

AP	=	Aspartic protease
BSA	=	Bovine serum albumin
DTT	=	Dithiothreitol
E64	=	*Trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane
MALDI–TOF/MS	=	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
PMF	=	Peptide mass fingerprinting
PMSF	=	phenylmethylsulfonyl fluoride
PNPE	=	H-Pro-Thr-Glu-Phe-p-(NO$_2$)-Phe-Arg-Leu:OH
PSI	=	Plant-specific insert
RP-HPLC	=	Reversed phase high-pressure liquid chromatography
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	=	Trichloroacetic acid
TFA	=	Trifluoroacetic acid

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

REFERENCES

[1]   Dunn, B.M. Structure and mechanism of the pepsin-like family of aspartic peptidases. *Chem. Rev.*, **2002**, *102*(12), 4431-4458.

[2[Devaraj, K.B.; Gowda, L.R.; Prakash, V. An unusual thermostable aspartic protease from the latex of *Ficus racemosa*. *Phytochemistry*, **2008**, *69*(3), 647-655.

[3]   Rawlings, N.D.; Barrett, A.J.; Bateman, A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.*, **2012**, *40*(D1) D343-D350.

[4]   Lufrano, D.; Faro, R.; Castanheira, P.; Parisi, G.; Verissimo, P.; Vairo-Cavalli, S.; Simöes, I.; Faro, C. Molecular cloning and characterization of procirsin, an active aspartic protease precursor from *Cirsium vulgare* (Asteraceae). *Phytochemistry*, **2012**, *81*, 7-18.

[5]   Rawlings, N.D.; Morton, F.R.; Kok, C.Y.; Kong, J.; Barrett, A.J. MEROPS: the peptidase database. *Nucleic Acids Res.,* **2008**, *36*, D320-D325.

[6]   Park, H.; Yamanaka, N.; Mikkonen, A.; Kusakabe, I.; Kobayashi, H. Purification and characterization of aspartic proteinase from sunflower seeds. *Biosci. Biotechnol. Biochem.*, **2000**, *64*, 931-939.

[7]   Cruz de Carvalho, M.H.; d'Arcy-Lameta, A.; Roy-Macauley, H. ;Gareil, M.; El Maarouf, H.; Pham-Thi, A.T.; Zuily-Fodil, Y. Aspartic protease in leaves of common bean (*Phaseolus vulgaris* L.) and cowpea *(Vigna unguiculata* L. Walp): enzymatic activity, gene expression and relation to drought susceptibility. *FEBS Lett.*, **2001**, *492* (3), 242-246.

[8]   Guevara, M.G.; Oliva, C.R.; Machinandiarena, M.; Daleo, G.R. Purification and properties of an aspartic protease from potato tuber that is inhibited by a basic chitinase. *Physiol. Plant.*, **1999**, *106*, 164–169.

[9]   Cordeiro, M.C.; Pais, M.S.; Brodelius, P.E. Tissue-specific expression of multiple forms of cyprosin (aspartic proteinase) in flowers of *Cynara cardunculus. Physiol. Plant.*, **1994**, *92*, 645–653.

[10]   Wang, L.; Wang, M.; Li, Q.; Cai, T.; Jiang, W. Partial properties of an aspartic protease in bitter gourd (*Momordica charantia* L.) fruit and its activation by heating. *Food Chem.*, **2008**, *108*, 496-502.

[11]   Brodelius, M.; Hiraiwa, M.; Marttila, S.; Karadaghi, S.A.; Picaud, S.; Brodelius, P.E. Immunolocalization of the saposin-like insert of plant aspartic proteinases exhibiting saposin C activity. Expression in young flower tissues and in barley seeds. *Physiol. Plant.*, **2005**, *125*, 405-418.

[12]   Bi, X.; Khush, G.S.; Bennett, J. The rice nucellin gene ortholog OsAsp1 encodes and active aspartic peptidase without a plant-specific insert and is strongly expressed in RT early embryo. *Plant Cell Physiol.*, **2005**, *46*, 87-98.

