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Purification and Characterization of a Milk-Clotting Aspartic Proteinase from Globe Artichoke (*Cynara scolymus* L.)

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The study of proteinase expression in crude extracts from different organs of the globe artichoke (*Cynara scolymus* L.) disclosed that enzymes with proteolytic and milk-clotting activity are mainly located in mature flowers. Maximum proteolytic activity was recorded at pH 5.0, and inhibition studies showed that only pepstatin, specific for aspartic proteinases, presented a significant inhibitory effect. Such properties, in addition to easy enzyme inactivation by moderate heating, make this crude protease extract potentially useful for cheese production. Adsorption with activated carbon, together with anion exchange and affinity chromatography, led to the isolation of a heterodimeric milk-clotting proteinase consisting of 30- and 15-kDa subunits. MALDI-TOF MS of the 15-kDa chain determined a 15.358-Da mass, and the terminal amino sequence presented 96% homology with the smaller cardosin A subunit. The amino terminal sequence of the 30-kDa chain proved to be identical to the larger cardosin A subunit. Electrophoresis evidenced proteinase self-processing that was confirmed by immunoblots presenting 62-, 30-, and 15-kDa bands.

KEYWORDS: Globe artichoke; *Cynara scolymus*; milk-clotting activity; plant aspartic proteinase; plant rennet

INTRODUCTION

Cynara scolymus L. (globe artichoke) is an edible plant widely distributed in the Mediterranean regions, besides being plentifully cultivated in the United States, Argentina, and New Zealand. The immature flower (head) constitutes the edible part of the globe artichoke, and a variety of organs have been extensively used in folk medicine against dyspeptic and hepatic disorders. It belongs to the Asteraceae family (Compositae), which also includes the cardoon (*Cynara cardunculus* L.). This is similar to the globe artichoke except that it is more robust, has large spiny leaves, and fails to produce an edible head.

Proteinases play an important role in biotechnology because proteolysis modifies the chemical, physical, biological, and immunological properties of proteins. Some plant proteinases are used in the food industry, in manufacturing cheeses and drinks, meat tenderizing, cookie baking, and the production of protein hydrolysates (*1*). All enzymes employed commercially in milk coagulation are aspartic proteinases (APs; EC 3.4.23), characterized by being more active at acidic pH, and specifically inhibited by pepstatin, presenting high levels of homology in their amino acid sequences and preserving three-dimensional structure (*2*, *3*). A number of APs have been detected and purified from the seeds, leaves, and flowers of several plants, and their features are revised in references *3* and *4*. In some countries such as Portugal and Spain, APs of Asteraceae flowers are used in the production of homemade cheeses with organoleptic features different from those obtained with bovine chymosin or microbial rennins (*5*). Mainly aqueous extracts of cardoon flowers are used, the APs of which have been thoroughly studied from the biochemical, kinetic, molecular, and structural points of view (*6*−*15*) as on casein proteolysis or during cheese ripening (*5*, *16*−*21*). Two cardoon groups of APs have been described: proteinases isolated under alkaline conditions are termed "cynarases" or "cyprosins" (*7*, *8*, *11*, *15*) and those isolated at acidic pH from fresh stigmas of *C. cardunculus* grown from selected seeds, named "cardosins" (*9*, *10*, *12*−*14*). In some regions of Spain, *Cynara humilis* flowers have also been used to make artisanal cheese and their APs assayed in their action on ovine and caprine caseinates (*20*, *21*). Besides, milk-clotting APs have been isolated from other members of the Asteraceae family such as *Centaurea calcitrapa*, *Sylibum marianum*, and *Onopordum turcicum* (*22*−*24*).

In this paper the milk-clotting enzymes present in plants of globe artichoke (*C. scolymus* L.) are studied. A proteinase was isolated from mature flowers and purified by anion exchange and affinity chromatography. The amino terminus of the two chains obtained was sequenced and compared with other proteinases from flowers.

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MATERIALS AND METHODS

Plant Material and Reagents. *C. scolymus* L. cv. Green Globe heads were obtained from plants grown from seeds in Nogoyá, province of Entre Ríos, Argentina. BSA, cysteine, E-64, EDTA, glycine, pepstatin, pepstatin−agarose, PMSF, prestain protein markers MW-SDS-70L (broad range) and MW-GF-70, sinapinic acid, and Tris were purchased from Sigma Chemical Co. (St. Louis, MO). Ampholytes (Pharmalyte pH 3−10), broad p*I* markers (3.5−9.3), DEAE-Sepharose Fast Flow, and Sephadex G-75 were purchased from Pharmacia Biotech (Uppsala, Sweden). Casein (Hammarsten type) was obtained from Research Organics Inc. (Cleveland, OH). Bromophenol blue dye marker was purchased from Mallinckrodt Chemical Works (Paris, KY). Acrylamide, bisacrylamide, Coomassie brilliant blue R-250, and low-range molecular weight standards were obtained from Bio-Rad (Hercules, CA). PVDF membrane Immobilon-NC Hahy, 0.45 μm, was provided by Millipore (Bedford, MA). Skim milk powder San Regime was from SanCor (Argentina). Horseradish peroxidase-conjugated goat antibody anti-rabbit IgG (H+L) was purchased from Zymed Laboratories Inc. (San Francisco, CA). All other chemicals were obtained from commercial sources and were of the highest purity available.

