BIOTECHNOLOGY LETTERS
Volume 17 No.6 (June 1995) pp.621-626
Received as revised 3rd May

THE VEGETABLE RENNET OF *CYNARA CARDUNCULUS* L. CONTAINS TWO PROTEINASES WITH CHYMOSIN AND PEPSIN-LIKE SPECIFICITIES

Paula Veríssimo, Cristina Esteves, Carlos Faro* and Euclides Pires

Departamento Bioquímica, Faculdade de Ciências e Tecnologia, Universidade de Coimbra. Apartado 3126, 3000 Coimbra, Portugal.

ABSTRACT

The flowers of cardoon (genus *Cynara*) are traditionally used in Portugal for cheese making. In this work the vegetable rennet of the species *Cynara cardunculus* L. was characterized in terms of enzymic composition and proteolytic specificity of its proteinases (cardosin A and cardosin B). Cardosin A was found to cleave insulin B chain at the bonds Leu15-Tyr16, Leu17-Val18 and Phe25-Tyr26. In addition to the bonds mentioned cardosin B cleaves also Glu13-Ala14, Ala14-Leu15 and Phe24-Phe25 indicating that it has a broader specificity. The kinetic parameters for the hydrolysis of the synthetic peptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe were also determined and compared to those of chymosin and pepsin. The results obtained indicate that in terms of specificity and kinetic parameters cardosin A is similar to chymosin whereas cardosin B is similar to pepsin. It appears therefore that the enzyme composition of cardoon rennet closely resembles that of calf rennet.

INTRODUCTION

The coagulant most widely used for cheese making is animal rennet extracted from the abdomasa of young ruminants (Fox,1987). This coagulant contains chymosin as the main enzyme component and pepsin which being a minor component usually accounts for a high proportion of the total proteolytic activity (Foltman, 1984). Worldwide increase in cheese production led to a shortage of animal rennet and for a long period there was an active search for rennet substitutes. With the advent of recombinant DNA technology, the production of chymosin for cheese making is no longer a problem (O'Sullivan and Fox, 1991; Harboe,1992).

Anyway the search for rennet substitutes from plant sources has gain a new interest due to the continuous growth of the vegetarian market.

Milk coagulants from plants are rarely used for cheese making. The most successful one is an enzyme preparation from the flowers of cardoon which have been used in Portugal since ancient times in the manufacture of traditional cheeses. An acid proteinase was first isolated from commercially available dried flowers (Faro *et al*,1987) and shown to promote milk clotting by cleavage of the sensitive bond Phe105-Met106 in κ-casein (Faro *et al*,1992; Macedo *et al*,1993). This enzyme preparation was further shown to be heterogeneous and several active forms were identified (Heimgartner *et al* 1990). Recent studies, however, showed the presence of two proteinases with different amino acid sequences in fresh flowers of *Cynara cardunculus* L., which are probably the products of two different genes, and were named cardosin A and cardosin B(Faro *et al*,1995).

In this work the vegetable rennet from the flowers of *Cynara cardunculus* L. is characterized in terms of enzyme composition and proteolytic specificity of the two proteinases.

MATERIAL AND METHODS

Material: Fresh flowers of *Cynara cardunculus* L. were collected from plants grown from seeds supplied by Botanic Garden of the University of Coimbra. The peptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe and oxidised insulin B chain were purchased from Sigma, USA.

Enzyme purification: Styles (0.2 gr) from fresh flowers of *Cynara cardunculus* L. were ground in a mortar and pestle under liquid nitrogen. The ground tissue was then homogenized in 1 ml 0.1M citric acid pH 3.0 and centrifuged at 12000g for 10 minutes. The supernatant (0.2ml) was applied to a HiLoad Superdex 200 column equilibrated and eluted with 25 mM Tris-Cl pH 7.6 (buffer A) at a flow rate of 1.0 ml/min. Each peak of absorbance was collected as a fraction and assayed for activity. The active fraction was applied to a Mono Q HR 5/5 column also equilibrated in buffer A. The protein was eluted with a linear gradient of NaCl (0-0.5M) in buffer A at a flow rate of 0.75 ml/min and the protein peaks were collected and assayed for activity.