[13]   Radlowski, M.; Kalinowski, A.; Adamczyk, J. Proteolytic activity in the maize pollen wall. *Physiol. Plant.*, **1996**, *98*, 172-178.

[14]   Tamura, T.; Terauchi., K.; Kiyosaki, T.; Asakura, T.; Funaki, J.; Matsumoto, L.; Misaka, T.; Abe, K. Differential expression of wheat aspartic proteinases, WAP1 and WAP2, in germinating and maturing seeds. *J. Plant Physiol.*, **2007**, *164*, 470-477.

[15]   Schaller, A.; Ryan, C.A. Molecular cloning of a tomato leaf cDNA encoding an aspartic peptidase, a systemic wound response protein. *Plant Mol. Biol.*, **1996**, *31*, 1073-1077.

[16]   Guilloteau, M.; Laloi, M.; Michaux, S.; Bucheli, P.; McCarthy, J. Identification and characterisation of the major aspartic proteinase activity in *Theobroma cacao* seeds. *J. Sci. Food Agric.*, **2005**, *85*, 549-562.

[17]   Simöes, I.; Faro, R; Bur, D.; Faro, C. Characterization of recombinant CDR1, an *Arabidopsis* aspartic proteinase involved in disease resistance. *J. Biol. Chem.*, **2007**, *282*, 31358-31365.

[18]   Vieira, M.; Pissarr, J.; Veríssimo, P.; Castanheira, P.; Costa, Y.; Pires, E.; Faro, C. Molecular cloning and characterization of cDNA encoding cardosin B, an aspartic proteinase accumulating extracel-

lularly in the transmitting tissue of *Cynara cardunculus* L. *Plant Mol. Biol.*, **2001**, *45*, 529-39.

[19] Guevara, M.G.; Almeida, C.; Mendieta, J.R.; Faro, C.J.; Veríssimo, P.; Vieyras Pires, E.; Daleo, G.R. Molecular cloning of a potato leaf cDNA encoding an aspartic protease (StAsp) and its expression after *P. infestans* infection. *Plant Physiol. Biochem.*, **2005**, *43*, 882-889.

[20] Huang, G.J.; Huang, S.S.; Chen, H.J.; Chang, Y.; Chang, S.J.; Chang, H.Y.; Hsieh, P.C.; Chang, M.J.; Lin, Y.C.; Lin, Y.H. Cloning and expression of aspartic proteinase cDNA from sweet potato storage roots. *Bot. Stud.*, **2009**, *50*, 149-158.

[21] Simões, I.; Faro, C. Structure and function of plant aspartic proteinases. *Eur. J. Biochem.*, **2004**, *271*, 2067-2075.

[22] Mutlu, A.; Gal, S. Plant aspartic proteinases: enzymes on the way to a function. *Physiol. Plant.*, **1999**, *105*, 569-576.

[23] Masson, O.; Bach, A.S.; Derocq, D; Prébois, C.; Laurent-Matha, V.; Pattingre, S.; Liaudet-Coopman, E. Pathophysiological functions of cathepsin D: Targeting its catalytic activity versus its protein binding activity? *Biochimie*, **2010**, *92* (11), 1635-1643.

[24] Mendieta, J.R.; Pagano, M.; Muñoz, R.R.; Daleo, G.R.; Guevara, M.G. Antimicrobial activity of potato aspartic peptidases (StAPs) involves membrane permeabilization. *Microbiology*, **2006**, *152*, 2039-2047.

[25] Rocha, G.; Fernández, G.; Parisi, M. Estudios de caracterización cinética y fisicoquímica de una proteinasa aspártica aislada de frutos maduros de *Salpichroa origanifolia. Información Tecnológica*, **2010**, *21*(2), 21-28.

[26] Rocha, G.; Kise, F.; Rosso, A.; Parisi, M. Péptidos con actividad antimicrobiana obtenidos por hidrólisis enzimática de proteínas lácteas con extractos de frutos de *Salpichroa origanifoli*a. *Información Tecnológica*, **2013**, *24*(2), 23-30.

