Acta Farm. Bonaerense **16** (1): 37-42 (1997)
Recibido el 3 de diciembre de 1996
Aceptado el 24 de febrero de 1997

Partial Characterization of a Milk Clotting Proteinase isolated from Artichoke *(Cynara Scolymus* L., Asteraceae)

Berta E. LLORENTE [1] *, Cristina B. BRUTTI [1], Claudia L. NATALUCCI [2] y Néstor O. CAFFINI [2]

[1] *Laboratorio de Cultivo de Tejidos Vegetales, Depto. de Cs. Básicas, Universidad Nacional de Luján, CC 221, 6700 Luján, Argentina.*

[2] *LIPROVE, Depto. de Cs. Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata CC 711, 1900 La Plata, Argentina*

SUMMARY. The presence of proteinases in *Cynara scolymus* L. ("artichoke") has been investigated by determining the proteolytic and milk clotting activities of crude extracts of different parts of the inflorescence in various stages of development, as well as of leaves and roots. Although all the preparations showed a certain extent of proteolytic activity, only those of adult leaves, pappus, and immature and mature flowers were able to clot milk. The extract of the upper (violet) part of mature flowers exhibited optimum activity at acid pH values (90% of maximum activity at pH 3.5 - 5.0) which was strongly inhibited by pepstatine A, suggesting the presence of aspartic proteinases. This extract had a low thermal stability at temperatures above 45 °C, which could be a useful property in cheese making process, as it could be quickly inactivated by moderate heating.

RESUMEN. "Caracterización parcial de una proteinasa coagulante de la leche aislada del alcaucil *(Cynara scolymus* L., Asteraceae)". Se ha estudiado la presencia de proteinasas en el alcaucil *(Cynara scolymus* L.) midiendo la actividad proteolítica y la capacidad coagulante de la leche de preparaciones crudas de diferentes partes de la inflorescencia en distintos estadios de desarrollo, así como en raíces y hojas. Si bien se detecta actividad proteolitica en todas las preparaciones, sólo las de hojas adultas, de papus y de flores inmaduras y maduras son capaces de coagular la leche. El extracto de la parte superior (violeta) de las flores maduras exhibe un perfil de pH que es óptimo en la zona ácida (90% de máxima actividad entre pH 3,5 y 5,0), pero la actividad es fuertemente inhibida por pepstatina A, lo que sugeriría la presencia de una o más proteinasas aspárticas. La estabilidad térmica del extracto es baja a temperaturas superiores a 45 °C, circunstancia que puede resultar de utilidad en la producción de quesos, ya que la enzima puede ser inactivada a temperaturas moderadas.

INTRODUCTION

Proteolytic enzymes are widely employed in food industry for cheese and beer manufacture, tenderization of meat, bread manufacture, production of emulsifiers, and other uses [1]. The coagulant most widely used for cheese making is ani-

KEW WORDS: Artichoke, Asteraceae, *Cynara scolymus*, Milk clotting, Proteinase
PALABRAS CLAVE: Alcaucil, Asteraceae, *Cynara scolymus*, Coagulante de la leche, Proteinasa

* Corresponding author

mal rennet, extracted from the abdominal mass of young ruminants. However, in recent years the use of enzymes of the group of acid proteinases from microorganisms has become fairly common, for economic, religious or cultural reasons. Still more recently, chymosin (the main proteinase of animal rennet) has been produced by microorganisms using recombinant DNA techniques [2]. Shortage of animal rennets, world-wide increase in cheese consumption and continuous growth of the vegetarian market encouraged researches on proteases from plants sources that could serve as functional substitutes for chymosin [1,3,4].

The flowers of cardoon *(Cynara cardunculus* L.) are traditionally used in the Mediterranean region for cheese making [4-7]. Different species are described for the genus *Cynara,* but only *C. cardunculus* L. is referred to be used in cheese making [8]. The present paper deals with the presence of milk clotting activity in different organs of the artichoke *(Cynara scolymus* L., Asteraceae).

MATERIALS AND METHODS

Plant material

Plants of *Cynara scolymus* c.v. Green Globe in different stages of development were collected in Nogoyá, Entre Ríos Province, Argentina, since October to December. They were scrupulously cleaned with tap water and divided into different parts: roots, young and adult leaves, midribs, inflorescence stems and leaves, immature flowers, receptacles, pappus, and upper part of mature (violet) flowers.

