Journal of Dairy Research (2005) **72** 271–275. © *Proprietors of Journal of Dairy Research 2005*
doi:10.1017/S0022029905000749 *Printed in the United Kingdom*

Extraction and partial characterization of a coagulant preparation from *Silybum marianum* flowers. Its action on bovine caseinate

Sandra Vairo-Cavalli, Santiago Claver, Nora Priolo* and Claudia Natalucci

LIPROVE, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, C.C. 711, B1900AVW, La Plata, Argentina

Received 16 December 2003 and accepted for publication 11 November 2004

Keywords: Aspartic proteolytic activity, casein hydrolysis, milk clotting, rennet substitute, *Sylibum marianum*.

An outstanding parameter in cheese making is the type of coagulant, which greatly influences the characteristics of the final products. Proteolysis is the most important set of biochemical changes during ripening of most cheeses, and is carried out, in different magnitude, by proteolytic agents originated in milk, rennet (or rennet substitute), and starter and non-starter micro-organisms (Silva & Malcata, 2000). The demand for alternative sources of milk coagulants, to replace the expensive and limited natural rennet supplies, has increased (Esteves et al. 2001). All commercial enzymes employed as milk coagulant are aspartic proteinases, which are most active at acidic pH and preferentially cleave peptide bonds between residues with hydrophobic side-chains (Silva & Malcata, 1999).

Because of the presence of aspartic proteinases, aqueous crude extracts from flowers of *Cynara cardunculus* (Veríssimo et al. 1995, 1996), *Cynara humilis*, and/or *Cynara scolymus* are traditionally employed in the Iberian Peninsula as vegetable rennet for cheesemaking (Reis et al. 2000). Milk clotting activity was also proved in flowers of *Centaurea calcitrapa* and *Onopordum turcicum* (Tamer, 1993; Domingos et al. 1998). All these species are included within the *Asteraceae* family and furthermore in the same tribe: *Cardueae* Cass. = *Cynareae* Less. (Ariza Espinar & Delucchi, 1998).

When a potential rennet substitute is studied, it is particularly important to evaluate adequately the degradation patterns of the caseins because of their effects on yield, consistency, and flavour of the final cheese (Fox, 1989). It is important to guarantee a well-balanced breakdown of curd proteins (caseins) in order to avoid formation of undesired attributes in cheese such as low viscosity and high bitterness (Visser, 1993).

One of the most frequently used methods to monitor proteolytic processes on caseins is urea-polyacrylamide gel electrophoresis. On the other hand, tricine-SDS polyacrylamide gel electrophoresis improves the separation, identification and quantification of casein hydrolysates because it allows the visualization of large and small peptides (Pardo & Natalucci, 2001), with the additional advantage of allowing the estimation of molecular masses. Both methods are then suitable to characterize the performance of vegetable rennet in different ways.

This preliminary study had the following objectives: the partial characterization of (i) the aspartic proteolytic activity present in flowers of *Silybum marianum* (L.) Gaertn. (*Asteraceae*); and (ii) the hydrolytic profile of bovine caseins.

Materials and Methods

Enzyme extract preparation

Fresh flowers of *Silybum marianum* (L.) Gaertn. were ground in a mortar under liquid nitrogen, homogenised in 0·1 M-citric acid-0·1 M-sodium citrate buffer (pH 3·0) containing 1·0 mM-EDTA (1 g per 3 ml), stirred for 30 min and centrifuged at 5000 ***g*** for 20 min at 4 °C. Ten ml of supernatant was applied to a Pharmacia K 15/30 column packed with Sephadex G-25 Fine (Amersham Biosciences, SE-751 84 Uppsala, Sweden) equilibrated with 50 mM-citric acid-50 mM-sodium citrate buffer (pH 3·0). Elution was performed with the same buffer at 0·45 ml/min flow rate and the enzyme extract (EE) was obtained.

Protein content was measured following the method of Bradford (1976), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO 63178, USA) as standard.

Milk clotting activity

Milk clotting activity (MCA) was measured following the procedure described by the International Dairy Federation, 1992. Skim milk powder, (San Regim, SanCor, Sunchales, 2322, Argentina) was reconstituted by dissolving 12 g in 100 ml of 10 mM-$CaCl_2$ (pH 6·5). EE (100 µl) was added to 1 ml milk at 30 °C and the clotting time was measured. One rennet unit (RU) is the amount of enzyme that coagulates 10 ml milk at 30 °C in 100 s (Barros et al. 2001).

