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Phytochemistry 66 (2005) 41–49
PHYTOCHEMISTRY
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Purification of cynarases from artichoke (*Cynara scolymus* L.): enzymatic properties of cynarase A

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Received 28 July 2004; received in revised form 6 October 2004
Available online 25 November 2004

Abstract

Aspartic proteinases from flowers of *Cynara cardunculus* have been extensively studied and long used as coagulants in the manufacture of several traditional Spanish and Portuguese cheeses. These endopeptidases are called cardosins or cynarases, depending on the authors. However, the proteinases of another plant of the genus *Cynara*, the artichoke (*Cynara scolymus*), are less known, probably because the flower of this plant is usually consumed as a vegetable. In the study described here, three proteinases (cynarases A, B and C) with milk-clotting properties were purified from the stigma of artichoke. All three proteinases are glycoproteins and composed of a one large and one small subunit. The enzymatic properties of cynarase A, a glycoprotein containing N-linked high mannose type glycans, which express maximum activity at pH 5.0 and 70 °C, were studied in detail. Catalytic and inhibition studies indicated that this cynarase is of the aspartic acid type. The results indicate artichoke extract could also be used in the milk industry in the same way as the extract obtained from the flower of *C. cardunculus*.

Keywords: *Cynara scolymus* L.; Artichoke; Aspartic endopeptidase; Proteinase purification; Steady-state kinetics; Spectroscopy

1. Introduction

Aspartic proteinases or endopeptidases are a class of enzymes (EC 3.4.23) involved in a number of physiological and pathological processes such as blood pressure homeostasis (renin), retroviral infection (human immunodeficiency virus proteinase), hemoglobin degradation in malaria (plasmepsin), intracellular proteolysis (cathepsin A), and digestion (pepsin) (Dunn, 2002). The number of industrially used enzymes of plant origin is small, proteinases being the most widely used due to their application in the food, pharmaceutical and detergent industries, as well as, in the preparation of leather and wool (Aehle, 2004). The most widely utilized plant proteinases are the cysteine endopeptidases papain, bromelain and ficin. Additionally, aspartic proteinases play an important role in the food industry, e.g., the cheese industry or in soya and cocoa processing. Cardosins are aspartic proteinases from the flowers of *Cynara cardunculus* L. (Verissimo et al., 1996), whose milk-clotting activity has been exploited in Spain and Portugal for many years. The molecular and enzymatic properties of cardosin A, the most abundant of the cardosins, have been studied in detail (Verissimo et al., 1996; Frazao et al., 1999; Sarmento et al., 2003). This proteinase share a number of common characteristics with other aspartatic proteinases: all have an acid pH optimum, are

Abbreviations: Con A, concanavaline A; LCA, *Lens culinaris* agglutinin; WGA, wheat germ agglutinin; WGA-suc, wheat germ agglutinin succinilated; RCA, *Ricinus communis* agglutinin; IEF, isoelectric focusing; PVP, polyvinyl pyrrolidone.

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doi:10.1016/j.phytochem.2004.10.005

heterodymerics and are inhibited by pepstatin. Moreover, this two chain enzyme was shown to cleave κ-casein in the same peptide bond (Phe105-Met106) as chymosin. Cardosins from *C. cardunculus* L. have also been named cyprosins or cynarases by other authors (Heimgartner et al., 1990; Brodelius et al., 1995; Cordeiro et al., 1998; White et al., 1999) who isolated three active proteinases fractions (cynarases 1, 2 and 3).

Globe artichokes (*Cynara scolymus* L.) are perennial, frost sensitive, thistle-like plants with edible flower buds, which sprout from the terminal portion of the main stem and on lateral stems. Each unopened flower bud resembles a deep green pine cone, 7–10 cm in diameter, round, but slightly elongated. Several pointed, leathery green bracts fold around a purple-blue flower. The base of each bract is the fleshy edible portion, along with the fleshy centre of the artichoke on which the flower and bracts are borne. Cynarases have been isolated from artichoke (Verissimo et al., 1998), but because the flowers of this plant are usually consumed as a vegetable, the properties and possible applications of these cynarases are less known. In the present work, we detected high proteinase activity in the stigma of artichoke. Therefore, we describe how to purify milk clotting proteinases from dried flowers of *C. scolymus*, and our initial studies of the physical and catalytic properties of the major isoenzyme present in these flowers, cynarase A.

