Food Chemistry **35** (1990) 89–97

Chemical Characterization of Proteases Extracted from Wild Thistle (*Cynara cardunculus*)

Rolando Campos, Ricardo Guerra, Marlene Aguilar, Oro Ventura & Lavinia Camacho

Institute of Nutrition and Food Technology (INTA), University of Chile, PO Box 15138, Santiago, Chile.

(Received 28 November 1988; revised version received and accepted 8 February 1989)

ABSTRACT

Three protease fractions were obtained by purification of a thistle extract with ammonium sulfate. The optimum pH and temperature on the proteolytic activity of the crude extract was found to be 5·7 and 37°C, respectively. The crude extract had a lower clotting activity and a stronger proteolytic activity than commercial rennets. However, the protease fractions showed a sharp increase in clotting activity and a decrease in proteolytic activity compared to the extract, indicating the proportion of the extract proteolytic activity within the fractions. Therefore, the ammonium sulfate fractionation gives clotting activity and proteolytic activity in a ratio close to a commercial fungal rennet extracted from Mucor miehei. *The electrophoretic pattern of the fractions showed qualitative differences in the number and intensity of bands. However, the major differences were detected in the 50% ammonium sulfate fraction, which also had the highest milk-clotting activity. Therefore, correlations between the specific milk-clotting activity and the number and intensity of the electrophoretic bands of the 50% fraction were established.*

INTRODUCTION

Rennet extract from the abomasa of milk-fed calves has been the traditional coagulant for cheese-making. However, an acute shortage of rennet during

Food Chemistry 0308-8146/89/$03·50

recent years has stimulated a search for new protease sources (Green, 1972; Chen & Zall, 1986*b*). Microbial rennets extracted from some bacteria and mold cells have been found to be appropriate substitutes for calf rennets (Richardson *et al.*, 1967; Vanderpoorten & Weckx, 1972; Yamamoto, 1975). Other researchers have investigated the use of vegetable proteases for cheese-making (Whitaker, 1959; Vieira de Sa & Barbosa, 1972; Ogundiwin & Oke, 1983; Iturbe & Lopez, 1986).

Kosikowsky (1977) and Badia and Canut (1983) reported the use of wild thistle as the rennet source for the elaboration of Mato and Serra cheeses, both produced in certain rural areas of Spain and Portugal. Vieira de Sa and Barbosa (1972) and Barbosa *et al.* (1981) found that curd properties and ripening behavior of French- and Italian-type cheeses were affected by the use of thistle rennet. However, little information about chemical characterization of the enzyme is available.

Thistle, or 'cardo de Castilla', is a wild vegetable grown extensively in the arid regions of Chile and Argentina. The main agricultural activity in these regions is goat's cheese production. Thistle can be used for clotting goat's milk, but the characterization of the coagulant must be known in order to design the cheese-making process. Therefore, the aims of this study were to evaluate the factors that affect the enzymatic activity of thistle extract and to carry out the chemical characterization of the coagulant.

MATERIALS AND METHODS

Coagulant standards

Freeze-dried calf rennet and fungal coagulant of *Mucor miehei*, purchased from Hansen, Copenhagen, Denmark, were used as control rennets.

Coagulant extract

Thistle flowers were collected at the end of the flowering period and frozen at −20°C until used. Flowers were blended with de-ionized and distilled water at 7% in a Waring blender for 2 min at 0°C, filtered through gauze and centrifuged at 450 *g* for 5 min. The supernatant, with a pH range of 5·5 to 5·7, corresponded to the crude extract, which was purified by saturation with 40, 50 and 80% solid ammonium sulfate until noticeable precipitates were formed. The purification started with the crude extract, and each supernatant was used for the next precipitation. Final precipitates were dissolved in water and analyzed for protein and enzymatic activity determination.

Protein determination

Protein was determined by a modification of the Lowry method described by Albro (1975).

