Effect of storage and lyophilization on ovine and caprine CASEIN degradation by extracts of *Cynara Cardunculus* L.

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Introduction

Increasing costs and shortage of supply of traditional rennets have urged the search for new rennet sources (Green, 1972). Although several plant proteases have recently been isolated, purified and extensively characterized, only the protease extracted from the flowers of wild thistle (*Cynara* spp.) has been successfully used in the manufacture of goat and ewe's milk in several rural areas of Portugal and Spain (Macedo *et al.*, 1993). Caseinolytic and proteolytic activities are important parameters in evaluation of rennet action during cheese manufacture. In this work, such activities were determined for extracts of *Cynara cardunculus* L., fresh or lyophilized and reconstituted either in water or citrate buffer (pH=5.4), stored up to 4 weeks at 4 °C.

Methodology

Caseinolytic activity

Rennet clotting time (R.C.T., min) was measured using low-heat skim milk powder by dissolving 12 g in 100 mL of 10^{-2} M $CaCl_2$ (pH= 6.5) at 30 °C. One rennet unit (R.U.) was defined as the amount of crude enzyme extract needed to coagulate 10 mL of reconstituted skim milk at 30 °C in 100 sec.

Proteolytic activity

Whole ovine or caprine Na-caseinate from Sigma were dissolved in 200 mM phosphate buffer (pH=6.5) to a final concentration of 1%. The reaction was started with the addition of enzyme extract to the casein solution. Aliquots were taken at selected time intervals and added to 2-mL of 5% trichloroacetic acid to quench the reaction. Samples were centrifuged at 12,000g and the absorbance of the clear supernatant was read at 280 nm.

Mathematical modelling

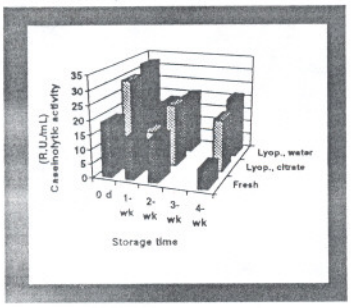
Mathematical modelling of the proteolytic breakdown of caseins was based on a postulated mechanism encompassing rearrangement of proteases in the crude extract of *C. cardunculus* between three isomeric forms, each of them able to catalyze the breakdown of ovine and caprine caseins into smaller peptides.

Urea-Polyacrylamide gel electrophoresis

Urea-PAGE was performed on the hydrolysates using the stacking gel system of Andrews (1983) with modifications (Shalabi and Fox, 1987); the gels were stained with Coomassie Blue G-250 using the method of Blakesley and Boezi (1977). Quantification of intact β- and α_s-caseins was done by densitometry.

Results and Discussion

Caseinolytic activity



Fresh extracts stored at 4 °C lost 23% of clotting power by 1 week of storage, whereas lyophilized extracts lost 44% and 61% (when reconstituted in water and in citrate, respectively); however, when assessed over the whole storage period, lyophilized extracts showed lower activity loss (34 and 38%) when compared to fresh extracts (65%). Initially, the chelating effect of citrate ions may decrease the renneting properties of the lyophilized extracts. However, along storage this effect may be overiden by the decrease in pH. Acidification is known to solubilize the Ca phosphate (thus increasing ionic calcium levels) and decrease clotting times (de la Fuente *et al.*, 1997).

Proteolytic activity

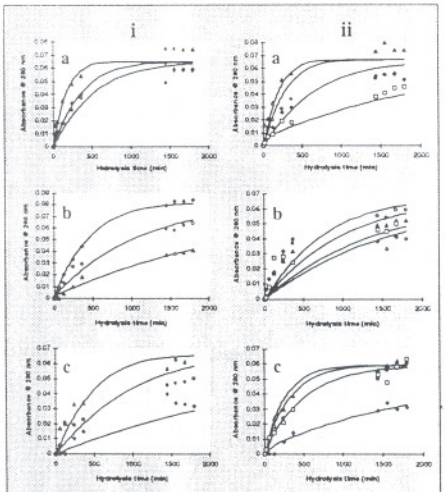


Figure 2. Non-linear fittings to the experimental data of the mechanistic model accounting for rearrangement of proteases in crude extracts from *C. cardunculus* between three isomeric forms coexisting in (a) fresh, (b) lyophilized and reconstituted in water, and (c) lyophilized and reconstituted in citrate buffer, for ovine [i] and caprine [ii] caseins; extracts were stored at 4 °C for 0 days days (♦), 7 days (●), 14 days (▲), and 28 days (■).