2. Results and discussion

2.1. Purification of cynarases from artichoke

Three proteinases with milk clotting activity were isolated from dried flowers of *C. scolymus.* The purification of proteinases from stigma tissue is hindered by the small amount of tissue available per artichoke and the strong purple pigmentation of these tissues. Initially, we tried various adsorbents (bentonites, PVP, and ion exchangers) as well as precipitation methods (ammonium sulfate) to remove the colored material or to concentrate proteinases. None of these methods were successful because they reduced the recovery of enzyme activity or were unable to remove a substantial amount of the colored material. However, ultrafiltration of the crude extract through a 10,000 Mw cut off filter was found to be successful for its clarification. This operation removed most of the purple color of the initial extract. After ultrafiltration, the protein mixture was applied to a Q-Sepharose Fast Flow pre-column. The bulk of the proteinases were displaced from the column by one-step elution with 0.5 M sodium chloride. A step gradient was used in this purification step to avoid over dilution of the enzyme. The fraction containing proteinase activity was dialysed and applied to a Q-Sepharose Fast Flow column. Three peaks with proteolytic activity were obtained yielding essentially pure proteinases (Fig. 1). The proteinases displaced from this column at 30%, 35% and 50% of sodium chloride were recognized as cynarases A, B and C, respectively (Table 1). Their combined content (more than 0.2% of the total protein in this plant tissue) suggests the great physiological importance that these enzymes might have. Although the function of these proteinases in the flowers of *Cynara* species is still uncertain, they may be involved in sexual reproduction of the plant or have a defense role against pathogens and/or insects. It is known that they accumulate in protein storage vacuoles in the stigmatic papillae of the flowers (Ramalho-Santos et al., 1997).

2.2. Molecular mass and pI

Preparations were judged to be homogeneous when a double band was observed on a Coomassie blue-stained reducing SDS–PAGE gel (Fig. 2(a)). This result shows that the three cynarases are each composed of two subunits of different sizes, which Mr_s were estimated (Table 2). The native Mr_s of the enzyme estimated by the gel-filtration method (40,800, 43,400 and 46,000 for cynarases A, B and C, respectively), were consistent with the SDS–PAGE results, indicating that the enzymes are dimeric proteins (Table 2). Silver stained IEF of purified proteinases resulted in a single band with pIs values of 3.18, 3.25 and 3.38 for cynarases A, B and C, respectively (Fig. 2(b) and Table 2).

2.3. Carbohydrate composition

The first indication that the three cynarases are glycoproteins was obtained by SDS–PAGE and blotting followed by subsequent carbohydrate staining (Fig. 2(c)). Both subunits of cynarase A and B are glycosylated, while only the larger subunit of cynarase C is glycosylated. This glycosylation pattern was similar to that found for the three cynarases obtained from *C. cardunculus* (Heimgartner et al., 1990). Plant aspartic proteinases characterized at the molecular level contain one or more consensus *N*-glycosylation site (Costa et al., 1997). For instance, the glycosyl content of cardosin A from *C. cardunculus* is described by 19 sugar rings attached to Asn-67 and Asn-257 (Frazao et al., 1999).

The carbohydrate structures of glycoproteins are recognized by specific lectins. In order to partially characterize the sugar composition of artichoke cynarases, the purified proteinases were incubated with Con-A, LCA, WGA, WGA-suc, and RCA and the results compared with those obtained with a control in which the proteinase was incubated with Sepharose-4B. The initial proteinase activity non-retained on the different lectins after overnight incubation at 4 °C can be observed in Fig. 3. The strong affinity of the three cynarases for

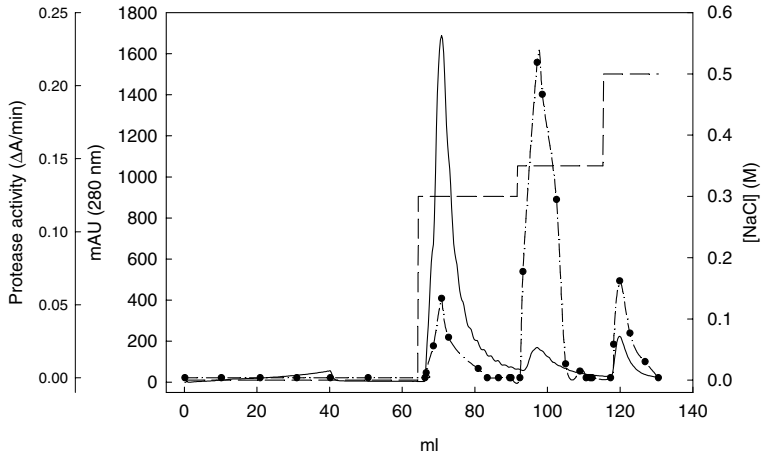

Fig. 1. Anion-exchange chromatography of cynarases on a Q-Sepharose fast flow column. Continuous line represents the absorbance at 280 nm; dashed line the NaCl concentration and data points proteinase activity.

Table 1
Summary of the purification procedure of cynarases from 100 g of dry stigmas of artichoke flowers

	Protein (mg)	Activity (Units)	Specific activity (Units/mg)	Yield (%)	Purification (-fold)
Crude extract	2700	17,500	6.5	100	1
Ultrafiltration	2160	14,000	6.5	80	1
Q-Sepharose FF	18.4	7150	388	40.8	59.7
Cynarase A (0.30 M NaCl)	4.3	875	203	5.0	31.2
Cynarase B (0.35 M NaCl)	2.2	2945	1338	16.8	205
Cynarase C (0.50 M NaCl)	0.6	740	1233	4.2	190

Con A suggests that these enzymes are glycoproteins containing *N*-linked high mannose type glycans. Therefore, Con A affinity chromatography could also be a valuable method for the final step of cynarase purification. Moreover, to the other lectins, cynarase A showed high affinity for WGA, which recognizes residues of *N*-acetylglucosamine (GlcNAc), sialic acid and, to a lesser extent *N*-acetylneuraminic acid (NeuNAc). The non-binding of cynarase A to WGA-suc confirms that the enzyme present rests of sialic acid. Fig. 3 shows different glycosylation patterns for the three cynarases isolated from artichoke.