Crude protein extract

Crude extracts of the different tissues were obtained by homogenising fresh tissue (10 g) in a blender with cold (-20 °C) acetone (30 ml) in order to obtain an acetone powder [9]. One g of this powder was extracted at 4 °C for 60 min with 50 ml of 0.1 M potassium phosphate buffer (pH 6.0) containing 5 mM EDTA and 5 mM cysteine, with gentle stirring. The suspension was centrifuged at 16000 x g for 30 min at 4 °C and the precipitate was discarded. The supernatant was collected and immediately frozen at -20 °C until its analysis.

Protein content

Protein concentration was determined according to Bradford [10]. Bovine serum albumin was used as standard.

Proteolytic activity

Casein

Proteolytic activity was determined using 0.1 ml de enzyme solution and 1.1 ml of 1% (w/v) casein solution (1 g casein was treated with 1.5 ml of 0.1 M NaOH and then 100 ml of 0.1 M potassium phosphate buffer was added; the resulting suspension was boiled for 20 min, filtered, adjusted to pH 6.0 if necessary and brought to 100 ml with distilled water). The reaction mixture was incubated in a water-bath at 37 °C for 30 min. The reaction was stopped by addition of 1.8 ml of 5% (w/v) trichloroacetic acid. Blanks were prepared by combining the trichloroacetic acid with the enzyme, then adding the substrate. The tubes were centrifuged at 4000 x g for 20 min and the absorbance of supernatants was mea-

sured at 280 nm. An arbitrary enzyme unit (U_{cas}) was defined as the amount of enzyme that produces an increase of one absorbance unit per minute in the assay conditions.

Haemoglobin

When bovine haemoglobin was used, this substrate was prepared by a modification of the Anson procedure [11]. The reaction mixture contained 250 µl of a dilution of the enzyme in a suitable buffer and 1.25 ml of haemoglobin solution. The reaction was carried out at 37 °C for 10 min and stopped by the addition of 2.5 ml of 5 % (w/v) trichloroacetic acid. The mixtures were centrifuged at 4000 x g for 20 min and the absorbance of supernatants was measured at 280 nm. One unit of enzyme activity was defined as the amount of enzyme required to cause an unit increase in absorbance at 280 nm per minute, under the assay conditions.

Azocasein

Azocasein was the substrate employed for inhibition assays with 1,10-phenanthroline, as this inhibitor of metallo-proteases shows high absorbance at 280 nm.. The substrate was prepared by a modified procedure [12] of the Charney & Tomarelli's technique [13]. The reaction mixture containing 250 µl of 2% azocasein in 0,1 M Tris-HCl (pH 8,0) and 150 µl of the enzyme extract was incubated at 37 °C. The reaction was stopped by the addition of 1.2 ml of 10 % trichloroacetic acid, the mixture was lain down for 15 min and centrifuged at 8000 x g for 3 min. Then, 1.2 ml of the supernatant was mixed with 1.4 ml of 1 M NaOH and the absorbance at 440 nm was measured. One unit of enzyme activity was defined as the amount of enzyme required to cause an unit increase in absorbance at 440 nm per minute, under the assay conditions.

Milk clotting activity

Crude extract (0.5 ml) was added to 3 ml of 10 % (w/v) solution of cow skimmed milk powder in 0.01 M calcium chloride. Clotting activity was determined at 37 °C. In order to better visualize curd formation, a narrower tube filled with dilute blue ink was inserted into the test tube. Clotting time was determined by direct observation of flocculation and gel formation against the blue-dye tube. Controls were prepared by adding extraction buffer to milk solution.

Effect of inhibitors

The effect of inhibitors on proteolytic activity was determined by preincubating the protease preparation with the inhibitor at 37 °C for 30 min and the residual activity estimated on casein or azocasein at pH 6.0. Cystein (5 mM), E-64 (10 (M), pepstatine A (1 (M), 1,10-phenanthroline (10 mM), and phenylmethylsulfonyl fluoride (1 mM) were assayed. Controls were prepared by pre-incubating the protease preparation with the appropriate solvent used to dissolve the inhibitors and activators.

Effect of pH on enzyme activity

Proteolytic activity of crude preparations from the upper part of mature (vio-

let) flowers was measured on bovine haemoglobin using 0.1M sodium citrate-citric acid (pH: 2.7-5.5), 0.1 M potassium phosphate (pH: 6.0-8.0), and 0.1 M boric acid-potassium chloride-sodium hydroxide (pH: 8.0-10.0) buffers.