Preparation of Crude Extracts. Different organs of globe artichoke were homogenized for 3 min in an electric mixer (Sorval) with 1:3 (w/v) 0.1 M potassium phosphate buffer, pH 6, cold. Homogenates were filtered and centrifuged at 16000*g* for 20 min at 4 °C. The supernatant was filtered through Whatman no. 1 paper and kept at −20 °C until analysis.

Milk-Clotting Activity. Standardized cow's skim milk powder in 10% solution (w/v) in 10 mM $CaCl_2$, pH 6 (3 mL), was added to 500 μL of crude extracts and incubated at 37 ± 0.2 °C in a test tube according to the method of Arima (*25*). The time elapsing between the mixing of reagents and the initial appearance of solid material was measured. One milk-clotting unit (MCU) was defined as the amount of enzyme required to clot 1 mL of skim milk at 37 °C in 40 min.

Proteolytic Activity Assay. Caseinolytic activities were determined by mixing 0.1 mL of crude or purified extracts and 1.1 mL of 1% casein (w/v) in 0.1 M potassium phosphate buffer, pH 6. The reaction was carried out at 37 °C and stopped 30 min later by the addition of 1.8 mL of 5% trichloroacetic acid (w/v). Each test tube was centrifuged at 4000*g* for 20 min and supernatant absorbance measured at 280 nm in 10-mm quartz cuvettes. Hemoglobinolytic activity was determined according to the method described in ref *26* after the pH had been adjusted to 4 with 0.2 M citrate−citric buffer, pH 3. The azocasein substrate was prepared in 0.1 M Tris-HCl, pH 6, and the proteolytic activity measured as described in ref *26*. Arbitrary enzyme units of casein (caseinolytic unit, Ucas) and hemoglobin (hemoglobinolytic unit, Uhem) were defined as the amount of enzyme that produces an increase of 1 absorbance unit per minute at 280 nm, whereas the azocaseinolytic unit (Uazoc) was determined at 440 nm.

Protein Quantification. Protein concentration was determined according to the Coomassie blue dye-binding method (*27*), using BSA as standard. In chromatographic fractions, the protein content of eluates was detected by measuring the absorbance at 280 nm.

Activator and Inhibitor Effects. The effects of cysteine (5 mM), E-64 (10 μM), pepstatin (1 μM), and PMSF (1 mM) on proteolytic activity were evaluated by preincubating crude extracts for 30 min at 37 °C and measuring residual caseinolytic activity at pH 6. The inhibition capacity of 1,10-phenanthroline (10 mM) was determined by using azocasein as substrate, because this metalloproteinase inhibitor shows high absorbance at 280 nm. Controls were prepared by preincubating the enzymatic preparation with the solvent used to dissolve activators and inhibitors.

Thermal Stability. To determine the effect of heating, flower samples were incubated for 0, 5, 10, 20, 40, 60, 90, 120, and 180 min at 37, 45, 55, and 65 °C in 0.1 M potassium phosphate buffer, pH 6. Residual caseinolytic activity after each incubation assay was measured as described.

pH Profile of Proteolytic Activity. Proteolytic activity versus pH was measured on bovine hemoglobin (pH range of 2.7−10) and on azocasein in the pH range of 4−8. The pH was controlled by adding 0.1 M sodium citrate−citric acid (pH 2.7−5.5), 0.1 M potassium phosphate (pH 6−8), and 0.1 M boric acid−potassium chloride−sodium hydroxide (pH 8−10) in various proportions.

Purification of Extracts from Globe Artichoke Flowers. Crude extracts from flowers were treated for 10 min with 2.5, 5, and 10% (w/v) of activated charcoal; mixtures were kept on ice for 30 min and centrifuged at 16000*g* for 60 min at 4 °C. Supernatant purification was carried out by anion exchange chromatography (DEAE-Sepharose Fast Flow). The column was equilibrated and washed with 60 mL of 50 mM potassium phosphate buffer, pH 6, and bound material eluted (12 mL h^{-1}) with a linear gradient of sodium chloride (0.1−0.35 M) in the same buffer. Eluted fractions (2 mL each) were collected and assayed for hemoglobinolytic activity and absorbance at 280 nm. Fractions containing proteinase activity were pooled and loaded onto a column (10 × 1.2 cm) packed with 5 mL of pepstatin−agarose. The column was washed with 0.1 M sodium acetate buffer, pH 5, and 0.1 M NaCl until absorbance values (280 nm) approaching zero were obtained. Elution of AP was performed with 0.1 M Tris-HCl buffer, pH 8, and 1 M NaCl at 30 mL h^{-1}.

Gel Filtration. The molecular mass (M_r) of the active fraction obtained by anion exchange was estimated on gel filtration chromatography (Sephadex G-75). The column was equilibrated and washed with 0.1 M potassium phosphate buffer, pH 6, and bound material eluted with the same buffer at 30 mL h^{-1}. Activity was followed by reading the absorbance at 280 nm.