Overall proteolytic activity (measured by precipitation with TCA) tended to decrease with storage for the fresh and lyophilized extracts reconstituted in water (Fig. 2), but increased when the extracts were reconstituted in citrate. The lower pH in these extracts (pH=5.4) may be enough to solubilize the colloidal calcium and destabilize the micelle structure, thus increasing proteolysis along the storage process.

Urea-PAGE

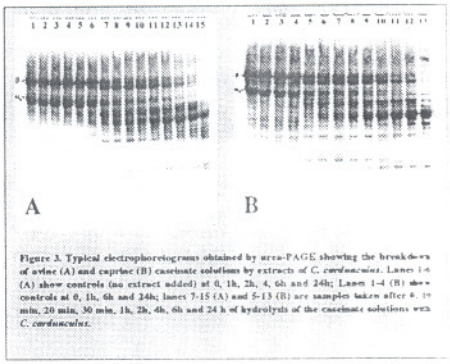


Figure 3. Typical electrophoretograms obtained by urea-PAGE showing the breakdown of ovine (A) and caprine (B) caseinate solutions by extracts of *C. cardunculus*. Lanes 1-6 (A) show controls (no extract added) at 0, 1h, 2h, 4, 6h and 24h; Lanes 1-4 (B) show controls at 0, 1h, 6h and 24h; lanes 7-15 (A) and 5-13 (B) are samples taken after 0, 10 min, 20 min, 30 min, 1h, 2h, 4h, 6h and 24 h of hydrolysis of the caseinate solutions with *C. cardunculus*.

Densitometer

CASEINS	0-d old		1-wk old		2-wk old	
OVINE	β-	α_s-	β-	α_s-	β-	α_s-
Fresh	100	100	84.3	86.5	57.2	79.2
Lyop.-H2O	97	67.1	61.6	96	54.8	88.3
Lyop.-cit.	94.8	89.2	44.6	89.7	78.5	89.2
CAPRINE	β-	α_s-	β-	α_s-	β-	α_s-
Fresh	91.3	100	83.9	96.8	45.7	78.1
Lyop.-H2O	87.7	83.4	91.2	83.1	61.6	98.3
Lyop.-cit.	96.4	81	83.8	84	82.3	95.8

Urea-PAGE analysis (a measure of major peptides formed by enzymatic action) shows that ovine and caprine casein degradation decreased by ca. 25% with lyophilization, and was mostly accounted for by a decrease in the degradation of α_s-caseins. The loss of water due to the lyophilization process seems to drive the protease towards a greater affinity for the more hydrophobic region of the micelle structure; however, with storage β-casein revealed to be more resistant to degradation.

Conclusions

- Lyophilized extracts tended to have less apparent decreases in clotting power than fresh extracts when stored, and more intense decreases in the proteolytic activities, thus suggesting that the lyophilization process does, in fact, increase the C/P (coagulative to proteolytic) ratio of the crude extract.
- Lyophilization also seemed to render β-casein more resistant to degradation, which is an advantage since it is a source of potential bitter peptides in cheese.
- Lyophilized extracts seem to be a suitable alternative to fresh extracts that have been employed for ages in the manufacture of traditional cheeses from ewe's and goat's milks, especially when reconstituted in water since they maintain a more favorable C/P ratio.

References

- Andrews, A. T. J. (1983). Proteases in normal bovine milk and their action on caseins. *J. Dairy Res.*, 50: 45-55.
- Blakesley, R. W. and Boezi, J. A. (1977). A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G-250. *Anal. Biochem.*, 82: 55-581.
- de la Fuente, M. A., Requena, T. and Juárez, M. (1997). Salt balance in ewe's and goat's milk during storage at chilling and freezing temperatures. *J. Agric. Food Chem.*, 45: 82-88.
- Green, M. L. (1972). Assessment of swine, bovine, and chicken pepsin as rennet substitutes for cheddar cheese-making. *J. Dairy Res.*, 39: 261-265.
- Macedo, A., Malcata, F. X. and Oliveira, J. C. (1993). The technology, chemistry and microbiology of Serra cheese: a review. *J. Dairy Sci.*, 76: 1725-1739.
- Shalabi, S. I. and Fox, P. F. (1987). Electrophoretic analysis of cheese: comparison of methods. *Ir. J. Food Sci. Technol.*, 11: 135-151.