*For correspondence; e-mail: priolo@biol.unlp.edu.ar

Stability at 37 °C of MCA was tested by incubation of EE in a thermostatized chamber for 0, 14, 30, and 60 min. The reaction was stopped at 0 °C and the residual MCA was measured as described above.

MCA was measured with increasing quantities of NaCl (0·0–1·0 M) to determine the effect of salt.

The effect of $CaCl_2$ on MCA was determined on 120 g skim milk powder/l reconstituted with increasing concentration of calcium chloride solution (0·0–0·1 M).

The EE was preincubated for 30 min at 37 °C in the presence of the following inhibitors: 1 mM PMSF (phenyl methyl sulphonyl fluoride), 0·1 mM E-64 (*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane), and 100, 50, 10 and 2 μM pepstatin A, in separate aliquots. The remaining MCA was determined. A control assay was done without inhibitors and the resulting activity was taken as 100%. The inhibitors were purchased from Sigma Chemical Co.

Proteolytic activity

Denatured haemoglobin (Hb) was prepared by stirring 20 ml of a 25 g Hb/l (Sigma) suspension with 2·5 ml 0·6 M-HCl during 30 min. The pH was adjusted to 4·0 with 0·5 M-NaOH and final concentration was 20 g/l. The solution was centrifuged to eliminate any precipitate. The reaction mixture was prepared by mixing 0·1 ml of the EE with 0·5 ml Hb. The reaction was carried out at 37 °C and stopped with 1 ml 50 g trichloroacetic acid/l (TCA). Initial velocities were taken. Test tubes were centrifuged at 5000 ***g*** for 20 min and the absorbance of the supernatant was measured at 280 nm (Anson, 1938). Blanks were made by addition of TCA prior to the EE. One unit of enzyme activity (EA) was defined as the amount of enzyme required to cause an increase of one unit in absorbance at 280 nm per minute, under the assay conditions.

The effect of pH on the proteolytic activity was measured at different pH values.

Enzymatic caseins hydrolysis

Whole bovine casein was prepared via isoelectric precipitation of bovine skim milk and freeze-dried (Souza & Malcata, 1998). The caseinate was dissolved to a final concentration of 10 g/l in 0·1 mM-monobasic sodium phosphate-0·1 M-dibasic sodium phosphate buffer (pH 6·5) containing 1 g NaN_3/l to prevent protein degradation by adventitious microflora, and stabilized at 30 °C. The reaction was started by addition of 450 μl of the caseinate to 45 μl of the EE (44 ± 4 mg protein/l). The reaction was quenched at different times by addition of 500 μl TCA (50 g/l). The samples were left to precipitate overnight at 4 °C and then centrifuged at 10 000 ***g***. The precipitates were redissolved in 450 μl of two different sample buffers: (a) for urea-PAGE, 0·062 M-Tris-0·1 M-HCl, pH 7·6, 8 M-urea, 2 ml 2-mercaptoethanol/l and 0·02 g bromophenol blue/l, the pH was adjusted with 1 M-NaOH and then samples were vortexed four times for 30 s; (b) for tricine-SDS-PAGE, 0·0625 M-Tris-0·1 M-HCl pH 6·8, 20 g SDS/l, 5 ml 2-mercaptoethanol/l, 0·02 g bromophenol blue/l and 100 g glycerol/l, the pH was adjusted with 1 M-NaOH and heated at 100 °C for 5 min.

Controls containing sodium caseinate and sodium azide at the same concentrations without addition of enzyme were also sampled.

Hydrolitic profile

Electrophoresis was performed using a Mini Protean III cell (Bio-Rad). Urea-PAGE was performed according to Shalabi & Fox (1987). The gels were pre-run at 50 mA for 10 min. After sample loaded, the power supplied was set at 100 V at the stacking gel and then increased to 200 V at the resolution gel. Tricine-SDS-PAGE was done following the method of Schägger & von Jagow (1987). This electrophoresis was performed at 30 V in the stacking gel, then it was increased 15 V per min for 4 times, finally it was maintained constant (90–100 V). Gels were stained with Coomassie Blue G-250 (Bio-Rad Bulletin, 2003). Quantification of intact caseins and polypeptides was done by gel scanning and densitograms were performed with the software Scion Image Beta 4·02 for Windows (Scion Corporation, Frederick, Maryland 21701, USA).