Polyacrylamide gel electrophoresis: SDS-PAGE was performed in a Pharmacia PhastSystem using 20% homogeneous gels as described in the manufacture manual.

Cleavage of oxidised insulin B chain: Oxidised insulin B chain (5 mg/ml) was incubated with each cardosin (ratio enzyme/substrate, 1:500) in 0.1M formic acid adjusted to pH 3.1 with NaOH. After 3h at 37°C, the reaction mixture was centrifuged and the peptide fragments were separated by reversed-phase HPLC using a Vydac C18 column. The chromatography was carried out at room temperature and the column was equilibrated with 0.1% TFA. The peptides were eluted with a linear gradient of acetonitrile (0-80%) in 0.1% TFA at a flow rate of

1.5 ml/min. The isolated peptides were then characterized by amino acid composition and N-terminal amino acid sequencing.

Sequence analysis: N-terminal amino acid sequences were determined by Edman degradation using an Applied Biosystems 473-A sequencer.

Kinetic studies: The kinetic parameters for the hydrolysis of the synthetic peptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe were determined according to Martin (1984) . Enzyme preparations were incubated at 37°C with the substrate in 50 mM sodium acetate pH 4.7, 0.2M NaCl, 4% DMSO and the rate of hydrolysis of the bond Phe(NO2)-Nle for different substrate concentrations was monitored at 310nm in Perkin Elmer Lambda 2 UV/Vis spectrophotometer using the operating software. The hydrolysis of the synthetic peptide used at Phe(NO_2)-Nle bond was confirmed by reversed-phase HPLC. The enzyme concentration was determined by active site titration with pepstatin A. The kinetics parameters were calculated graphically from the plots of 1/initial velocity vs 1/substrate concentration.

RESULTS AND DISCUSSION

In order to estimate the relative amount of each cardosin in the milk curdling enzyme preparation of *Cynara cardunculus* L., an acidic extract obtained from fresh flowers of this cardoon was analysed by ion-exchange chromatography. Although the acidic extraction yields an enzyme preparation free of protein contaminants, there is a need to remove non-protein contaminants before the ion-exchange step. For analytical purposes this was achieved by gel filtration on Superose 6 which is quicker and requires less starting material than Superdex 200. The chromatographic pattern on Mono Q is shown in fig.1. As shown elsewhere the first peak (cardosin A_0) was inactive under the assay conditions used. SDS-PAGE of the two active peaks revealed that the material of the second peak migrated as two bands with molecular masses of 31 kDa and 15 KDa whereas the third peak produced two bands with molecular masses of 34 kDa and 14.4 KDa respectively. These proteinases were therefore recognized as cardosin A and cardosin B. The relative amount of cardosin B was determined by integration of the peak areas and for this variety of cardoon was about 25 % of the total protein. However the proportion of this proteinase varies between the types of cardoon, ranging from 0 to about 25%; *Cynara humilis* L. which is also used for cheese making contains only cardosin A and often the commercially available preparations of cardoon are obtained essentially from the flowers of this species.