[27] Mongelli, E.; Pampuro, S.; Coussio, J.; Salomón, H. Ciccia, G. Cytotoxic and DNA interaction activities of extracts from medicinal plants used in Argentina. *J. Ethnopharmacol.*, **2000**, *71*, 145-151.

[28] Boeris, M.A.; Toso, R.E.; Skliar, M. Actividad antiinflamatoria de *Salpichroa origanifolia. Acta Farm. Bonaerense*, **2004**, *23*(2), 138-141.

[29] Mareggiani, G.; Picollo, M.I.; Zerba, E.; Burton., G.; Tettamanzi, M.C.; Benedetti-Doctorovich, M.O.V.; Veleiro, A.S. Antifeedant activity of withanolides from *Salpichroa origanifolia* on *Musca domestica. J. Nat. Prod.*, **2000**, *63*, 1113-1116.

[30] Bado, S.; Mareggiani, G.; Amiano, N.; Burton, G.; Veleiro, A.S. Lethal and sublethal effects of withanolides from Salpichroa origanifolia and analogues on *Ceratitis capitata, J. Agric. Food Chem.*, **2004**, *52*, 2875-2878.

[31] Anson, L.The estimation of pepsin, trypsin, papain and cathepsin with haemoglobin. *J. Gen. Physiol.*, **1938**, *22*, 79-89.

[32] Parisi, M.G.; Moreno, S.; Fernández, G. Isolation and characterization of a dual function protein from *Allium sativum* bulbs which exhibits proteolytic and hemagglutinating activities. *Plant Physiol. Biochem.*, **2008**, *46*, 403–413.

[33] Dunn, B.M.; Jiménez, M.; Parten, B.F.; Valler, M.J.; Rolph, C.E.; Kay, J. A systematic series of synthetic chromophoric substrates for aspartic proteinases. *Biochem. J.,* **1986**, *237*, 899-906.

[34] Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of Protein-Dye Binding. *Anal. Biochem.*, **1976**, *72*, 248-254.

[35] Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **1970**, *227*, 680-685.

[36] Blum, H.; Beier, H.; Gross, H.J. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **1987**, *8*, 93-99.

[37] Kleiner, D.E.; Stetler-Stevenson, W.G. Quantitative zymography: detection of picogram quantities of gelatinases. *Anal. Biochem.*, **1994**, *218*, 325-329.

[38] Barrett, A.J.; Rawlings, N.D.; Woessner, J.F. Handbook of Proteolytic Enzymes. Academic Press, New York, **1998**.

[39] Salvesen, G.; Nagase, H. In: *Inhibition of proteolytic enzymes, Proteolytic Enzymes: A Practical Approach*; R.M. Beynon, J.S. Bond, eds.; IRL Press: Oxford, UK, **1989;** pp 83.

[40] Chazarra, S.; Sidrach, L.; López-Molina, D.; Rodríguez-López, J.N. Characterization of the milk-clotting properties of extracts from artichoke (*Cynara scolymus*, L.) flowers. *Int. Dairy J.*, **2007**, *17*(12), 1393-1400.

[41] Barrett, A.J.; Kirschke, H. Cathepsin B, cathepsin H and cathepsin L. *Method Enzymol.*, **1981**, *80*, 535-561.

[42] Helsens, K.; Martens, L.; Vandekerckhove, J.; Gevaert, K. MascotDatfile: An open-source library to fully parse and analyse MASCOT MS/MS search results. *Proteomics*, **2007**, *7*, 364–366.

[43] Mancone, C.; Amicone, L.; Fimia, G.M.; Bravo, E.; Piacentini, M.; Tripodi, M.; Alonzi, T. Proteomic analysis of human very low-density lipoprotein by two-dimensional gel electrophoresis and MALDI-TOF/TOF. *Proteomics,* **2007**, *7*, 143–154.

[44] Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.*, **2011**, *28*, 2731-2739.