2.4. Enzymatic properties of cynarase A

2.4.1. pH studies

Cynarase A exhibited a broad pH activity profile (Fig. 4(a)). While maximum activity was around pH 5.0, about 40% of this optimal activity was observed at pH 3.0. At pH 7.0 the enzyme showed about 10% of the activity observed at pH 5.0. The pH optima of aspartic proteinase normally lies between 1.5 and 5.0 and in the case of artichoke cynarase A, the optimum pH was similar to that found for a proteinase obtained from *C. cardunculus* (Heimgartner et al., 1990). The enzyme also showed a high stability over a wide pH range when incubated at room temperature for 60 min. There was 90–100% retention of activity upon incubation within the pH range of 3.0–7.0 (Fig. 4(b)).

2.4.2. Temperature studies

The hydrolysis of [H–Pro–Thr–Glu–Phe–*p*-(NO_2)–Phe–Arg–Leu–OH] by cynarase A was optimal at 70 °C (Fig. 5(a)). At higher temperatures there was a rapid decline in activity. Thermostability of the enzyme was examined by measuring the residual activity of aliquots of the enzyme incubated at different temperatures. Although the enzyme has a high temperature optimum, its stability at 50 °C and above was studied. There was a total retention of activity after 60 min of incubation at 50 °C (Fig. 5(b)). However, at 60 °C the enzyme was rapidly inactivated.

2.4.3. Catalytic properties

The kinetic parameters of cynarase A acting on [H–Pro–Thr–Glu–Phe–*p*-(NO_2)–Phe–Arg–Leu–OH] were determined at the pH optimum of the enzyme. The steady state rate increased with increasing substrate concentration. A typical hyperbolic response was observed (Fig. 6). The apparent K_m and V_{max} (70.3 μM and 8.2 μM min^{-1}, respectively), were determined by non-linear regression. Taking into account the enzyme concentration, the catalytic constant (k_{cat}) was calculated to be 144 s^{-1}. The values of these kinetic constants for

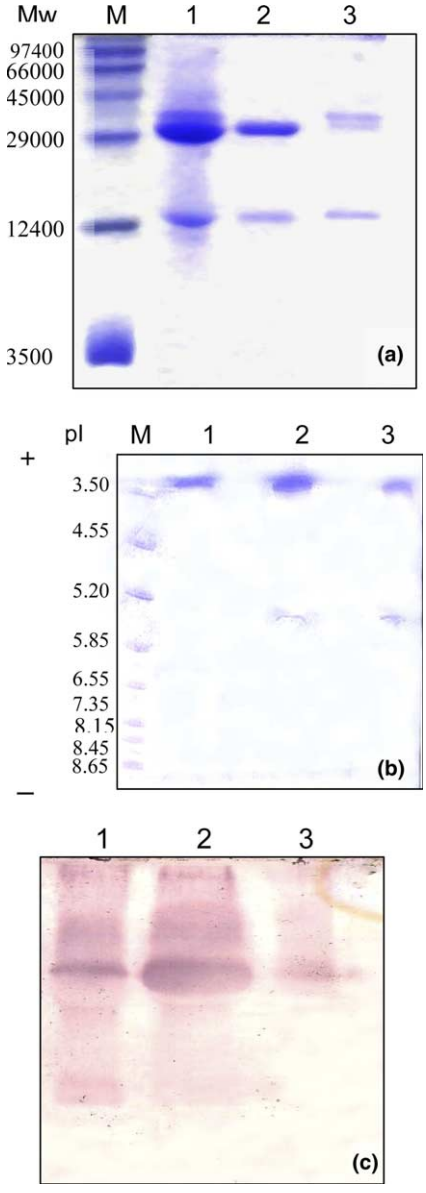

Fig. 2. (a) SDS–PAGE, (b) IEF and (c) periodate-blot analysis of artichoke cynarases. Lane 1, cynarase A; Lane 2, cynarase B; Lane 3, cynarase C; M, markers.

cynarase A were intermediate among that of pepsin and chymosin, under the same assay conditions (Table 3).

2.4.4. Inhibition studies

Pepstatin, a tight binding inhibitor specific for aspartic proteinases (Rich and Sun, 1980), inhibited cynarase A at relatively low concentrations. Therefore, it is confirmed that the catalytic site of cynarase A from artichoke contains two aspartic acid residues and that it belongs to the group of aspartic proteinases (Verissimo et al., 1998). A complete steady-state kinetic analysis revealed that the tight binding inhibition constant (K_I) of cynarase A for pepstatin was 30 nM. This value was intermediate among that of pepsin and chymosin, under the same assay conditions (Table 3).