Enzymatic activity

Proteolytic activity was determined by the casein digestion method at 37°C and pH 8·0, according to Arnon (1970), but without cystine and EDTA. A unit of enzyme activity was defined as that which caused an increase of one absorbance unit at 280 nm/min. To determine the optimum temperature for proteolytic activity, increasing protein concentrations (50, 70, 100, and 200 μl) of the thistle extract were incubated in Tris (50 mM, pH 8·0), at temperatures of 22–38°C for 10 min, and using casein as the substrate. The reaction was stopped with trichloroacetic acid (5%). pH stability was determined by incubating increasing protein concentrations of the extract in sodium acetate (50 mM for pH values under 5·7) and in Tris (50 mM sodium chloride, 25 mM, for pH values over 6·0) at 37°C for 10 min with 1% casein substrate. The pH values were in the range of 3·9–8·0.

Milk-clotting activity was determined at 37°C, based on the method of Balls and Hoover (1937). A medium containing 5 ml pasteurized cow's milk, 0·25 ml calcium chloride (10 mM), and thistle protein dissolved in 0·5 ml sodium acetate (1·25 M, pH 5·7), was used. One unit of enzyme was defined as the concentration of protein able to clot 1 ml of cow's milk in 1 min, under the assay conditions. Results were expressed as a linear regression between clotting time reciprocal and the respective protein concentration. Milk-clotting activity corresponded to the slope of the curve, while the number of enzymatic units was represented by the time reciprocal. Standards for comparison were calf and microbial rennets.

Optimum temperature for clotting activity was measured at milk pH and at a constant protein concentration of 0·12 mg/ml. Temperature range was 21–80°C.

Polyacrilamide gel electrophoresis

Protein electrophoresis was done in a polyacrilamide gel on a gradient (11–14%). Buffer stock of the electrode formed by Tris (1·2%), glycine (5·76%, pH 8·3), was four times diluted, and SDS, from a 20% stock, added up to a final concentration of 0·1%. Sample solubilization buffer was formed by 2·5 ml of Tris (6·1%)–SDS (0·4%) (pH 6·8) solution, 2·5 ml SDS (20%), 0·1 ml EDTA (0·1 M), dissolved with distilled water. An aliquot of the solubilization buffer was blended with 0·05 ml β-mercapto-ethanol and

0·1% bromophenol. The sample was added and the mixture placed in a boiling bath for 1 min. Fixing solution was 50% methanol:10% acetic acid. Gels were stained with coomassie blue R-250 and destained by 10% methanol:7% acetic acid. Electrophoresis was done for 2 h at 150 V, samples containing 15 μg protein per slot. Fixation time corresponded to 12 h, while staining was done for 1 h. Gel destaining was carried out under agitation at 55°C (Zingales, 1983).

RESULTS AND DISCUSSION

Effect of temperature and pH on the enzymatic activity of thistle extract

The effect of temperature on the proteolytic activity is described in Fig. 1(a), showing the change of the initial reaction rate as a result of temperature increase. Each point of the curve corresponded to the slope of a curve where proteolytic activity of the crude extract was plotted at increased protein concentrations and at the same temperature. Each point represents an average value of five experimental determinations. As is shown in this figure, increasing incubation temperature has a positive effect on the proteolytic activity of thistle extract, with a peak at 37°C. The temperature dependence is a typical behavior of many enzymes, but this is modified when temperatures are related to milk clotting activity. Figure 2 illustrates the effect of incubation temperature on milk clotting time. Protein concentration of 0·12 mg/ml was maintained against increased temperatures. Milk-clotting time decreased at increased temperatures, showing an increase