[45] Kervinen, J. In: *Phytepsin. Handbook of Proteolytic Enzymes*; Neil D. Rawlings and Guy S. Salvesen, eds.; Academic Press: Oxford, **2013**; Vol. *1*, pp. 118-124.

[46] Guevara, M.G.; Daleo, G.R.; Oliva, C.R. Purification and characterisation of an aspartic protease from potato leaves. *Physiol. Plant.*, **2001**, *112*, 321–326.

[47] Sidrach, L;. Garcia-Cánovas, F.; Tudela, J.; Rodriguez-López, J.N. Purification of cynarases from artichoke (*Cynara scolymus* L.): enzymatic properties of cynarase A. *Phytochemistry*, **2005**, *66*, 41-49.

[48] Raposo, S.; Domingos, A. Purification and characterization of milk-clotting aspartic proteinases from *Centaurea calcitrapa* cell suspension cultures. *Process Biochem.*, **2008**, *43*, 139-144.

[49] Vairo-Cavalli, S.; Lufrano, D.; Colombo, M.L.; Priolo, N. Properties and application of phytepsins from thistle flowers. *Phytochemistry*, **2013**, *92*, 16-32.

[50] Fujinaga, M.; Chernaia, M.M.; Tarasova, N.I.; Mosimann, S.C.; James, M.N.G. Crystal-structure of human pepsin and its complex with pepstatin. *Protein Sci.*, **1995**, *4*, 960–972.

[51] Rich, D.H.; Sun, E.T.O. Mechanism of Inhibition of Pepsin by Pepstatin. Effect of Inhibitor Structure on Dissociation Constant and Time-Dependent Inhibition. *Biochem. Pharmacol.*, **1980**, *29*, 2205-2212.

[52] Carles, C.; Ribadeau-Dumas, B. Kinetics of the action of chymosin (rennin) on a peptide bond of bovine α_{s1}-casein, comparison of the behavior of this substrate with β-casein and κ-casein. *FEBS Lett.*, **1985**, *185*, 282-286.

[53] Ben-Khaled, H.B.; Ghorbel-Bellaaj, O.; Hmidet, N.; Jellouli, K.; El-Hadj Ali, N.; Ghorbel, S.; Nasri, M. A novel aspartic protease from the viscera of Sardinelle (*Sardinella aurita*) Purification and characterization. *Food Chem.*, **2011**, *128*, 847-853.

[54] Kumar, A. Khan, F. Rao, M. Biochemical Characterization of a Novel Aspartic Peptidase from *Plutella xylostella*, Diamondback moth (Lepidoptera: *Plutellidae*) Midgut: Inactivation by an Aspartic Peptidase Inhibitor. *Int. J. Innov. Biol .Res*., **2012**, *1*(1), 9-19.

[55] Verissimo, P.; Esteves, C.; Faro, C.; Pires, E. The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities. *Biotechnol. Lett.,* **1995**, *17*, 621–626.

[56] Sarmento, A.C.; Lopes, H.; Oliveira, C.; Vitorino, R.; Samyn, B.; Sergeant, K.; Debyser, G.; Beeumen, J.; Domingues, P.; Amado, F.; Pires, E.; Domingues, M.R.M.; Barros, M.T. Multiplicity of aspartic proteinases from *Cynara cardunculus* L. *Planta*, **2009**, *230*, 429-439.

[57] Kervinen, J.; Sarkkinen, P.; Kalkkinen, N.; Mikola, L.; Saarma, M. Hydrolytic specificity of the barley grain aspartic proteinase. *Phytochemistry*, **1993**, *32*, 799–803.

[58] Guevara; M.G.; Veríssimo, P.; Pires, E.; Faro, C.; Daleo, G.R. Potato aspartic proteases: induction, antimicrobial activity and substrate specificity. *J. Plant Pathol.*, **2004**, *86*, 233–238.

[59] Foltmann, B. On the amino acid composition of prorennin, rennin and of peptides liberated during the activation of prorennin. *C R Travaux Lab Carlsberg*, **1964**, *34*, 275-286.