Table 2
Estimated Mrs and pI_s of proteinase subunits

M_r ($\times 10^{-3}$)				p*I* Native
Cynarase	Native	Large subunit	Small subunit	
A	42,900	29,650	13,250	3.18
B	46,000	34,500	11,500	3.25
C	45,500	32,250	13,250	3.38

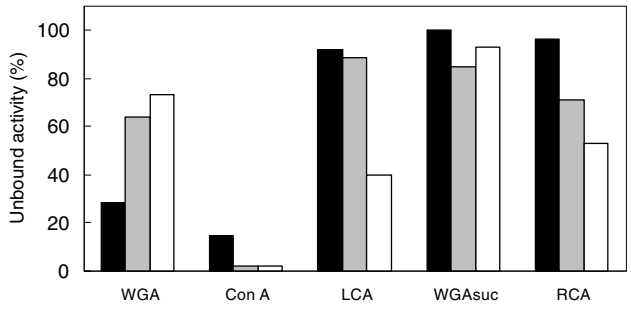

Fig. 3. Interaction of cynarases A (black bars), B (grey bars) and C (white bars) with immobilized lectins.

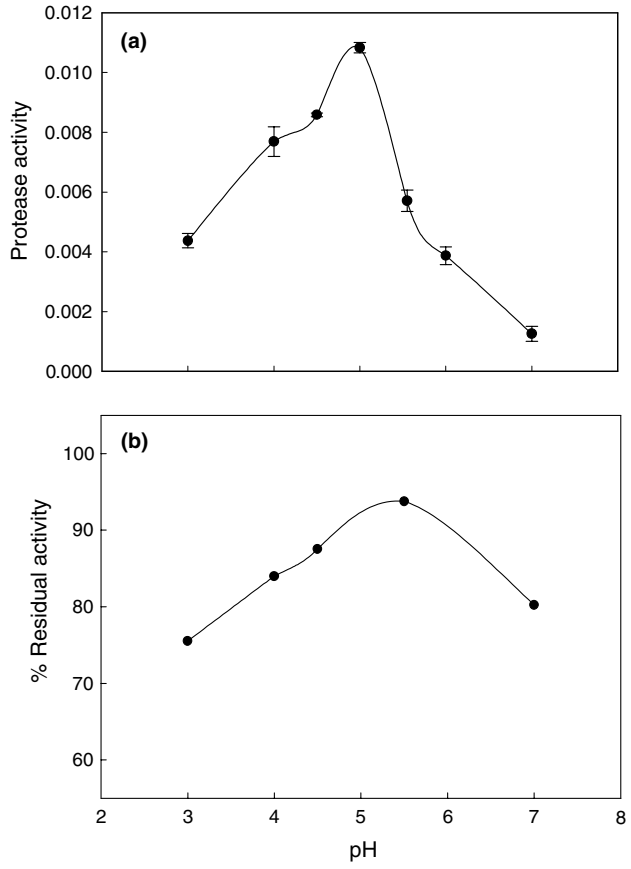

Fig. 4. Effect of pH on the catalytic activity (a) and stability (b) of cynarase A. Experimental conditions and buffer used are specified in the Section 3.

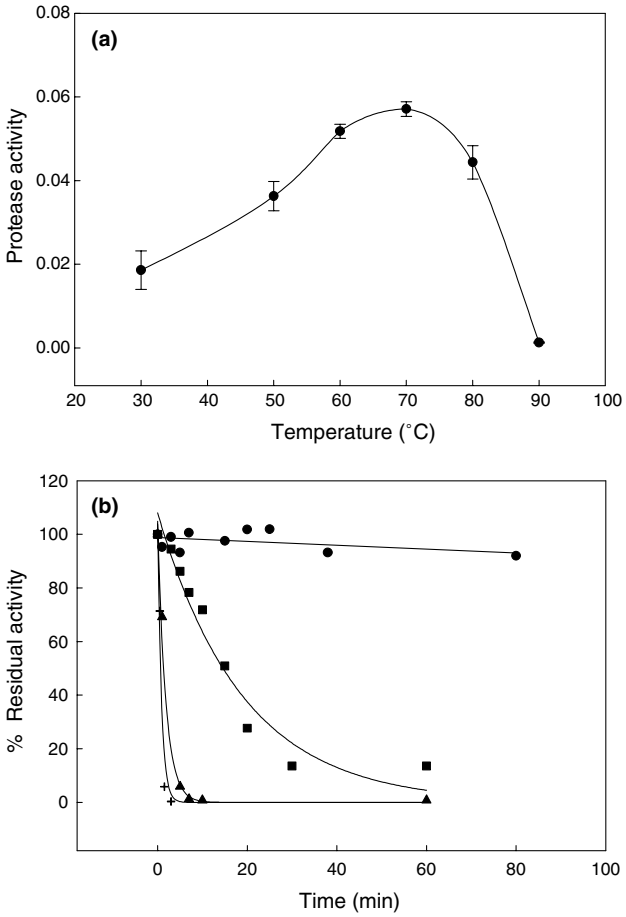

Fig. 5. (a) Effect of temperature on the reaction rate of cynarase A. (b) Thermal stability of this proteinase at different temperatures (●) 50; (■) 60; (▲) 70 °C; (+) 80 °C. Experimental conditions are specified in the Section 3.