Electrophoresis. SDS-PAGE of enzyme samples was performed in a Miniprotean II cell (Bio-Rad) on 14% gels (stacking 5%) according to the procedure of Laemmli (*28*). Samples were treated in denaturing buffer with SDS and β-mercaptoethanol and boiled for 5 min before SDS-PAGE. Electrophoresis was run at 150 V until the bromophenol blue dye marker disappeared from the separating gel. Gels were stained with Coomassie brilliant blue R-250 and destained by repeated washing in methanol/acetic acid/water (2.5:1:10). Molecular mass markers used were Sigma MW-SDS-70L: bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (36 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), trypsinogen from bovine pancreas (24 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa).

Isoelectrofocusing (IEF) and Zymograms. IEF was developed on immobilized pH gradient gels of 10% polyacrylamide containing broad pH range ampholytes (3.5−9.3) in a Mini IEF Cell (model 111, Bio-Rad). Samples were concentrated by cold (−20 °C) acetone precipitation, centrifuged at 16000*g*, and the protein sediments redissolved with 100 mL of deionized water. About 3−10 μg of protein was loaded in each case. Focusing was carried out under constant voltage conditions in a stepped procedure: 100, 200, and 450 V for 15, 15, and 60 min, respectively. Gels were fixed, stained by Coomassie brilliant blue R-250. Pharmacia broad p*I* markers (3.5−9.3) were trypsinogen (p*I* 9.30), lectins from *Lens culinaris* (p*I* 8.65, 8.45, and 8.15), myoglobin basic band (p*I* 7.35), myoglobin, acidic band (p*I* 6.85), human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), β-lactoglobulin A (p*I* 5.20), trypsin inhibitor (p*I* 4.55), methyl red dye (p*I* 3.75), and amylglucosidase (p*I* 3.50).

To confirm the proteolytic activity of bands separated by their p*I*, zymograms were made according to the method of Westergaard (*29*). An agarose gel (1%) was imbibed in 2% hemoglobin solution in 50 mM citric−citrate buffer, pH 4, and then washed twice with distilled water. Unstained IEF gels were placed in contact for 40 min at 50 °C with the agarose gel and then stained with Coomassie Blue R-250. Proteolytic activities became visible as clear bands on the stained agarose gels.

Polyclonal Antibodies and Western Blotting. Antibodies to the isolated 15-kDa chain were obtained by cutting the SDS band, emulsifying it with complete Freund's adjuvant, and injecting it subcutaneously into two male New Zealand rabbits. After 20 days, a booster dose of the same amount of isolated chain was administered. Antibody presence and titer were confirmed by immunoelectrophoresis and an indirect ELISA. Antiserum was prepared from blood, 1 week after the last injection. For Western blotting analysis, protein samples (10−20 μg) separated for SDS-PAGE (14%) were electroblotted onto nitrocellulose membranes in 25 mM Tris base buffer, 192 mM glycine, and 20% methanol, pH 8.1−8.4, at 100 V for 1 h in a Mini Trans-Blot

Table 1. Characteristics of Crude Extracts from Various Organs of Globe Artichoke

extract	protein (μg mL^{-1})	specific proteolytic activity (Ucas[a] mg of protein^{-1})	cysteine relative activation (%)	relative inhibition (%) pepstatin (1 μM)	relative inhibition (%) E-64 (10 μM)	specific milk-clotting activity (MCU[a] mg of protein^{-1})
roots	216 ± 54 cde	0.33 ± 0.01 fg	86	18	78	0
rhizome	187 ± 40 de	0.26 ± 0.03 g	35	0	50	0
young leaves (<20 cm)	340 ± 29 c	0.50 ± 0.03 f	230	0	92	0
adult leaves (>50 cm)	288 ± 81 cd	2.39 ± 0.22 b	45	32	45	44.92 ± 7.31 b
midribs	115 ± 25 e	0.88 ± 0.06 e	80	0	70	0
receptacles	247 ± 22 cde	1.50 ± 0.03 c	36	0	50	0
inflorescence stems	281 ± 34 cd	1.43 ± 0.07 c	39	0	55	0
inflorescence leaves	128 ± 29 e	1.10 ± 0.06 d	190	0	90	0
immature flowers (<10 mm)	1024 ± 101 b	1.11 ± 0.06 d	6	60	14	23.02 ± 1.04 c
mature flowers (>50 mm)	1800 ± 108 a	5.45 ± 0.09 a	2	95	7	147.65 ± 7.29 a

[a] MCU (milk-clotting unit): amount of enzyme required to coagulate 1 mL of skim milk at 37 °C in 40 min. Ucas (caseinolytic unit): amount of enzyme that produces an increase in 1 absorbance unit per minute at 280 nm at pH 6 and 37 °C, with casein as substrate. Data are means (± standard deviation) of five determinations, and each experiment was repeated twice. In each column, values followed by different letters show significant differences between means at $P \leq 0.01$ using Tukey's test.

(Bio-Rad). Membranes were washed thoroughly with phosphate-buffered saline, pH 7.2 (PBS), containing 0.3% Tween-20 and coated with 3% low-fat milk to avoid nonspecific adsorption. After 1 h of incubation at 37 °C, they were submerged (3 h with agitation) into a 1:5000 dilution of the rabbit serum against the 15-kDa chain in PBST (3% Tween-20 in PBS). Membranes were washed three times with PBST and blotted with horseradish peroxidase-conjugated goat anti-rabbit IgG (H+L) at a 1:1000 dilution for 1 h. After washing, peroxidase activity was visualized with 3,3′-diaminobenzidine in the presence of 0.1% H_2O_2.