[60] Sanger, F. Tuppy, H. The amino-acid sequence in the phenylalanyl chain of insulin: II The investigation of peptides from enzymatic hydrolysate. *Biochem. J.*, **1981**, *49*, 481-490.

[61] Kervinen, J.; Törmäkangas, K.; Runeberg-Roos, P.; Guruprasad, K.; Blundell, T.; Teeri, T.H. Structure and possible function of aspartic proteinases in barley and other plants. *Adv. Exp. Med. Biol.*, **1995**, *362*, 241–254.

[62] Mazorra-Manzano, M. Yada, R. Expression and characterization of the recombinant aspartic proteinase A1 from *Arabidopsis thaliana. Phytochemistry*, **2008**, *69*, 2439-2448.

[63] Muñoz, F.F.; Mendieta, J.R.; Pagano, M.R.; Paggi, R.A.; Daleo, G.R.; Guevara, M.G. The swaposin-like domain of potato aspartic

protease (StAsp-PSI) exerts antimicrobial activity on plant and human pathogens. *Peptides*, **2010**, *31*, 777-785.

[64]   Bryksa, B.C.; Bhaumik, P.; Magracheva, E.; De Mour, D.C.; Kurylowicz, M.; Zdanov, A.; Dutcher, J.R;. Wlodawer, A.; Yada, R.Y. Structure and Mechanism of the Saposin-like Domain of a Plant Aspartic Protease. *J. Biol. Chem*., **2011**, *286*, 28265-28275.

[65]   Castanheira, P.; Samyn, B.; Sergeant, K.; Clemente, J.C.; Dunn, B.M.; Pires, E.; Van Beeumen, J.; Faro, C. Activation, proteolytic processing, and peptide specificity of recombinant cardosin A. *J. Biol. Chem*., **2005**, *280*, 13047-13054.

[66]   Payie, K.; Tanaka, T.; Gal, S.; Yada, R. Construction, expression and characterization of a chimaeric mammalian-plat aspartic proteinase. *Biochem. J.,* **2003**, *372*, 671-678.

[67]   Yamauchi., Y.; Sugimoto, T.; Sueyoshi, K.; Oji,. Y. Emergence of proteases in germinating cucumber cotyledons and their roles in the two-step degradation of storage protein. *Plant Cell Physiol*., **1996**, *37*, 279–284.

[68]   Timotijevic, G.S.; Milisavljevic, M.D.; Radovic, S.R.; Konstantinovic, M.M.; Maksimovic, V.R. Seed-specific aspartic proteinase feap12 from buckwheat (*Fagopyrum esculentum* Moench). *Arch. Biol. Sci.*, **2010**, *62*(1), 143-151.

[69]   An, C.; Takekawa, S.; Okazawa, A.; Fukusaki, E.; Kobayashi, A. Degradation of a peptide in pitcher fluid of the carnivorous plant *Nepenthes alata* Blanco. *Planta*, **2002**, *215*, 472-477.

[70]   Terauchi, K.; Asakura, T.; Nishizawa, N.K.; Matsumoto, I.; Abe, K. Characterization of the genes for two soybean aspartic proteinases and analysis of their different tissue-dependent expression. *Planta*, **2004**, *218*, 947-957.

[71]   Guo, R.; Xu, X.; Carole, B.; Li, X.; Gao, M.; Zheng, Y.; Wang, X. Genome-wide identification, evolutionary and expression analysis of the aspartic protease gene superfamily in grape. *BMC Genomics*, **2013**, *14*, 554.

[72]   Thompson, J.D.; Higgins, D.G.; Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position- specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **1994**, *22*(22), 4673–4680.

[73]   Nicholas, K.B. Nicholas Jr, H.B. Deerfield, D.W. GeneDoc: analysis and visualization of genetic variation. *EMBNEW.NEWS*, **1997**, *4*, 14.

[74]   Saitou, N.; Nei, M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **1987**, *4*, 406-425.

[75]   Felsenstein, J. Confidence Limits on Phylogenies: An Approach using the Bootstrap. *Evolution*, **1985**, *39*(4), 783-791.