2.5. Milk clotting properties of dried flower of artichoke

The crude and dialyzed extract of dried flowers of artichoke showed milk clotting properties. In milk, the primary soluble proteins are the whey proteins, α-lactalbumin and β-lactoglobulin. The insoluble proteins found in the colloidal particles include casein micelles. It has been described that milk clotting in the presence of milk-clotting aspartic proteinases occurs in two separate steps (Chitipinityol and Crabbe, 1998; Sousa et al., 2001). The first step starts with the cleavage of κ-casein at the Phe105-Met106 bond, which results in a release of a hydrophilic glycopeptide (106–169 components), which passes into the whey, and *para*-κ-casein, which remains in the micelles. *Para*-κ-casein becomes positively charged at neutral pH and causes the diminishes the electric repulsive forces between casein micelles. The hydrolysis of other proteins in milk, including α_{S1}-casein, α_{S2}-casein, β-casein and α-lactalbumin monomer has been reported to occur with a much slower rate of proteolysis (Miranda et al., 1989). This initial phase destroys the stability of the micelles and is followed by a non-enzymatic secondary phase, in which the aggregation of *para*-κ-casein and other casein components occurs in association with Ca^{2+} ions and eventually results in the formation of a gel (Merin et al., 1989; Esteves et al., 2001). Therefore, clot formation is Ca^{2+} dependent. Moreover, there are several factors that influence the milk-clotting process, including pH, temperature, ionic strength, enzyme concentration and the presence of salts (Bringe and Kinsella, 1986; Okigbo et al., 1985). In order to study the possible industrial application of artichoke extracts in the milk industry, we studied casein digestion, which constitutes the main process in the first phase of milk clotting. Casein digestion by artichoke extracts was compared with other commercial milk-clotting proteinases. Although the aspartic proteinases present in crude and dialyzed extracts of artichoke were shown to cleave κ-casein at the same peptide bond as chymosin and other fungal and microbial proteinases (Fig. 7), the major differences were found in the hydrolysis of α- and β-caseins. However, the extent to the hydrolysis of these three milk caseins by artichoke extract was similar to that obtained with aqueous extracts from *C. cardunculus* (Silva et al., 2002), which are widely employed for cheese making.

In conclusion, three cynarases (cynarases A, B and C) with milk-clotting activity have been purified from dried flowers of *C. scolymus*. Although the three proteinases are each composed of one large and one small subunit they show, in some respects, different properties. They are eluted at different salt concentrations from an anion-exchanger corresponding to their different isoelectric points, and the sizes of the subunits as well as their glycosylation patterns are also different. The results indicate the possibility of the use of extracts from dried flowers of *C. scolymus* in the cheese-making industry. The optimal conditions for cheese clotting using these extracts and to what extent they affect the overall properties of such cheeses are under investigation in our laboratory.

3. Experimental

3.1. Plant material and chemicals

Dry flowers of artichoke (*C. scolymus* cv. Blanca) were picked in Murcia (Spain). The stigmas were separated from the rest of the flower and stored at room temperature until use. All the chemicals used are commercially available and of reagent grade. Azocasein, α_S-, β- and κ-caseins as well as chymosin and pepsin were purchased from Sigma (Madrid, Spain). The peptide contains a phenylalanyl-*p*-nitrophenylalanine sequence at the reactive site, [H–Pro–Thr–Glu–Phe–*p*-(NO_2)–Phe–Arg–Leu–OH], which was obtained from Bachem (Basel, Switzerland). Pure chymosin from