Results and discussion

Proteins with milk clotting activity were obtained with buffer at pH 3·0 from fresh flowers of *S. marianum*. Gel filtration of crude extract on Sephadex G-25 was needed to remove lower molecular weight compounds such as phenolic pigments. Proteolytic and MCA were retrieved in the void volume: protein content was 264 ± 4 mg/l, clotting activity was 0·083 ± 0·003 RU/ml, and proteolytic activity was 0·128 ± 0·002 EA/ml.

To elucidate the nature of the proteolytic activity involved in MCA the effect of a set of inhibitors (PMSF a serine protease inhibitor, E-64 a cysteine protease inhibitor, and pepstatin A an aspartic protease inhibitor) was tested, though inhibition was only promoted (100% of inhibition) by pepstatin A at every concentration assayed. Pepstatin is one of the most specific inhibitors known in enzymology and is highly selective for the aspartic peptidases (Dunn, 2001). Thus, this behaviour indicated that the MCA present in the partially purified preparation was due to aspartic peptidase activity.

In the presence of 50 mM-NaCl clotting activity decreased 53 ± 2% with respect to clotting activity without salt. Additional increments of NaCl decreased enzyme activity even more, reaching zero when NaCl concentration was 250 mM (Fig. 1A). The same behaviour is observed for milk clotting enzymes isolated from seeds and flowers of *Onopordum turcicum* (Tamer, 1993).

On the other hand, MCA rose when the $CaCl_2$ content was increased up to 30 mM (Fig. 1B). This result was

https://doi.org/10.1017/S0022029905000749 Published online by Cambridge University Press

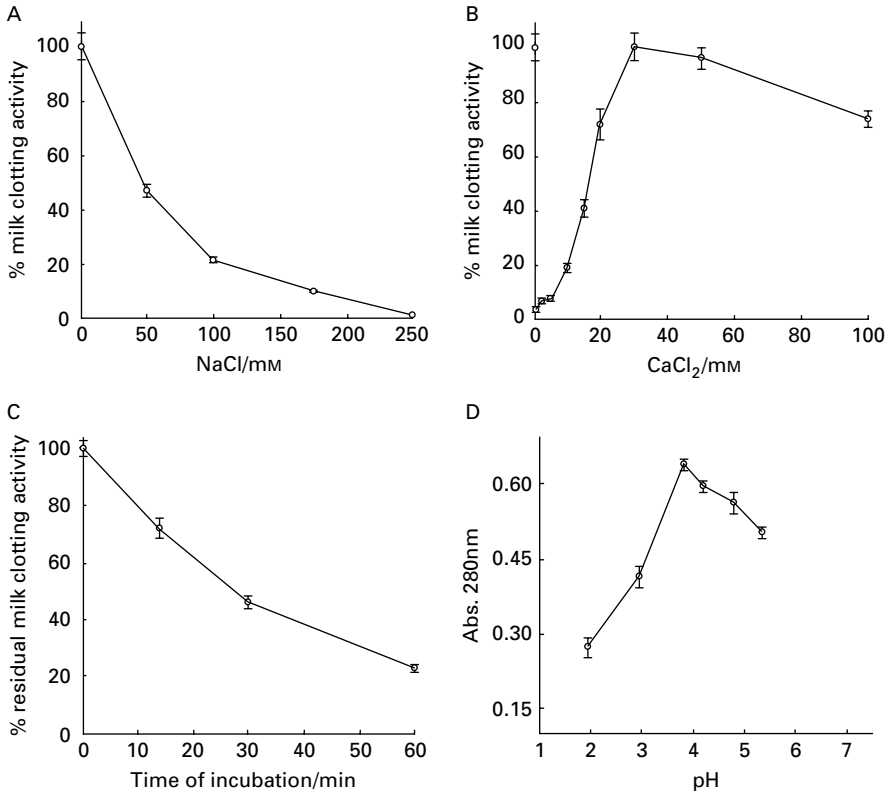

Fig. 1. (A) Effect of NaCl on milk clotting activity. (B) Effect of $CaCl_2$ on milk clotting activity at pH 6·5. (C) Residual milk-clotting activity measured after incubation of the enzyme preparation at 37 °C. (D) pH profile of the proteolytic enzyme preparation using haemoglobin as substrate. All assays were performed by triplicate, vertical bars are the standard deviation.