Amino-Terminal Sequence and Mass Spectrometry. A sample of the purified AP was electrophoresed on a 14% SDS−polyacrylamide gel, electroblotted onto a PVDF membrane, and washed several times with deionized water. N-Terminal amino acid sequences were determined by Edman's automated degradation using a Beckman LF3000 protein sequencer equipped with a PTH-amino acid analyzer System Gold (Beckman). Homology with other proteinases was determined using the BLAST network service (*30*). Mass spectrometry was performed by mixing 1−10 mM of sample with sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as matrix and dissolving the mixture in 0.1% trifluoroacetic acid in water/acetonitrile 2:1. One microliter was spotted onto a sample slide, allowed to evaporate to dryness, and processed in a MALDI-MS/TOF Bruker instrument (model Biflex) with a pulsed nitrogen laser (337 nm), in linear positive ion mode, using a 19-kV acceleration voltage. Trypsinogen ($M_w = 23981$) was used as standard.

Statistical Analysis. Calculations of means, standard deviations, and one-way analysis of variance (ANOVA) with Tukey's post-hoc means comparison test were performed using the Statistical Software Package for Windows 11.0 (SPSS Inc., Chicago, IL). Significant differences between media were determined at the 1% confidence level.

RESULTS AND DISCUSSION

Organ Expression of Proteinases. Proteinase expression in different globe artichoke organs was evaluated by measuring the proteolytic activity on casein, milk-clotting activity, protein content, and action of activators and inhibitors of the crude extracts from different parts of the inflorescence, as well as from leaves, roots, and rhizomes (**Table 1**). According to the analysis of variance, mature flowers had statistically significant differences at $P \leq 0.01$ in protein content, specific proteolytic activity on casein, and specific milk-clotting activity with respect to other organs analyzed. Highest relative proteolytic activities on casein at pH 6 were obtained in mature flowers (100%), followed by immature flowers (12%) and adult leaves (7%). Clotting activity presented the same pattern with relative values of 100, 9, and 5%, respectively, and was not detected in other organs analyzed during the assay time (300 min). As the protein content of the extracts from the different organs varied from 115 to 1800 μg mL^{-1} and mature flowers showed the higher protein content, values of specific milk-clotting activity (MCU mg of protein^{-1}) must be considered for comparative purposes; accordingly, mature flower extracts are still the most active ones (100%), followed now by adult leaf extracts (30.4%) and immature flowers (15.6%). Some samples (receptacles, inflorescence stems, and inflorescence leaves) were most active or showed nonstatistically significant differences ($P \leq 0.01$) in specific activity of casein degradation from immature flower, but they do not have any milk-clotting activity. That substantial parts of these activities (50−90%) were sensitive to E-64 may, therefore, be attributed to the action of cysteine proteinase. Proteolytic activity of the samples from mature flowers, immature flowers, mature leaves, and roots was inhibited by pepstatin (1 μM), in the percentages indicated in **Table 1**. The activities of different globe artichoke organs were compared with those obtained in a species of the same genus, *C. cardunculus* L. Agreement was observed in organs exhibiting proteolytic and clotting activity, except for mature leaf extracts, in which (*7*) milk-clotting proteinases were not detected. Reference *6* highlights that the styles of Australian cardoon flowers exhibit high caseinolytic activity, whereas leaf extracts showed scant activity. Other authors report that cardosins are present in mature stigmas (*13*, *14*), whereas cyprosins are immunolocalized in the epidermal cell layer of styles (*7*).

Characterization of Milk-Clotting Proteinase from Flowers. Because only mature globe artichoke flowers showed high clotting activity, subsequent work was focused on them. Although the properties of the crude enzyme preparation may well be derived from a combination of enzymes and nonenzymatic proteins, it was regarded as essential to characterize the crude extracts due to their potential use in food technology, as in industrial applications enzyme purity is usually of minor importance compared to the cost (*31*).

After 3 h at 37 °C, caseinolytic activity remained practically unchanged and was still high (70% of residual activity) after 3 h at 45 °C. Exposure to the enzyme at 55 °C led to a rapid decrease in residual activity (56% at 10 min and 18% at 3 h). Activity at 65 °C dropped abruptly to 27% in 5 min (**Figure 1**). Some plant proteinases were investigated as suitable rennet substitutes for cheesemaking, but in general they present a high proteolytic activity that results in bitter peptides in ripened cheese (*19*). The thermal behavior of the proteinase present in globe artichoke flowers that is inactivated by moderate heating supports its use in the production of cheeses.