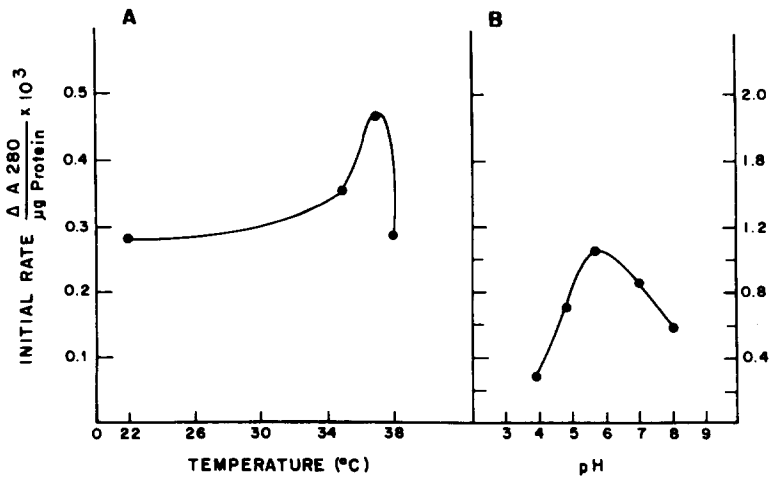

Fig. 1. Proteolytic activity of thistle extract as affected by temperature (A) and pH (B).

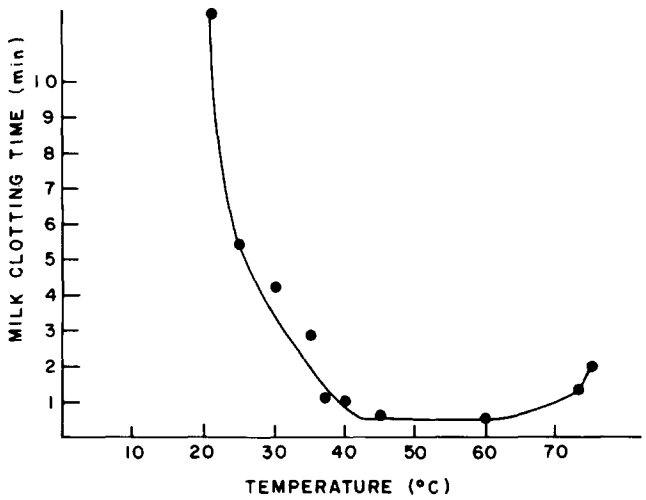

Fig. 2. Effect of temperature on milk clotting activity of thistle extract.

in the reaction rate. This trend was observed in the temperature range of 40–60°C. However, milk-clotting rate decreased at temperatures over 70°C, indicating a probable protein denaturation.

The initial reaction rates, which are related to different pH values of the thistle extract, are plotted in Fig. 1(b). It was found that the clotting activity of the vegetable coagulant was pH-dependent, showing an optimum milk-clotting rate at pH 5·7. According to Le Jaouen (1982), this acidity condition must be created to make some types of soft cheeses. Therefore, modifications of temperature and pH in the traditional cheese-making process must be made in order to attain an appropriate clotting activity of the thistle extract in κ-casein.

Enzymatic activity of thistle extract and purified protease fractions

Figure 3 illustrates the comparative characteristics of milk-clotting time of ammonium sulfate fractions, thistle crude extract and commercial rennets. Calf and fungal rennets had specific clotting activities of 18·7 units/mg and 9·91 units/mg, respectively, which were higher than that of thistle crude extract. Effectively, Table 1 shows that the crude extract had a milk clotting activity of 0·57 units/mg. However, this was lower than the ammonium sulfate purified fractions. Although the 50% fraction had the lowest yield, its specific clotting activity was stronger than the other fractions. Besides the economic and simplistic advantages of the ammonium sulfate fractionation process, our results indicate that clotting activity significantly increased in the separate fractions. In fact, the purification procedure resulted in an