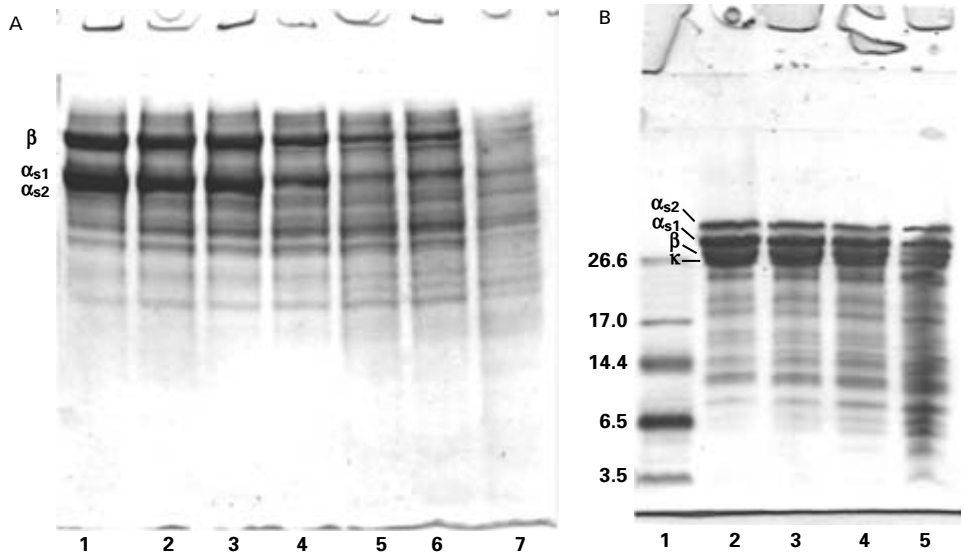

Fig. 2. (A) Urea-PAGE electrophoretogram (resolution gel was T=12·5% and C=4%, pH 8·9, overlaid by a T=4%, C=3%, pH 7·6 stacking gel) shows the degradation patterns of bovine caseins. Lane 1–7: caseins after incubation for 0, 30 min, 1, 3, 6, 12, and 24 h, respectively. (B) Tricine SDS-PAGE electrophoretogram (resolution gel was T=14·5%, C=3%, overlaid by a T=10%, C=3% separating gel, and then by a T=4%, C=3% stacking gel) showing the degradation patterns of bovine caseins. Lane 1: polypeptide molecular weight markers (Bio-Rad): bacitracin (1·4 kDa), insulin β chain, oxidized (3·5 kDa), aprotinin (6·5 kDa), α-lactalbumin (14·4 kDa), myoglobin (17·0 kDa), and triosephosphate isomerase (26·6 kDa). Lanes 2: intact casein. Lanes 3–5: whole casein after 6, 12, and 24 h of digestion, respectively. In both cases proteins were stained with Coomasie Blue G.

https://doi.org/10.1017/S0022029905000749 Published online by Cambridge University Press

consistent with those found for several clotting enzymes on different milk types (de Kruif, 1999; Castillo et al. 2002). Most authors believe that the main effect of $CaCl_2$ addition is on aggregation (Castillo et al. 2002). Clotting activity fell with concentrations of $CaCl_2$ higher than 30 mM (Fig. 1B) probably due to the increment of the ionic strength; a similar behaviour is also found by Famelart et al. (1999) for renneted milk; the effect of $CaCl_2$ increase is typically biphasic, with an initial decrease and a following increase in rennet clotting times.

To study the effect due to instability of the enzymatic preparation itself the sample was exposed to 37 °C for different periods of time (Fig. 1C). The EE lost 28±4% of the residual MCA after 15 min incubation. Only 23±1% of initial activity was retained after 60 min at 37 °C.