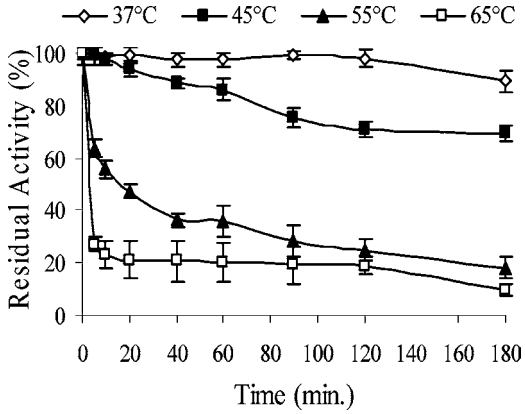

Figure 1. Thermal stability of globe artichoke flower crude extract. Determinations of residual activity were made on casein at pH 6. Data are means of five determinations, and each experiment was repeated twice. Error bars represent mean standard deviation.

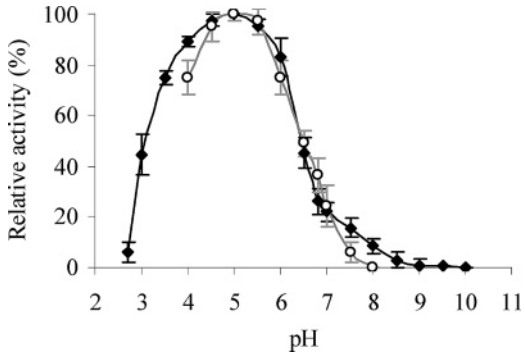

Figure 2. Effect of pH on proteolytic activity of globe artichoke flower crude extracts. Activity determinations were made on denatured hemoglobin at pH 2.7–10 (◆) and azocasein at pH 4–8 (○). Given values correspond to the pH of reaction mixtures. Data are averages of two independent assays ($n = 5$). Error bars represent mean standard deviation. The pH was controlled by adding 0.1 M sodium citrate–citric acid (pH 2.7–5.5), 0.1 M potassium phosphate (pH 6–8), and 0.1 M boric acid–potassium chloride–sodium hydroxide (pH 8–10) in various proportions.

As in the case of APs, the maximal activity of the crude preparation of globe artichoke mature flowers was reached at acidic pH (>95% from 4.5 to 5.5) using bovine hemoglobin or azocasein as substrate (**Figure 2**). This is in reasonable agreement with the optimal pH for the activity of the cardoon proteinases, 5.0 (*13*) and 5.1 (*11*), although peak activity in Australian cardoon was at pH 6.0 (*6*).

Cysteine addition failed to modify proteolytic activity on casein at pH 6, whereas pepstatin produced practically total inhibition (95%) and E-64 inhibited peptidase activity by only 7% (**Table 1**). Furthermore, crude extracts from flowers were barely inhibited (<5%) by PMSF and 1,10-phenanthroline (data not shown), and therefore the presence of cysteine proteinases, serine proteinases, and metalloproteinases was considered to be negligible. The above data allow one to conclude that the proteinases present in the mature flowers of *C. scolymus* L. cv. Green Globe belong to the same group of APs as the proteinases isolated from other Asteraceae flowers (*7*−*11*, *13*−*15*, *21*−*24*).

Purification of Milk-Clotting AP from Flowers. The crude extracts obtained from globe artichoke flowers were treated with different concentrations of activated charcoal to remove phenolic compounds and materials liable to interfere with enzymatic activity and increase the viscosity of the sample. Only the treatment with 10% (w/v) carbon rendered translucent, blue

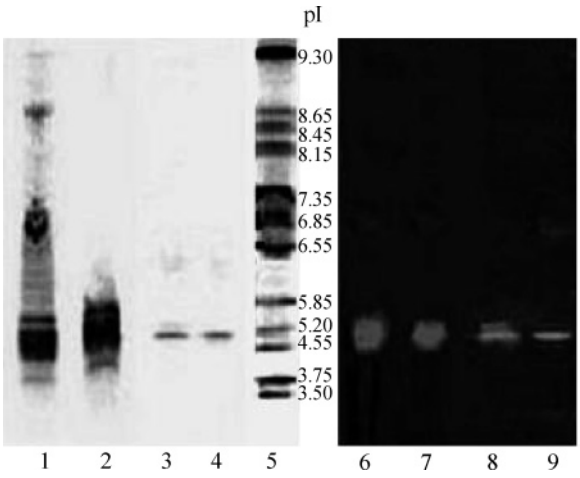

Figure 3. Isoelectric focusing (lanes 1–5) and zymogram (lanes 6–9) of globe artichoke flower extracts: lanes 1 and 6, crude extract; lanes 2 and 7, charcoal supernatant of crude extract; lanes 3 and 8, peak IV of DEAE-Sepharose enzyme purification; lanes 4 and 9, peak V of DEAE-Sepharose enzyme purification; lane 5, Pharmacia broad p*I* markers (3.5–9.3).

Table 2. Quantitative Purification Course of the Proteinase from Globe Artichoke Mature Flowers

purification step	protein (mg)	total activity (MCU[a])	specific activity (MCU[a] mg^{-1})	purification (*n*-fold)	yield (%)
crude extract	18.00	2666.7	147.65	1	100
charcoal supernatant	12.50	1846.2	147.70	1	69.2
DEAE-Sepharose peak V	6.24	1043.5	167.23	1.13	39.1
pepstatin–agarose peak 3	0.20	888.9	4444.50	30	33.3

[a] MCU (milk-clotting unit): amount of enzyme needed to coagulate 1 mL of skim milk at 37 °C in 40 min.

extracts. In this first step, high-p*I* peptides were eliminated (**Figure 3**), recovering from the crude extract 69% of clotting activity and protein (**Table 2**). IEF followed by zymogram analysis showed several bands with hemoglobinolic activity at pI = 4, thus evidencing the proteolytic heterogeneity of the sample under study (**Figure 3**). Preliminary experiments for validating the methods to remove interfering compounds showed that charcoal adsorption provided substantially better results than acetone or ammonium sulfate precipitation.