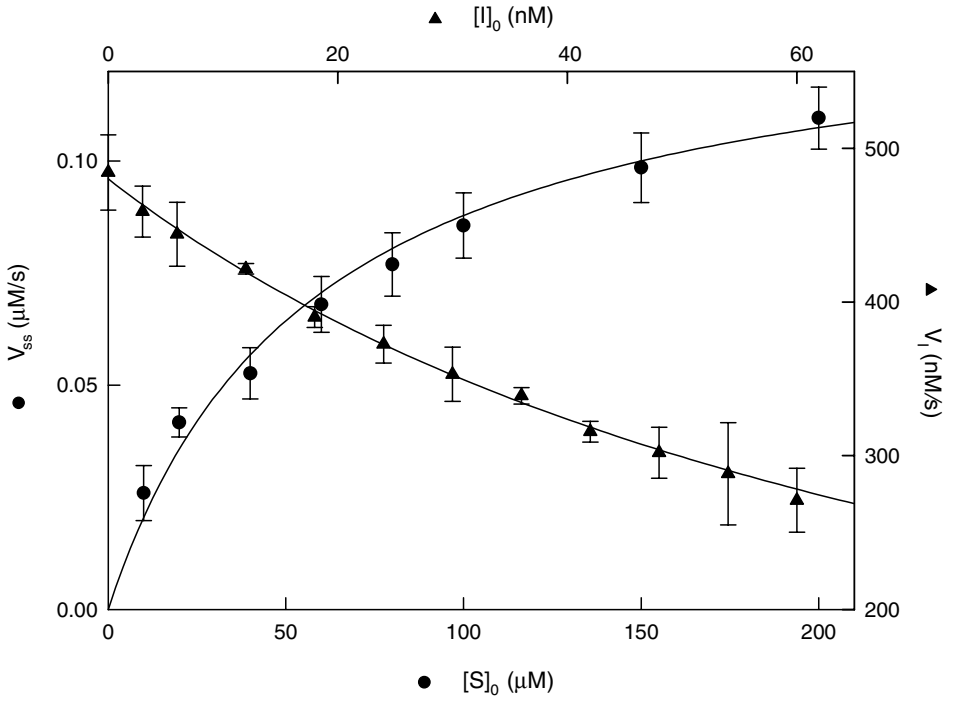

Fig. 6. (a) Hyperbolic dependence of the steady state rate of cynarase A versus substrate concentration. (b) Inhibition of cynarase A by pepstatin. Assay conditions: 50 mM sodium acetate buffer pH 5.0 at 25 °C.

Table 3
Kinetic characterization of pepsin, chymosin and cynarase A[a]

Enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($nM^{-1}\ s^{-1}$)	K_I (nM)
Pepsin	17.89 ± 0.80	104.5 ± 7.3	171.2 ± 14.0	57.2 ± 18.9
Chymosin	0.20 ± 0.02	174.7 ± 28.3	1.2 ± 0.2	10.5 ± 2.3
Cynarase A	2.63 ± 0.30	57.5 ± 16.4	45.7 ± 13.9	30.0 ± 8.7

[a] Assay conditions: 50 mM sodium acetate buffer pH 5.0 at 25 °C.

genetically-engineered yeast and milk-coagulating fungal proteinase were obtained from the National Centre for Biotechnology Education (Reading, UK). Microbial and calf stomach (chymosin 80% and pepsin 20%) rennet were purchased from Caglio Star (Spain). Sepharose 4B and agarose-immobilized lectins such as concanavalin-A (Con A), wheat germ agglutinin (WGA), wheat germ agglutinin succinilated (WGA-suc) *Ricinus comunis* agglutinin (RCA) and *Lens culinaris* agglutinin (LCA) were purchased from Sigma. Milli-Q (Millipore, Madrid, Spain) pure water was used throughout this research.

3.2. Purification steps

3.2.1. Homogenization

Tissue homogenization, centrifugation and dialysis were carried out between 4 and 8 °C. Fast protein liquid chromatography purification steps were performed at room temperature. Stigmas (100 g) were ground in a food mixer and homogenized in 800 ml 50 mM aqueous citrate buffer (pH 3.0) containing 1 M NaCl to prevent non-specific proteinase binding to the filter membrane in the following ultrafiltration step. The ground homogenate was filtered through muslin to remove most of the solid residue. After centrifugation of the homogenate at 24,000 rpm for 20 min, the supernatant was filtered through a Whatman 4 filter paper. The resulting solution constituted the crude extract, which was concentrated approximately 10× through a 10,000 Mw cut off ultrafilter (Pellicon-2 PLCGC10, Millipore Corp.). This concentrated solution was dialyzed by ultrafiltration against 25 mM Tris–HCl buffer, pH 7.6, also and used for further purification steps.

3.2.2. Ion-exchange chromatography

This last fraction was directly loaded in a Q-Sepharose Fast Flow pre-column (Amersham) equilibrated with 25 mM Tris–HCl buffer, pH 7.6. After sample application, the enzymes were displaced with a step gra-

