Effect of storage and lyophilization on ovine and caprine casein degradation by extracts of *Cynara cardunculus* L.

F. K. Tavaria, M. J. Sousa, and F. X. Malcata*
Escola Superior de Biotecnologia, Universidade Católica Portuguesa
Rua Dr. António Bernardino de Almeida, 4200 Porto

*To whom correspondence should be addressed

SUMMARY

Caseinolytic and proteolytic activities are important parameters to evaluate rennet action in cheese manufacture. These activities were determined for extracts of *Cynara cardunculus,* fresh or lyophilized and reconstituted either in water or citrate buffer (pH 5.4), stored up to 4-weeks at 4°C. Ovine and caprine casein degradation was followed by urea polyacrylamide gel electrophoresis in attempts to characterize the evolution of the extract activity throughout the storage time.

INTRODUCTION

Rennet extracts from the abomasa of milk-fed calves have provided the first (and best) coagulant for general cheesemaking. However, increasing costs and shortage of supply in recent years has urged the search for new rennet sources (Green, 1972). Even though several plant proteases have recently been isolated, purified, and extensively characterized, only the protease extracted from the flowers of the wild thistle (i.e. *Cynara* spp.) has been successfully used in the manufacture of cheese from goat and ewe's milk (Macedo *et al.*, 1993) in several rural areas of Portugal and Spain.

Application of enzymes in biotechnological processes is limited frequently by their rates of deactivation. Several environmental factors promote changes in an enzyme structure that reduce its catalytic activity. The aim of this work was to study the effects of storage on the activity of the *Cynara* spp. enzyme system in order to effectively predict and control the characteristics of the final cheese product.

MATERIALS AND METHODS

Enzyme: Dried flowers of the wild thistle (*C. cardunculus* L.) were obtained from local shops in Serra da Estrela region. The crude extract was prepared by grinding the stylets of the flowers in 0.1 M citrate buffer (pH 5.4) and centrifuging at 6,000 g for 5 min.

Storage: Extracts were either fresh or lyophilized before storage and used promptly (0 d of storage) or kept at 4°C for different storage times (1, 2, or 4 wk). Lyophilized extracts were, after lyophilization, reconstituted either in water or citrate.

Milk clotting activity: Rennet clotting time (RCT, min) was measured using low-heat skim milk powder as substrate. The RCT was obtained for the ratio of 0.1 ml of crude extract to 2 ml of reconstituted skim milk powder. One rennet unit (R.U.) was defined as the amount of crude enzyme extract needed to coagulate 10 ml of reconstituted low-heat skim milk at 30°C in 100 sec.

Hydrolysis of caseins: Whole ovine or caprine Na-caseinate (1g/100mL), obtained from Sigma, were dissolved in 200 mM phosphate buffer (pH=6.5) and warmed up to 30°C in a thermostatted water bath. The reaction was started with addition of enzyme solution (526 mL of crude extract per 10 mL of casein solution). Aliquots of 1 mL were taken (to determine the proteolytic activity) at selected time intervals and added to 2 mL of 5% trichloroacetic acid (TCA) to quench the reaction. After resting for 10 min, the samples were centrifuged at 12,000 g and absorbance of the clear supernatant was read at 280 nm. The activity was referred to the initial amount of protein in the extract as determined by the Coomassie method.

Aliquots of 750 µl were also taken and mixed with an equal volume of double concentrated sample buffer (McSweeney *et al.*, 1993) and vortexed for 30 sec prior to analysis by urea polyacrylamide gel electrophoresis (Urea-PAGE).

Urea-PAGE: Urea-PAGE was performed using a vertical slab-gel unit (12.5 % T and 4 % C, pH 8.9) (Andrews, 1983) with modifications (Shalabi and Fox, 1987); the gels were stained with Coomasie Blue G-250 using the method of Blakesley and Boezi (1977). Quantification of intact β-casein (i.e., β_1- and β_2-caseins),and α_s-casein (i.e., α_{s3}, α_{s2}, and α_{s1}-caseins) was done by densitometry.

RESULTS AND CONCLUSIONS

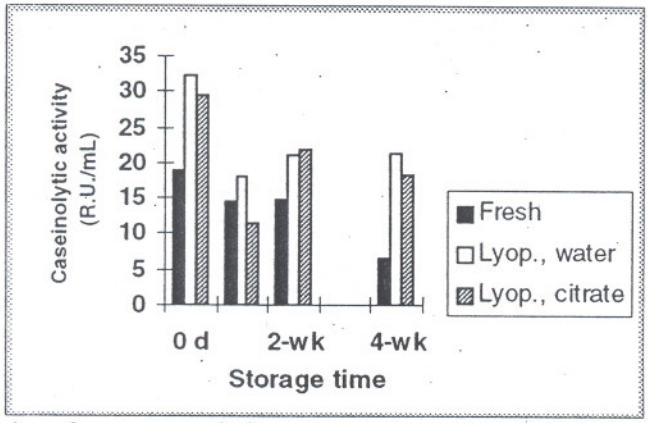

FIG. 1 Clotting activity of extracts of *Cynara cardunculus* either fresh or lyophilized, after storage at 4°C up to 4 weeks.