Chromatography on an anion exchange column of the carbon-treated enzymatic preparation yielded five peaks (**Figure 4**). Activity measurements showed that the greater part of total activity loaded onto the column was eluted in peaks IV and V, which present the same specific hemoglobinolytic activity (2.6 Uhem mg^{-1}) and similar values of specific clotting activity (158.20 and 167.23 MCU mg^{-1}, respectively). Fraction V retained the blue pigment. Although fraction III had a high specific activity on hemoglobin (9.50 Uhem mg^{-1}), it failed to clot the milk and was therefore discarded.

IEF with pH 3−10 ampholytes and the corresponding zymograms obtained by immersion of the gel in hemoglobin pH 4 solution indicate that both fractions present proteolytic activity at pI 4 (**Figure 3**). In fraction IV, the presence of a second band was observed that indicated heterogeneity, so that analysis was continued only for fraction V. The molecular mass of this fraction determined in triplicate by means of molecular exclusion chromatography in Sephadex G-75 was 60 ± 3 kDa.

Because pepstatin, a specific inhibitor of aspartic proteinases, binds reversibly to the active enzyme, an affinity purification

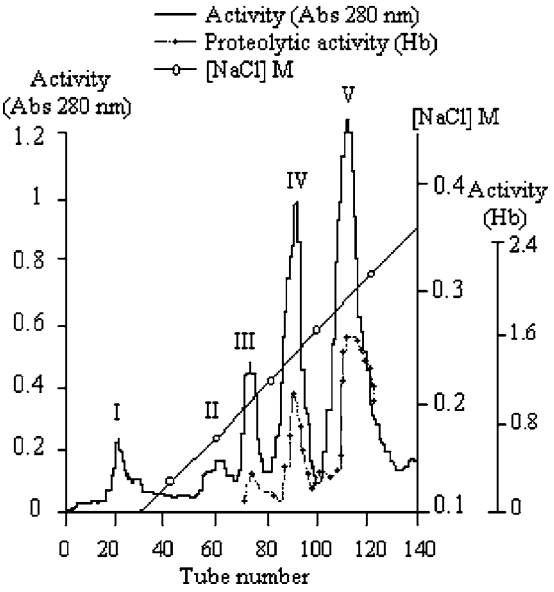

Figure 4. Anion exchange chromatography (DEAE-Sepharose Fast Flow) of crude extract from globe artichoke flowers treated with 10% (w/v) charcoal.

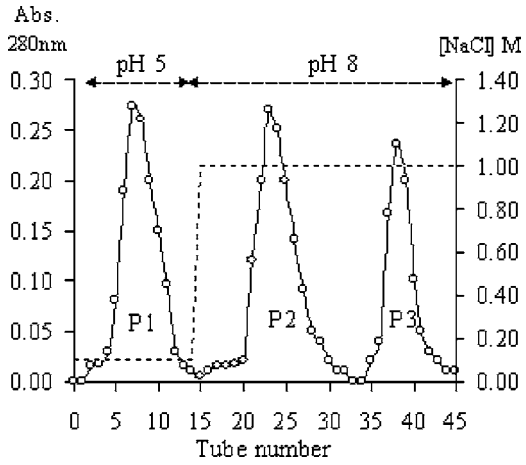

Figure 5. Affinity chromatography in pepstatin–agarose of peak V of DEAE-Sepharose enzyme purification step: (○) absorbance at 280 nm; (- - -) NaCl concentration.

step using a pepstatin−agarose column was used to further purify peak V, which resolved in three fractions: P1, P2, and P3 (**Figure 5**). The proteins that failed to bind to pepstatin were eluted with acetic acid−sodium acetate buffer, 0.1 M, pH 5 (P1), the bluish pigment eluted with Tris-HCl buffer, 0.1 M, pH 8, containing 1 M NaCl (P2), and the proteinase strongly bound to the pepstatin separated as P3. Clotting activities of P3 and P2 were 88.9 and 22.2 MCU mL^{-1}, respectively, whereas P1 failed to clot the milk in the 300 min of the assay. For further analysis, it was considered to be reasonable to use P3, which had the highest specific activity and presented no associated bluish pigment. The strategy used in affinity chromatography by varying both pH and NaCl concentration allowed 30-fold purification with an 85% yield of the milk-clotting enzyme obtained as peak V in ion exchange chromatography (**Table 2**).

Affinity chromatography on pepstatin−agarose has been chosen and recommended as a technique to purify several APs from seeds (*32*−*37*), but in ref *22* it is reported that attempts to purify cenprosin, an AP from *Centaurea calcitrapa* flowers, were unsuccessful because the enzyme could not be eluted. On the other hand, DEAE-Sepharose purification of globe artichoke flowers rendered a very low yield, in agreement with the results obtained during the purification of AP from *Arabidopsis thaliana* seeds (*35*). Such findings suggest that ion exchange chromatography may not be necessary for the purification of seed APs, but it could be required during the first steps of the purification of APs from flowers. Preliminary assays carried out in our laboratory support the above affirmation. The lower yield in affinity chromatography with regard to the one obtained by other authors (*34*, *35*, *37*) may be attributed to differences in proteinase content among seeds and flowers.