The effect of pH on the hydrolysis of acid-denatured haemoglobin is shown in Fig. 1D. Optimum proteolysis was achieved around pH 3·8, as observed for other aspartic proteinases such as calf, pig and lamb chymosins (Foltmann & Szecsi, 1998). Proteinases extracted from *Centaurea calcitrapa* flowers express maximum activity between pH 4·0 and 5·0 (Domingos et al. 1998), while cardosins A and B isolated from flowers of *Cynara cardunculus*, exhibit maximal activity at a less acidic pH range, between 5·0 and 5·5 (Faro et al. 1995).

The degradation pattern of the whole caseinate hydrolysed at different times is shown in urea-PAGE and tricine SDS-PAGE electrophoretograms (Figs. 2A and 2B, respectively). Two main groups of caseins were identified (α_s-caseins region and β-casein region) in urea-PAGE. Breakdown of either β-casein and α_s-caseins were observed after 3 h incubation (45% and 65% hydrolysis, respectively). These results are concordant with those obtained by other authors who report that β-casein of bovine milk is less susceptible than α_s-caseins to proteolysis by extracts of *Centaurea calcitrapa* (Tavaria et al. 1997) and *Cynara cardunculus* (Silva et al. 2002). The rate of hydrolysis was maximum for the first 3 h of reaction, especially for the α_s-caseins region. Afterwards the hydrolytic rate slowed down. Even though the degradation of α_s-caseins was faster than β-casein at the beginning of the reaction, they were degraded to approximately the same extent after 24 h incubation (28% and 34% respectively).

In tricine SDS-PAGE method, α_{s2}-, α_{s1}-, β- and κ-caseins could be visualized. The κ-casein was the casein fraction with highest mobility. The β-casein showed a medial mobility, whereas that with lowest mobility was the α_s-caseins region (Pardo & Natalucci, 2001). Also in this electrophoresis casein bands tended to disappear as incubation progressed. Bands with lower molecular mass than caseins appeared after 6 h (about 15·0, 8·0, and 6·7 kDa) and 12 h (around 24·0, 5·0, and 3·0 kDa). The intensity of these bands increased up to 24 h incubation, by which time a further three new bands (about 21·5, 17·0 and 13·0 kDa) had appeared. A band of around 10·0 kDa, present at the start intensified throughout the 24 h incubation.

The goal of this work was to find a new source of vegetable rennet with distinctive characteristics that could be useful in the dairy industry. From a technological point of view, the enzymes could be used both for milk clotting, as an alternative or in addition to calf rennet, or for the acceleration of cheese ripening in order to reduce time and costs of cheese storage.

S. Vairo-Cavalli is awarded fellowship by CONICET. C. Natalucci is member of the CIC Research Career. The authors acknowledge Dr. Néstor Caffini for the critical revision of the manuscript. Authors also thank Tolbiac S. R. L. and Mr José Madarnas for the supply of *Silybum marianum* seeds. The present work was supported by grants from ANPCyT, CIC, CONICET, CYTED and UNLP.

References

Anson ML 1938 The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. *Journal of General Physiology* **22** 79–89

Ariza Espinar L & Delucchi G 1998 280 *Asteraceae*, parte 11. Tribu XI. *Cardueae*. In: *Flora Fanerogámica Argentina*, pp. 24–25 (Ed. L Ariza Espinar) Córdoba: Pro Flora

Barros RM, Ferreiras CA, Silva SV & Malcata FX 2001 Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate. *Enzyme and Microbial Technology* **29** 541–547

Bio-Rad Bulletin 2423 2003 Bio-Safe™ Coomassie Stain. http://www.bio-rad.com/LifeScience/pdf/Bulletin_2423.pdf

Bradford MM 1976 A rapid and sensitive method for the quantitation of micrograms quantities of proteins utilizing the principle of protein dye binding. *Analytical Biochemistry* **72** 248–254

Castillo M, Payne FA, Hicks CL, Laencina J & López MB 2002 Effect of calcium and enzyme in cutting time prediction of coagulating goats' milk using a light scattering sensor. *International Dairy Journal* **12** 1019–1023

de Kruif CG 1999 Casein micelle interactions. *International Dairy Journal* **9** 183–188

Domingos A, Xue Z-T, Guruprasad K, Clemente A, Blundell T, Pais MS & Brodelius PE 1998 An aspartic proteinase from flowers of *Centaurea calcitrapa*. Purification, characterization, molecular cloning and modelling of its three-dimensional structure. In: *Aspartic Proteinases*, pp. 465–472 (Ed. MNG James) New York: Plenum Press

Dunn BM 2001 Determination of protease mechanism. In: *Proteolytic Enzymes: a Practical Approach*, 2nd edn, pp. 83–86 (Eds R Beynon & JS Bond) New York: Oxford University Press Inc.