Thermal stability

Thermal behavior of crude preparations of the upper part of mature (violet) flowers was determined by keeping enzyme solutions for 5, 10, 20, 40, 60, 90, 120, and 180 min at 37 °C, 45 °C, 55 °C, and 65 °C, respectively, and then measuring the residual activity as indicated above using casein as substrate.

RESULTS AND DISCUSSION

Studies on the expression of proteinases in C. scolymus have been carried out by studying the proteolytic and milk clotting activities. Crude extracts of different parts of the inflorescence in various stages of development, as well as of leaves and roots, have been used in these studies. The results obtained are summarised in Table 1.

Part of the plant assayed	Protein ($\mu g\ ml^{-1}$)	Proteolytic Activity ($U_{cas}\ ml^{-1}$)	Clotting time (min)
Roots	96	0.040	NC
Young leaves	151	0.170	NC
Adult leaves	128	0.440	180
Midribs	51	0.060	NC
Receptacles	110	0.210	NC
Immature flowers	455	0.660	90
Inflorescence stems	125	0.230	NC
Inflorescense leaves	57	0.080	NC
Pappus	302	1.480	26
Upper part (violet) of mature flowers	801	5.670	8

Table 1. Protein content, proteolytic activity and clotting time of crude extracts obtained from different parts of *Cynara scolymus* L.

Activity was measured at pH 6.0, with casein as substrate. Data are means of five determinations and each experiment was repeated twice. NC: no clotting activity observed during the assay time (300 min).

The protein content of the different extracts varied from 51 to 801 $\mu g\ ml^{-1}$. Roots, young leaves, midribs, receptacles, and inflorescence stems and leaves showed no significant caseinolytic activity. The highest relative activities were obtained in the upper part (violet) of mature flowers (100%), pappus (26%), immature flowers (11.6%), and adult leaves (7.8%).

Extracts from the upper part of mature (violet) flowers, pappus, immature flowers, and adult leaves clot milk at different rates. No milk clotting activity could be detected in roots, young leaves, midribs, receptacles and inflorescence stems and leaves within the assay time (300 min). Maximum activity was observed in the upper part (violet) of mature flowers, which may suggest an involvement in the senescence process, just as was indicated for *C. cardunculus* L. Conversely to the behavior observed in *C. scolymus,* leave extracts of *C. cardunculus* do not show milk clotting activity even after 480 min [8].

It was found that proteolytic activity was inhibited by pepstatine A (aspartic proteinases inhibitor) in samples proceeding from the upper part (violet) of mature flowers, immature flowers, pappus, and adult leaves, that is, samples that produce milk clotting. The addition of cystein (5 mM) does not modify the caseinolytic activity of these crude preparations, with the exception of the crude extract of adult leaves, which shows a moderate activity increase. On the other hand, cystein produces activity increase in crude extracts from roots, young leaves, midribs, inflorescence stems, receptacles, and inflorescence leaves. As the addition of E-64 (cysteine proteinases inhibitor) provoked partial inactivation in most samples, the possibility that cystein-proteinases could be present is under investigation. Phenylme-thylsulfonyl fluoride and 1,10-phenanthroline assays gave negative results, discarding the presence of serine- or metalloproteases (data not shown).

As in the case of aspartic proteinases, maximum activity of crude preparations of the uppper part (violet) of mature flowers was reached at acidic pH, (more than 90% between pH 3.5 and 5.0) using bovine haemoglobin as substrate (Figure 1).

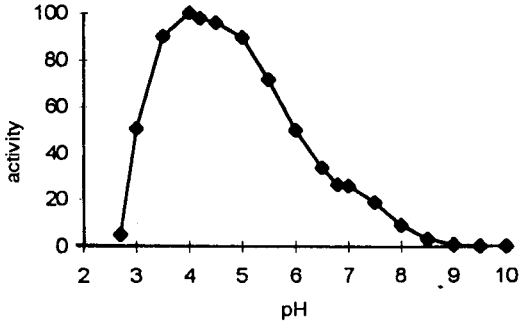

Figure 1. Effect of pH on the activity of crude preparations of violet parts of the flowers.