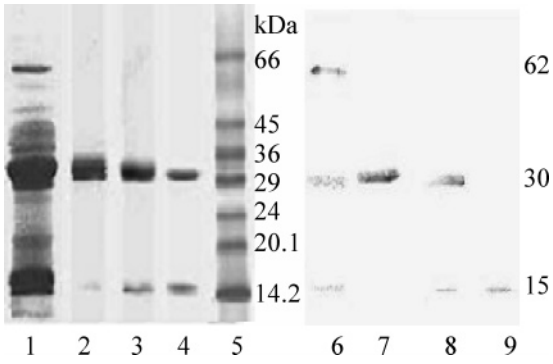

Figure 6. SDS-PAGE (lanes 1–5) and western blotting (lanes 6–9) of aspartic proteinase from globe artichoke flowers: lanes 1 and 6, crude extract; lanes 2 and 7, affinity chromatography of fresh P3; lanes 3 and 8, P3 stored at 4 °C during 24 h; lanes 4 and 9, P3 stored at 4 °C during 7 days; lane 5, Sigma MW-SDS-70L molecular weight markers. Immunodetection was performed with antibodies against the lower molecular peptide of globe artichoke proteinase. P3: peak 3 of affinity purification step.

Proteinase Processing. Band patterns in SDS-PAGE of the affinity chromatography eluate 3 (P3) varied according to the degree of sample handling, which indicated proteinase processing. **Figure 6** shows electrophoresis under denaturing conditions according to whether SDS-PAGE was carried out with fresh P3 samples or stored during 3 and 7 days at 4 °C. In the first case a protein fraction is obtained that is visualized as several bands from 30 to 34 kDa. At 3 days of storage a thin band is also observed in the 15-kDa area, and after 7 days, SDS-PAGE presented two well-defined protein bands at 15 and 30 kDa. Addition of the AP inhibitor pepstatin (1 μM) prevented autoproteolytic processing. On occasion, a minor protein band was visualized at ∼62 kDa, which may be a precursor form of the AP.

An aliquot of the affinity column eluate separated with SDS-PAGE was blotted to nitrocellulose, and immunodetection was carried out with an antiserum specific for the 15-kDa chain. Immunoblots confirmed the mentioned processing because in fresh samples antisera reacted strongly with a 30-kDa band, whereas in preserved preparations it reacted with a 15-kDa band. On the other hand, in the crude extract from mature flowers antigen−antibody reaction was observed at 62, 30, and 15 kDa (**Figure 6**).

The foregoing suggests that the milk-clotting proteinase isolated from globe artichoke flowers is synthesized as a 62-kDa proenzyme, the processing of which originates the 30- and 15-kDa peptides of the mature enzyme. This type of behavior agrees with that described in refs *3*, *38*, and *39*, where it is stated that all of the nonviral APs are synthesized as inactive zymogens, which maintain the N terminus bound to the active site, allowing temporal and spatial regulation of peptidase activity. Thus, the phytepsin (HvAP) of barley is synthesized

Table 3. Amino Acid Sequence Alignment of the Chains of the Globe Artichoke Flower Proteinase with cDNA-Derived Amino Acid Sequences from Flower APs[a]

plant species	sequence	identities (%)	ref.
Cynara scolymus L. (30 kDa chain)	1 DSGSAVVALTNDRDTSYFGE 20		
Cynara cardunculus L. (procardosin A)	69 DSGSAVVALTNDRDTSYFGE 88	100	(*9*)
Cynara cardunculus L. (procardosin B)	69 DSGSGIVALTNDRDTAYYGE 88	80	(*14*)
Centaurea calcitrapa (procenprosin)	69 DSDSDIIELKNYMDAQYYGE 88	55	(*22*)
Cynara cardunculus L. (procyprosin)	69 DSGSDIIALKNYMDAQYYGE 88	55	(*15*)
Cynara scolymus L. (15-kDa chain)	1 SSEELQVDCNTLSRMPNVSFTIGGKKF 27		
Cynara cardunculus L. (procardosin A)	416 SSEELQVDCNTLSSMPNVSFTIGGKKF 442	96	(*9*)
Centaurea calcitrapa (procenprosin)	423 ESAVDCNDLSSMPNIAFTIGGKVF 446	71	(*22*)
Cynara cardunculus L. (procardosin B)	417 SSAESIVDCNGISSMPNIAFTIGSKLF 443	63	(*14*)
Cynara cardunculus L. (procyprosin)	387 ESAVDCSSLSSMPNIAFTVGGKTF 410	62	(*8*)

[a] Identical amino acids are shaded, and substitutions are shown in black.

and translocated within the endoplasmic reticulum as a proenzyme that undergoes several proteolytic cleavages to produce the mature bicatenary form present in grains, leaves, and roots (*38*). These authors indicate that the processing mechanisms of the proenzymes allows different protein patterns to be observed depending on the transformation degree, resulting in turn from the interaction of several factors such as the pH and the presence of proteinases in the intracellular compartment traversed by the AP on the way to its final localization in the cell.