The extract activity expressed in R.U./mL, tended to decrease throughout storage time (Fig. 1), an observation expected in view of the spontaneous loss of the catalytically active conformation with elapsing time. However, the caseinolytic activity was consistently lower for fresh extracts at all times of storage then for lyophilized ones; lyophilization prevents decrease in activity probably because the protein structure is kept more rigid as a consequence of reduced water activity. Although there is a higher loss in caseinolytic activity of lyophilized extracts after 1 wk of storage (44% when reconstituted in water, and 61% when reconstituted in citrate) as compared with fresh extracts (23% only), the same qualitative trend does not hold for the whole storage time (4 wk), where the lyophilized extracts showed a lower loss in activity (34% and 38%, respectively) than fresh extracts (65% decrease).

Table 1. Percent degradation of ovine and caprine caseins after 6 h of hydrolysis as affected by lyophilization and storage.

	0-d old		1-wk old		2-wk old	
OVINE	**β-CN**	**α-CN**	**β-CN**	**α-CN**	**β-CN**	**α-CN**
fresh	100	100	84.3	86.5	57.2	79.2
Lyop., H_2O	97	67.1	61.6	96	54.8	88.3
Lyop., citrate	94.8	89.2	44.6	89.7	78.5	89.4
CAPRINE	**β-CN**	**α-CN**	**β-CN**	**α-CN**	**β-CN**	**α-CN**
fresh	91.3	100	83.9	96.8	45.7	78.1
Lyop., H_2O	87.7	83.4	91.2	83.1	61.6	90.3
Lyop., citrate	96.4	81	83.8	84	82.3	95.8

Both β- and α_s-caseins of ovine and caprine caseinate solutions acted upon by fresh and lyophilized extracts of proteinases of *C. cardunculus* (reconstituted in water or citrate) have undergone extensive degradation by 6 hr of hydrolysis (see Table 1). β-Casein of ovine caseinate was degraded slowlier and slowlier throughout the storage time (for both fresh and lyophilized extracts reconstituted in water), an observation that agrees with the aforementioned loss in enzymatic activity throughout storage; however, β-casein was more resistant than α_s-casein to proteolytic degradation in both ovine and caprine caseinates, which is consistent with data reported in the literature for actual cheeses using extracts of *C. cardunculus* as rennet (Sousa and Malcata, 1996). Lyophilization, which results in a compulsory loss of water, may drive the protease towards a greater affinity for the more hidrophobic region of the micelle structure which is accounted for by β-casein, and this could explain the higher degradation of β-casein when subject to enzyme previously exposed to lyophilization (see Table 1).

CONCLUSIONS

A general decrease in the clotting and proteolytic activities of extracts of *Cynara cardunculus* with storage time in terms of degradation of both β- and α_s-caseins was observed. According to Pires *et al.* (1994), a good rennet is characterized by a high caseinolytic activity and a low proteolytic activity; lyophilized extracts show this trend. A loss in the caseinolytic and proteolytic activities of the extracts is believed to be due to thermal deactivation of the enzyme. Lyophilized extracts from flowers of *Cynara cardunculus* are apparently a suitable alternative to fresh extracts which have been employed for ages in the manufacture of traditional cheeses in Portugal.

REFERENCES

Andrews, A. T. J. Proteinases in normal bovine milk and their action on caseins. *J. Dairy Res.* **1983**, 50, 45-55.

Blakesley, R. W.; Boezi, J. A. A new staining technique for proteins in polyacrylamide gels using Coomassie Brillant Blue G-250. *Anal. Biochem.* **1987**, 82, 55-581.

Green, M. L. Assessment of swine, bovine and chicken pepsin as rennet substitutes for cheddar cheese-making. *J. Dairy Res.* **1972**, 39, 261-265.

Macedo, A., Malcata, F. X., Oliveira, J. C. The technology, chemistry and microbiology of Serra cheese: a review. *J. Dairy Sci.* **1993**, 76, 1725-1739.

McSweeney, P. L.H., Olson, N.F., Fox, P.F., Healy, A., Hojrup, P. Proteolytic specificity of chymosin on bovine α_{S1}-casein. *J. Dairy Res.* **1993**, 60, 401-412.

Shalabi, S. I., Fox, P. F. Electrophoretic analysis of cheese: comparison of methods. *Ir. J. Food Sci. Technol.* **1987**, 11, 135-151.

Sousa, M. J., Malcata, F. X. Comparison of plant and animal rennets in terms of microbiological, chemical and proteolysis characteristics of ovine cheese. *J. Agric. Food Chem.* **1996**, 45, 78-81.