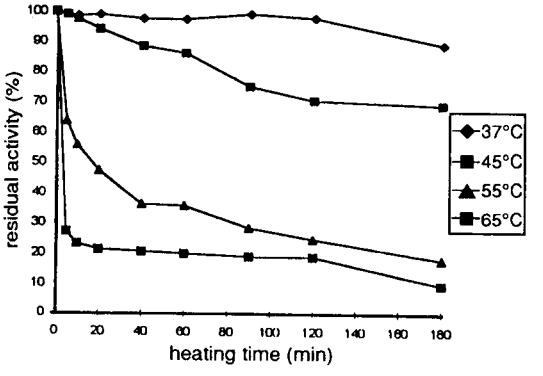

Figure 2. Thermal stability of violet part of the flowers.

Thermal stability of these crude preparations is shown in Figure 2. 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, but notably decreased at higher temperatures. Nevertheless, thermal behavior of the enzyme is a useful property in cheese making process, as it can be quickly inactivated by moderate heating.

Simultaneous studies on the purification of the aspartic proteinase as well as on its expression in tissue cultures is being achieved in our laboratories.

CONCLUSIONS

The presence of proteinases in *Cynara scolymus L.* ("artichoke") has been investigated by determining the proteolytic and milk clotting activities of crude extracts of different parts of the plant, as well as the effect on proteolytic activity of cysteine and different protease inhibitors. Higher activities were shown by extracts obtained from the upper part (violet) of mature flowers, pappus, immature flowers, and adult leaves, in this order. As evidenced by the inhibition assays, proteolytic activity is principally owed to aspartic proteinases, but the expression of cysteine proteinases (even in reproductive organs) should not be discarded.

The extract of the upper (violet) part of mature flowers exhibited optimum activity at acid pH values (90% of maximum activity at pH 3.5-5.0) which was strongly inhibited by pepstatine A, suggesting the presence of one or more aspartic proteinases. This extract had a low thermal stability above 45 °C, which could be a useful property in cheese making process, as it could be quickly inactivated by the use of moderate heating.

Acknowledgements. C.L. Natalucci and N.O. Caffini belong to the CIC Researcher Career. The present work has been supported by grants of CIC, Universidad Nacional de La Plata and Universidad Nacional de Luján. Authors wish thank to Marcelo Pardo and Cecilia Cimino for technical assistance.

REFERENCES

1. Padmanabhan, S., A. Chitre & N. Shastri (1993) *Die Nahrung* **37**: 99-101
2. Dalgleish, D.G. (1992) "The enzymatic coagulation of milk", in *Advanced Dairy Chemistry - 1: Proteins* (P.F. Fox, ed.). Elsevier Applied Science Publishers, London and New York. p. 581
3. Veríssimo P, C. Esteves, C. Faro & E. Pires (1995) *Biotechnology Letters* **17**: 621-6
4. Veríssimo P, C. Faro, A. Moir, Y. Lin, J. Tang & E. Pires (1996) *Eur. J. Biochem.* **235**: 762-8
5. Heimgartner, U., M. Pietrzak, R. Geertsen, P. Brodelius, A.C. Da Silva Figuereido & M.S.S Pais (1990) *Phytochemistry* **29**: 1405-10
6. Cordeiro, M.C., E. Jakob, Z. Puhan. M.S. Pais & P.E. Brodelius (1992) *Milchwissenschaft* **47**: 681-7
7. Cordeiro, M.C., Z.T. Xue, M. Pietrzak, M.S. Pais & P.E. Brodelius (1994) *Plant Mol. Biol.* **24**: 733-41
8. Cordeiro, M.C., M.S. Pais & P.E. Brodelius (1994) *Physiol. Plant.* **92**: 645-53
9. Priolo, N.S., L.M.I. López, M.C. Arribére, C.L. Natalucci & N.O. Caffini (1991) *Acta Alimentaria* **20**: 189-96
10. Bradford, M.M. (1976) *Anal. Biochem.* **72**: 248-54
11. Sarath, G., R.S. de la Motte & F.W. Wagner (1989) "Protease assay methods" in *Proteolytic Enzymes* (R.J. Beynon & J.S. Bond, eds.). IRL Press, Oxford, p. 27
12. López, L.M.I. (1995) "Aislamiento, purificación y caracterización de las proteasas presentes en el látex de frutos de *Maclura pomifera* (Raf.) Schneid. *(Moraceae)*". Ph.D. Thesis. Fac. Ciencias Exactas, Univ. Nac. La Plata, Argentina
13. Charney, J. & R.M. Tomarelli (1947) *J. Biol. Chem.* **171**: 501-5