In cardoon flowers a 64 kDa procardosin A was also identified, which by removal of a plant AP specific insert (PSI) and the prosegment, the 31- and 15-kDa polypeptides that constitute the mature enzyme are produced. The scheme of proteolytic processing proposed for procardosin A (*12*) consists of an initial cleavage site between the 31-kDa subunit and the PSI, producing two peptides of 35 and 30 kDa. From the latter, PSI would separate, releasing the 15-kDa chain, and finally the prosegment bound to the 35-kDa fraction would be eliminated, thus originating the 31-kDa subunit.

Similar heterodimeric APs have been found in cardoon flowers by Heimgartner (*11*), who reported the isolation, under alkaline conditions (pH 8.3), of three aspartic proteinases (cynarases), whereas two distinct APs were isolated by using citric acid (pH 3), which were named cardosins A and B (*13*). Extracts at pH 3 from *Centaurea calcitrapa* flowers also present an AP consisting of two 16- and 30-kDa chains, whereas extraction at pH 8.1 showed bands with major molecular masses that would represent the proenzyme (*22*).

Amino-Terminal Sequences and Mass Spectrometry. Eluate 3 (P3) from the affinity column was separated with SDS-PAGE and blotted onto a PVDF membrane, and the resulting peptides were eluted from the membrane and sequenced. Subunit N-terminal amino acid sequences were aligned with cDNA-derived amino acid sequences from flower APs (**Table 3**). The two chains of AP from globe artichoke flowers present a high degree of homology with cardosin A. The N-terminal sequence of the 30-kDa subunit presented from 55 to 100% identity with the AP sequences isolated from flowers (cyprosin, cenprosin, and cardosins). The 27 amino acids identified at the 15-kDa chain N-terminal present 96% homology with cardosin A (*9*), whereas sequence identities with cyprosin (8), cardosin B (14), and cenprosin (22) were 62, 63, and 71%, respectively.

The molecular mass of the smaller peptide from globe artichoke flowers obtained by spectrometry was 15.358 Da, using trypsinogen of MW 23.981 for calibration (**Figure 7**). Attempts to determine the molecular mass of the 30-kDa chain have been unsuccessful.

Plentiful investigations for new rennet sources have been carried out to allow the replacingement of bovine chymosin in cheese production. The microbial recombinant chymosin is a suitable substitute. Natural rennet extracted from plants could provide an alternative especially valued by vegetarian and environmentalist consumers. Although several plant proteinases are able to coagulate milk, most are inappropriate for the production of cheeses due to their high proteolytic activity, which degrades the clot-generating peptides, conferring a bitter

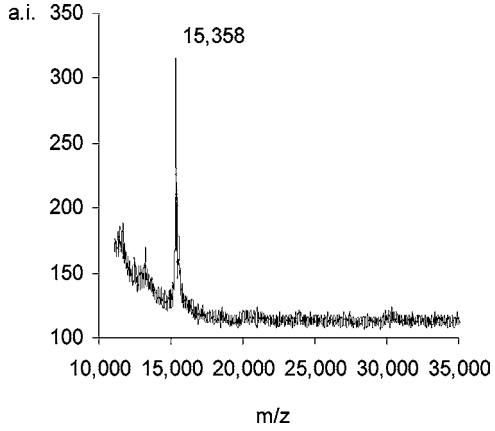

Figure 7. Mass spectroscopy of the 15-kDa unit of globe artichoke flower proteinase. The output of the mass spectrometer shows a plot of absolute intensity (a.i.) versus the mass-to-charge ratio (*m*/*z*).

flavor. An exception is the aqueous extract from cardoon flowers used for the manufacture of artisanal cheese in Portugal and Spain. As observed in this work, the protein extract from globe artichoke flowers has milk-clotting ability. Analysis of the crude extract indicates that the milk-clotting ability is due to an AP inactivated by moderate heating. This is a heterodimeric proteinase with amino-terminal sequences homologous to cardosin A isolated from cardoon. Therefore, we consider globe artichoke flower extract to be very promising in cheese manufacture. This application would grant an added value to the cultivation of this species, as poorly marketable inflorescences due to size or appearance (usually discarded) could be allowed to mature and used for this purpose. Research is under way in our laboratory on the milk-clotting properties of extracts from globe artichoke flowers and the proteolysis during cheese ripening in both bovine and goat milks.

ABBREVIATIONS USED

AP, aspartic proteinase; BLAST, basic local alignment search tool; BSA, bovine serum albumin; DEAE, diethylaminoethyl; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IEF, isoelectric focusing; MALDI-TOF MS, matrix-assisted laser desorption ionization−time-of-flight mass spectrometry; MCU, milk-clotting unit; MW, molecular weight; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline with Tween-20; PMSF, phenylmethanesulfonyl fluoride; PSI, plant aspartic proteinase specific insert; PVDF, poly(vinylidene difluoride); SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)-aminomethane; Uazoc, azocaseinolytic unit; Ucas, caseinolytic unit; Uhem; hemoglobinolytic unit.

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