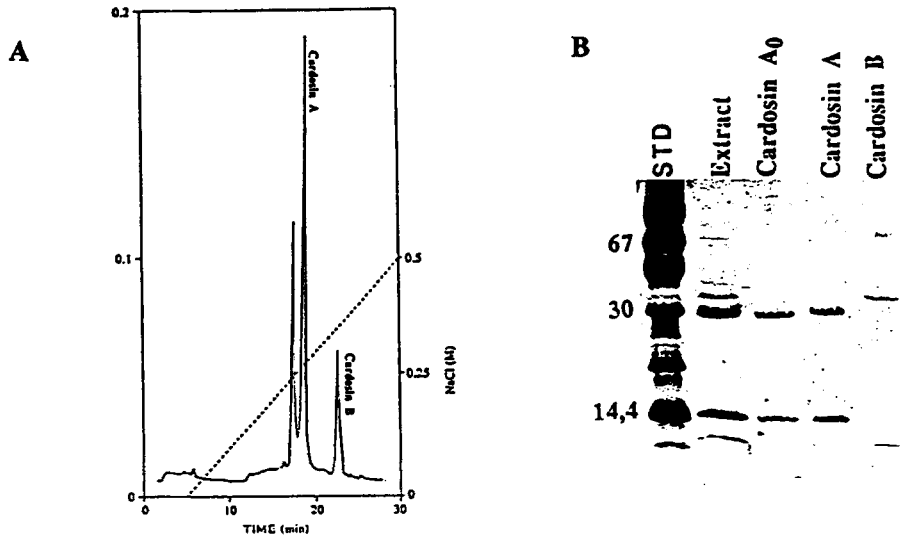

Fig. 1. **A.** Ion-exchange chromatography on Mono Q of the *Cynara cardunculus* L. rennet previously purified by gel filtration on Superose 6. **B.** SDS-PAGE analysis on a Phastgel homogeneous 20 of the enzyme components of the *Cynara cardunculus* L. rennet

The proteolytic specificity of the cardosins was investigated using oxidised insulin B chain as substrate. This peptide was incubated with each cardosin and the peptide fragments were then separated by reversed phase HPLC (fig.2). The analysis of the amino acid composition and amino acid sequencing of the individual peptides provides the identification of the cleavage sites by reconstruction of the complete sequence of insulin B chain. Cardosin A hydrolysed this peptide at bond Leu15-Tyr16, Leu17-Val18 and Phe25-Tyr26. Cleavage by cardosin B was found to occur at Glu13-Ala14, ala14-Leu15, Leu15-Tyr16, Leu17-Val18, Phe24-Phe25 and Phe25-Tyr26. The additional bonds hydrolysed by cardosin B represent genuine differences in specificity as longer incubation of insulin B chain with cardosin A did not generate additional peptides. These results suggest that cardosin B has a broader specificity than cardosin A although they share the same preference for peptide bonds with hydrophobic side chains. In a previous work the specificity of the acid proteinase from the flowers of cardoon was reported (Faro et al,1992). The bonds which were cleaved by the so called therein acid protease from the flowers of cardoon, are basically the same described in the present work for cardosin A. Since those authors had used commercial dried flowers is very likely that the material contained mainly flowers from Cynara humilis L. in which cardosin B was found to be absent (results not shown).

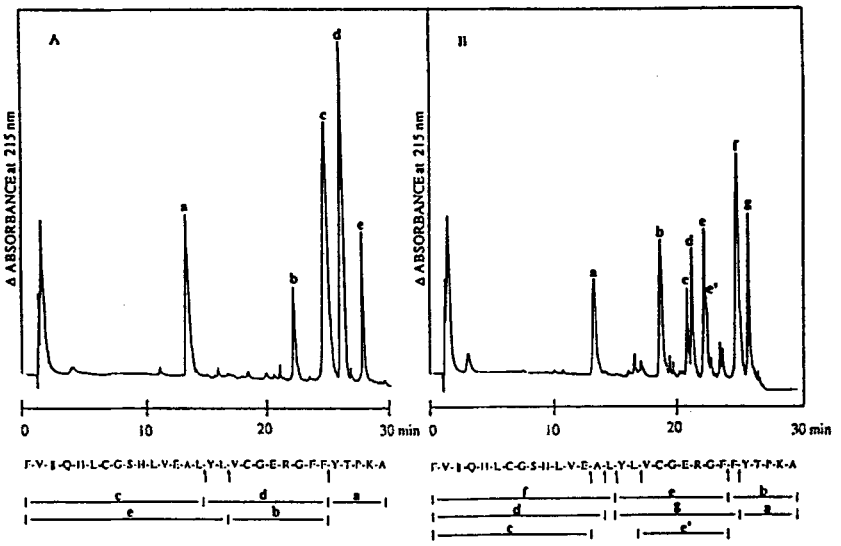

Fig.2. Cleavage of oxidised insulin B chain by cardosin A (panel A) and cardosin B (panel B). Oxidised insulin B chain was digested by each cardosin (250:1) at pH 3.1(see material and methods). The insulin fragments were isolated by reversed phase HPLC (chromatograms shown) and characterized by amino acid sequencing. The cleavage sites were identified by matching the sequences of the peptide fragments