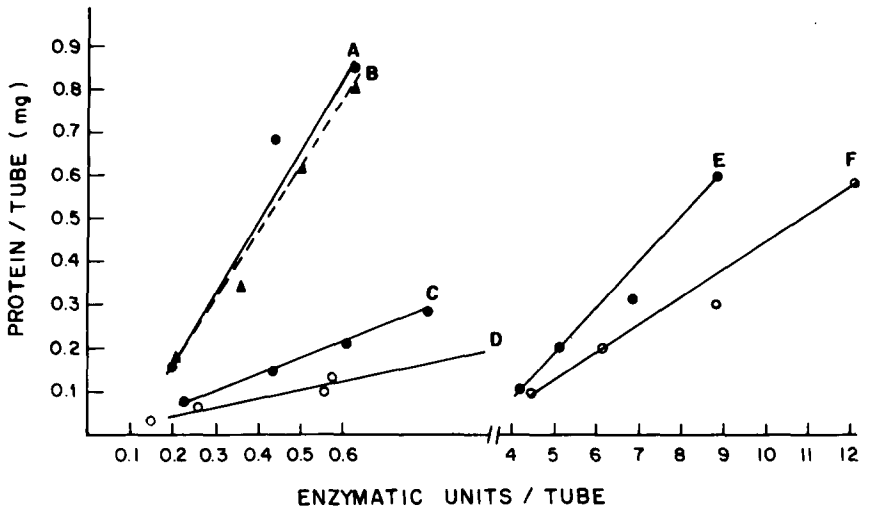

Fig. 3. Milk clotting activity of thistle extract (A), protease 40% (B), protease 50% (D), and protease 80% (C), as compared to fungal (E) and calf (F) rennets.

increase of clotting activity of 8-fold and 4·6-fold in 50 and 80% ammonium sulfate fractions, respectively.

As is shown in Table 1, there was an inverse relationship between clotting and proteolytic activity of the ammonium sulfate fractions. Fractionation caused a significant decrease of the proteolytic activity of the crude extract. Therefore, the ammonium sulfate purification process causes a rearrangement of the original proteolytic activity of the extract within the fractions. These results agree with Chen and Zall (1986*a*, *b*), who applied an ethanol fractionation to a clam viscera extract and found an increased clotting activity in one fraction as a result of the purification process. On the other hand, Aworh and Nakai (1986) determined a decrease of the total proteolytic activity in the fractions of sodom apple extract.

TABLE 1
Enzymatic Activity of Ammonium Sulfate Fractions as compared to Thistle Crude Extract

Thistle coagulant	*Yield (%)*	*Specific activity (units/mg)*		*Ratio (a)/(b)*
		Clotting (a)	*Proteolytic (b)*	
Crude extract	100·00	0·57	0·11	0·02
Fraction 40%	44·25	0·68	0·03	0·08
Fraction 50%	12·88	5·00	0·04	0·43
Fraction 80%	30·00	2·60	0·07	0·14

According to Green (1972) and Chen and Zall (1986b), the relative ratio of clotting activity to proteolytic activity is a useful indicator of the protease efficiency to be used as a coagulant for cheese-making. Based on an arbitrary ratio of 1 for calf rennet, Table 1 shows the relative ratio of clotting activity to proteolytic activity of the extract and its fractions. Ammonium sulfate fractionation caused an increase of this ratio in the separate fractions, as compared to that of the crude extract. However, the highest increase was experienced by the 50% ammonium sulfate fraction which attained a ratio closer to fungal rennet. The latter had a relative ratio of clotting activity to proteolytic activity of 0·43.

Most substitutes are significantly more proteolytic and less active for clotting milk than calf rennet. It is well known that these defects not only lower the cheese yield and retention of protein and fat by curd but also affect the ripening of the product and some sensory attributes. Clotting activity is related to the enzyme's ability to split κ-casein in the region of the Phe (105)–Met (106) peptide bond which is specific for cheese-making. According to our data, thistle extract shows a broader specificity and attacks several peptide bonds; that means it presented a low ratio of relative clotting activity to proteolytic activity. Therefore, modification in pH and temperature should be done in order to improve clotting conditions during cheese-making. Moreover, comparative experiments with the crude extract and the purified fractions should be carried out in order to establish the best process for the production of cheese made with thistle rennet.

It has been established that a further proteolytic activity is developed during ripening of cheeses made with vegetable proteases. According to the temperature curve of this study, a slower proteolytic activity of thistle protease should be expected during cheese ripening and would be visible if the enzyme acts jointly with an appropriate starter.