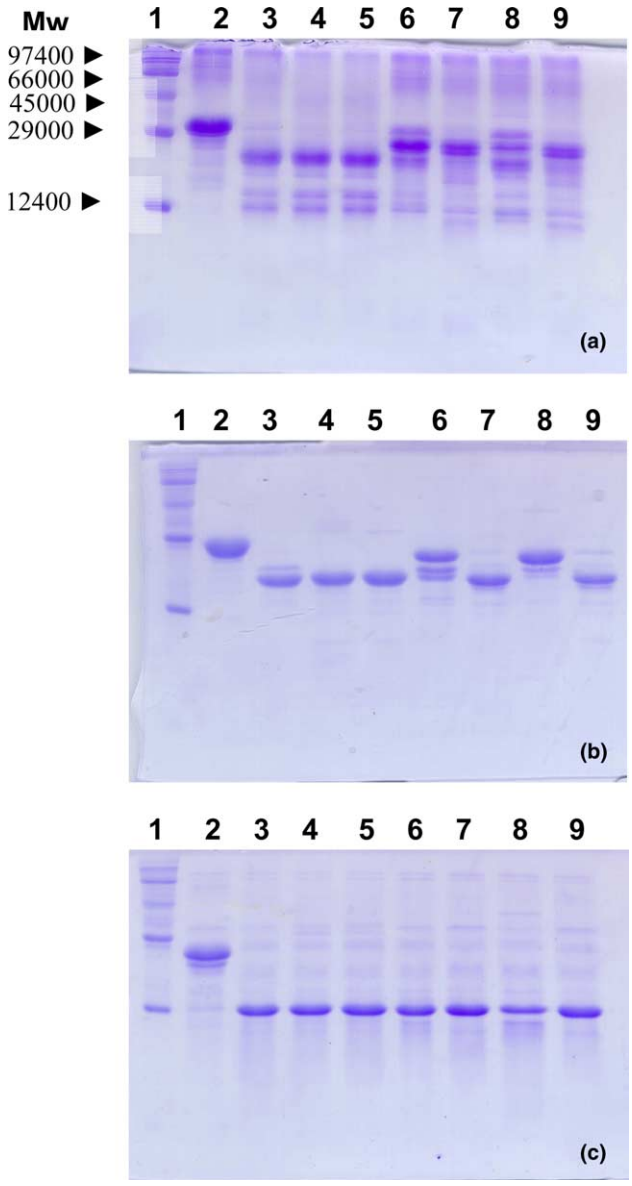

Fig. 7. SDS–PAGE electrophoretogram illustrating the degradation patterns of bovine α- (a), β- (b) and κ-caseins (c) by different coagulants. Lanes 1 and 2 contain protein markers (the same for panels a–c) and the corresponding casein, respectively. Lanes 3–9 correspond to the hydrolysis of caseins by the following coagulants: (3) crude extract of artichoke flowers; (4) dialyzed extract of artichoke flowers; (5) purified cynarase A; (6) calf stomach rennet; (7) microbial rennet; (8) pure chymosin from genetically-engineered yeast; (9) milk-coagulating fungal proteinase. Coagulants (proteinases) were used at catalytic concentrations and, therefore, they did not produce detectable protein bands after Coomassie staining.

dient of 0.5 M NaCl. This collected fraction was dialysed overnight at 4 °C in 25 mM Tris–HCl buffer, pH 7.60 and loaded in a Q-Sepharose Fast Flow column equilibrated in the same buffer. After sample application, the enzymes were displaced with step gradients of 0.30, 0.35 and 0.5 M NaCl in the previous buffer. Fractions of 1 ml were collected and assayed for proteinase activity using azocasein as substrate. The fractions showing proteinase activity were pooled and dialyzed against water, lyophilized and stored at −80 °C until required.

3.3. Electrophoresis

SDS–PAGE was performed in a vertical gel apparatus (Mini-Protean, Bio-Rad) with 15% pre-cast Tris–Gly gel (Bio-Rad), as described by Laemmli (1970). Samples containing approximately 10 μg of protein were applied to the gel and electrophoresed at 200 V for 45 min. Proteins were stained with 0.2% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 45 min. For glycosylation analysis, the proteinases were separated by SDS–PAGE on a 10% polyacrylamide gel and transferred to a nitrocellulose (0.2 μm) membrane by electroblotting in 25 mM Tris/HCl, 192 mM glycine, 0.01% SDS, 10% methanol, pH 8.3, at 70 V, 3 h. The GlycoTrack kit from Oxford Glycosystems was used for specific carbohydrate staining. IEF was performed using the Pharmacia Fast Gel system on pre-poured gels (pH 3–9) with the following standards: trypsinogen (p*I* 9.3), lentil lectin-basic (p*I* 8.65), middle (p*I* 8.45), and acidic (p*I* 8.15), myoglobin-basic (p*I* 7.35), human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), β-lactoglobulin A (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55) and amyloglucosidase (p*I* 3.50). The pIs of the standards were assumed to be as designated by the supplier (Pharmacia). Proteins were stained with 0.2% (w/v) Coomassie brilliant blue in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 45 min.

3.4. M_r determination by gel filtration

A Sephacryl S-75 16/60 Hi-Prep column (Pharmacia) was equilibrated at room temperature with 50 mM phosphate buffer, pH 7.0, containing 0.2 M NaCl. Purified cynarases and calibration proteins (alcohol dehydrogenase [150,000], BSA [66,000], carbonic anhydrase [29,000], and cytochrome *c* [12,400]; 1 mg each; supplied by Sigma) were applied on the column and eluted at a flow rate of 0.5 ml/min. The M_r of native cynarases was estimated from a semilogarithmic plot of the M_r values for the calibration proteins against the elution volume.

3.5. Protein determination

Protein concentrations were determined using the Bio-Rad protein assay procedure with bovine serum albumine as a standard.

3.6. Determination of proteinase activity

Two different methods for proteinase activity were used throughout this study. The first was a modification of the azocasein method used by Bendicho et al. (2002). The second method used the synthetic peptide [H–Pro–Thr–Glu–Phe–*p*-(NO_2)–Phe–Arg–Leu–OH] as substrate of aspartic endopeptidases. Enzyme preparation was incubated at room temperature with 0.2 mM substrate in 50 mM sodium acetate buffer, pH 5.0, and the rate of hydrolysis of nitrosyl product was monitored at 310 nm in a Perkin–Elmer Lammbda 2 UV/Vis spectrophotometer on-line interfaced with a compatible PC for further data analysis. A molar absorption coefficient of 1800 $M^{-1}\,cm^{-1}$ at 310 nm was used in the calculations (Dunn et al., 1984). One unit of the enzyme is defined as the amount of enzyme that hydrolysed 1 mol [H–Pro–Thr–Glu–Phe–*p*-(NO_2)–Phe–Arg–Leu–OH]/min at 25 °C under the above assay conditions.