Synthetic peptides have been used to characterize rennet preparations (Martin et al,1981). Among them the hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe was proposed and has been used by several authors for studying the proteolytic activity of milk-clotting enzymes. The kinetic parameters values obtained for the hydrolysis of this peptide by each cardosin are shown in Table I. It is apparent that the Kcat/km value for cardosin B is in the same range as that determined for pepsin, being both higher than the ones obtained for chymosin and cardosin A.

Table I- Kinetic parameters for the hydrolysis of the synthetic peptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe by cardosin A and B- comparison to chymosin and pepsin. The values for chymosin and pepsin are those reported by Martin (1984).

Enzyme	Km (mM)	Kcat (s^{-1})	Kcat/Km (mM^{-1} s^{-1})
Cardosin A	1.08	23.6	21.87
Chymosin	0.98	25.1	25.6
Cardosin B	0.08	85.5	1057
Pepsin	0.03	54	1640

Taken together the results above described indicate that in terms of specificity and kinetic parameters, cardosin A is similar to chymosin whereas cardosin B is similar to pepsin. In these aspects the vegetable rennet of *Cynara cardunculus* L. closely resembles calf rennet. Since these studies were carried out with soluble substrates, studies involving actual cheese making and maturation are required to establishe whether a cardoon rennet containning an appropriate ratio between cardosin A and cardosin B can be used as a suitable substitute for animal rennet. In addition, the results clearly showed that the enzymic composition of milk curdling enzyme preparations from different cardoon species can be analysed by the column chromatography procedure herein described followed by SDS-PAGE or eventually by measuring the specific activity towards the hexapeptide Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe. This approach is a straighforward method for routine analysis of cardoon rennets.

AKNOWLEDGEMENTS

This work was supported by JNICT cont. nº PMCT/C/BIO/909/90 and INIC. Paula Veríssimo and Cristina Esteves are the recipients of JNICT fellowships.

REFERENCES

Faro, C.J., Alface, J.S and Pires, E.V.(1987) *Cienc. Biol.* **12**,5A: 201

Faro, C.J., Moir, A.J.G. and Pires, E.V. (1992) *Biotech Lett* **14**: 841-846.

Faro, C.J., Veríssimo, P.C., Lin, Y., Tang, J. and Pires, E.V. (1995) in: "Aspartic proteinases: structure, function, biology and biomedical implications" K. Takahashi. (ed.) Plenum Press New York pg 373-377.

Fox,P.(1987) in "Cheese: chemistry, physics and microbiology" Vol. 1 P.F.Fox.(ed.) Elsevier Applied Science Publishers. London. pg 1-32.

Harboe M.K. (1992) *Bullet. Intern. Dairy Fed.* **269**:3-7

Heimgartner,U., Pietrzak, M., Geertsen, R., Brodelius, P., Figueiredo, A.C. and Pais, M.S.(1990) *Phytochemistry* **29**: 1405-1410.

Macedo, I.Q., Faro,C.J. and Pires,E.V.(1993) *J.Agric. Food Chem.* **41**. 1537-1541

Martin, P. (1984) *Biochem. Biophys. Acta* **791**: 28-36

Martin, P., Collin, J-C, Garnot, P., Dumas, B.R. and Mocquot, G. (1981) *J. Dairy Research* **48**. 447-456.

O'Sullivan, M. and Fox, P.F.(1991) *Food Biotechnology* **5**:19-32.