Esteves CLC, Lucey JA & Pires EMV 2001 Mathematical modelling of the formation of rennet-induced gels by plant coagulants and chymosin. *Journal of Dairy Research* **68** 499–510

Famelart MH, Le Graet Y & Raulot K 1999 Casein micelle dispersions into water, NaCl and $CaCl_2$: physicochemical characteristics of micelles and rennet coagulation. *International Dairy Journal* **9** 293–297

Faro C, Veríssimo P, Lin Y, Tang J & Pires E 1995 Cardosins A and B, aspartic proteinases from the flowers of cardoon. In: *Aspartic Proteinases: Structure, Function, Biology and Biomedical Implications*, pp. 374–377 (Ed. K Takahashi) New York: Plenum Press

Foltmann B & Szecsi PB 1998 Chymosin. In: *Handbook of Proteolytic Enzymes*, pp. 815–819 (Eds AJ Barrett, D Rawlings and JF Woessner) London: Academic Press

Fox PF 1989 Proteolysis during cheese manufacture and ripening. *Journal of Dairy Science* **72** 1379–1400

International Dairy Federation 1992 *Bovine rennets: Determination of total milk-clotting activity*. Brussels: IDF (FIL-IDF Standard no. 157)

Pardo M & Natalucci CL 2001 An improved method for the electrophoretic analysis of bovine caseins. *Acta Pharmaceutica Bonaerense* **21** 57–60

Reis PM, Lourenço PL, Domingos A, Clemente AF, Pais MS & Malcata FX 2000 Applicability of extracts from *Centaurea calcitrapa* in ripening of bovine cheese. *International Dairy Journal* **10** 775–780

Shägger H & von Jagow G 1987 Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range of 1 to 100 kDa. *Analytical Biochemistry* **166** 368–379

Shalabi SI & Fox PF 1987 Electrophoretic analysis of cheese: comparison of methods. *Irish Journal of Food Science and Technology* **11** 135–151

Silva SV & Malcata FX 1999 On the activity and specificity of cardosin B, a plant proteinase, on ovine caseins. *Food Chemistry* **67** 373–378

Silva SV & Malcata FX 2000 Comparative catalytic activity of two plant proteinases upon caprine caseins in solution. *Food Chemistry* **71** 207–214

Silva SV, Barros RM & Malcata FX 2002 Hydrolysis of caseins by extracts of *Cynara cardunculus* precipitated by ammonium sulfate. *Journal of Food Science* **67** 1746–1751

Souza MJ & Malcata FX 1998 Proteolysis of ovine and caprine caseins in solution by enzymatic extracts from flowers of *Cynara cardunculus*. *Enzyme and Microbial Technology* **22** 305–314

Tamer IM 1993 Identification and partial purification of a novel milk clotting enzyme from *Onopordum turcicum*. *Biotechnology Letters* **15** 427–432

Tavaria FK, Sousa MJ, Domingos A, Malcata FX, Brodelius P, Clemente A & Pais S 1997 Degradation of caseins from milk of different species by extracts of *Centaurea calcitrapa*. *Journal of Agriculture and Food Chemistry* **45** 3760–3765

Veríssimo PC, Esteves C, Faro CJ & Pires EM 1995 The vegetable rennet from *Cynara cardunculus* L. contains two proteinases with chymosin-like and pepsin-like specificities. *Biotechnology Letters* **17** 614–645

Veríssimo P, Faro C, Moir A, Lin Y, Tang J & Pires E 1996 Purification, characterization and partial amino acid sequencing of two novel aspartic proteinases from the flowers of *Cynara cardunculus* L. *European Journal of Biochemistry* **235** 762–768

Visser S 1993 Proteolytic enzymes and their relation to cheese ripening and flavour: An overview. *Journal of Dairy Science* **76** 329–350