3.7. Binding to lectins

The partial characterization of the sugar composition of cynarases was attempted by incubation of the purified proteinases with immobilized lectins. The lectin used and the terminal sugars recognized by them are as follow: Con A, αD-mannose; LCA, αD-mannose with fucose at the "fucosylation core" (α1-6-linked fucose near the *N*-glycosidic linkage); WGA, *N*-acetylglucosamine (GlcNAc), sialic acid, and to a lesser extent *N*-acetylneuraminic acid (NeuNAc); RCA-I, D-galactose-β-(1-4)-GlcNAc; and WGA-suc, mainly GlcNAc. Samples of or cynarase A (1 mg/ml) were mixed with lectin-free Sepharose 4B (control) or with immobilized lectins in 10 mM Tris–HCl buffer, pH 7.5, containing 1 M NaCl, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. After overnight incubation at 4 °C, cynarase A-lectin complexes were removed by centrifugation, and the unbound proteinase activity was measured. Proteinase activity in control assays was taken as the 100% value, and the percentage of proteinase binding was calculated from the difference between the activity in the supernatant of the control and lectin experiments.

3.8. pH optimum and pH stability

The enzymes were monitored at different pH values, using the specified buffers in the respective pH range of 3–7. For pH 3.0–5.5, 50 mM sodium acetate buffer; and for pH 5.5–7.0, 50 mM sodium phosphate buffer were used. Suitable controls at the respective pH values were also set up comprising the buffered substrate without enzyme. To test pH stability, the enzymes (0.5 mg/ml) in a volume of 0.1 ml of each specified buffer was incubated for 150 min at room temperature. At the end of the incubation period, aliquots of the incubated enzyme were assayed at pH 5.0 against the synthetic peptide.

3.9. Temperature optimum and temperature stability

The effect of temperature on the activity of cynarase A was studied using a final concentration of 0.2 mM substrate in 50 mM acetate buffer, pH 5.0. The buffered substrate was incubated at specific temperatures of 30–90 °C for 20 min. The enzyme (1.9 nM) was added to the reaction mixture and the assay performed as described above. The buffered substrate without the enzyme served as the control. Thermal stability of cynarase A was tested by incubating the enzyme in distilled water for varying intervals of time at different temperatures (50, 60, 70 and 80 °C). Aliquots of the enzyme were withdrawn at the end of specific time intervals and assayed for activity at room temperature.

3.10. Steady-state kinetics and kinetic data analysis

Steady-state kinetic constants were obtained by triplicate measuring the steady state rates of synthetic peptide substrate hydrolysis at 25 °C in 50 mM sodium acetate buffer, pH 5.0 with 23.5 nM cynarase A. The reciprocal of the variances of the rates were used as weighting factors in the non-linear regression fitting of rates vs. initial susbtrate concentration data to the Michaelis equation (Johnson, 1994). The fitting was carried out by using a Gauss–Newton algorithm (Marquardt, 1963) implement in the Sigma Plot program (SPSS, 2003).

3.11. Inhibition experiments

Cynarase A (26.4 nM) was preincubated with pepstatin dissolved in assay buffer for 30 min at 25 °C. Control containing no inhibitor was treated in the same way. The reaction was started by the addition of the chromogenic peptide [H–Pro–Thr–Glu–Phe–*p*-(NO_2)–Phe–Arg–Leu–OH]. The reciprocal of the variances of the rates were used as weighting factors in the non-linear regression fitting of rates vs. initial susbtrate concentration data to the Morrison equation (Szedlacsek and Duggleby, 1995; Kuzmic et al., 2000). The fitting was carried out by using a Gauss–Newton algorithm (Marquardt, 1963) implement in the Sigma Plot program (SPSS, 2003). Random error propagation was considered in the arithmetic calculations (Mannervik, 1982).

3.12. Caseins digestion

Commercial bovine α_S-, β-, κ-caseins (Sigma, Madrid) were dissolved up to 2 mg/ml in the desired buffer and incubated independently with cynarase A, crude en-

zyme extract or commercial clotting rennet (E/S 1/500 w/w) at 30 °C for 60 min. At selected times aliquots were taken and the reaction stopped by addition of equal volumes of denaturing solution and heated at 100 °C for 5 min. Samples were analyzed by SDS–PAGE according to the method described above.

Acknowledgements

This research was supported by grants from the Ministerio de Ciencia y Technología (Spain), project AGL2002-01255, to F.G.-C. and J.N.R.-L., the Fundación Seneca (Murcia, Spain), project PI-79/00810/FS/01 to J.T. and J.N.R.-L, and the Consejería de Economía, Industria e Innovación (Región de Murcia, Spain), project 2I04SU004 to F.G.-C., J.T. and J.N.R.-L. This research was also partially supported by the company ARTBIOCHEM, S.L. L.S. has a grant from the Ministerio de Educación y Cultura (Spain). L.S. and J.N.R.-L. are grateful to Ms. María Fernanda Montenegro for technical help with sugar analysis.

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