Electrophoretic pattern

Band resolution was carried out in a 10% polyacrilamide gel, with 15 μg/slot as the best level for an appropriate detection. Figure 4 shows that the gel electrophoresis yielded bands with a specific mobility and intensity. Each protease fraction had a different number of bands, explaining their specific clotting properties in casein. Proteins with molecular weights of 32 000, 15 000, and 60 000 were qualitatively similar.

We postulate that the procedure used for the purification contributed to obtaining proteins structured as oligomers with different numbers of monomer units. This gives support to the possibility of fractionation by precipitation, and to our finding of different band intensities. The best milk-clotting activity was obtained with fraction 50%, while the highest intensity

of its protein bands showed the best specific activity. Correlations of milk-clotting activity with the electrophoretic pattern indicate that the bands of the 50% ammonium sulfate fraction show qualitative differences in relation to the other fractions. In fact, that fraction shows two slight bands with molecular weights ranging from 23 000 to 24 000 d, while the other bands present a higher colour intensity than those of similar R_f of the other

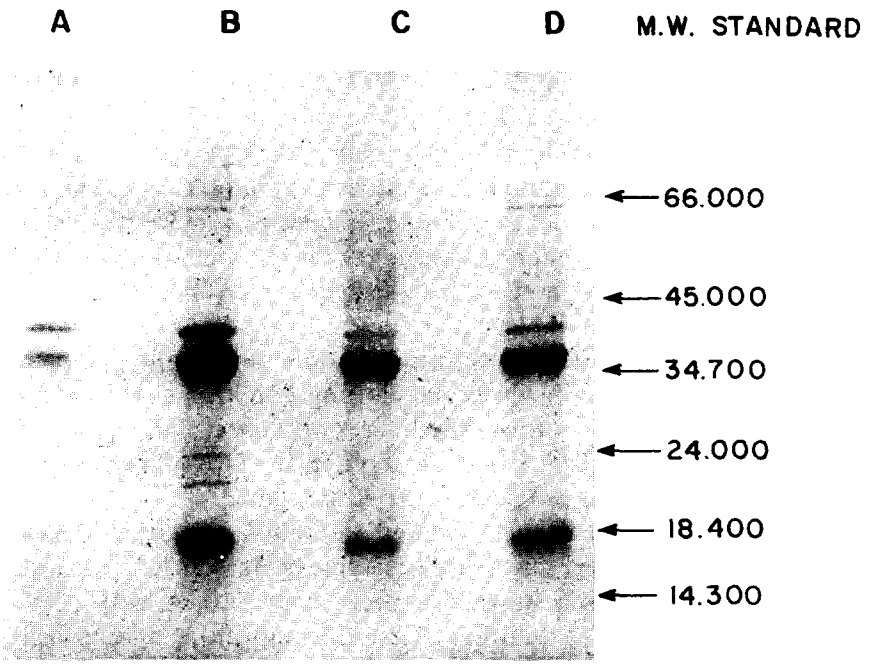

Fig. 4. SDS–PAGE in purified fractions of a crude thistle extract by ammonium sulfate precipitation. Fraction 80% (A); fraction 50% (B); fraction 40% (C); crude extract (D). molecular weight standards were: bovine albumin (66 000); egg albumin (45 000); pepsin (34 700); trypsinogen (24 000); β-lactoglobulin (18 400); lysosyme (14 300).

fractions. Direct evidence about the polypeptide responsible for milk-clotting phenomena is not available, because of the denaturing effect of electrophoresis on the native protein of the extract. However, even though the electrophoretic band of 38 000 d was apparently different in colour intensity between fractions (50% > 80% > 40%), it is related to the specific clotting activity of each fraction. Therefore, we conclude that the specific clotting activity is a function of the polypeptide numbers contained in the fraction, as well as of the concentration of these peptides.

ACKNOWLEDGEMENTS

This research was supported by the National Fund of Science and Technology (FONDECYT), Grant 567/87 and the Technical Department of Research (DTI) of the University of Chile, Grant A-2394-8723.

REFERENCES

Albro, P. W. (1975). Lowry alternative modified method. *Anal. Biochem.*, **64**, 485–93.

Arnon, R. (1970). Papain. In *Methods in Enzymology* (Vol 19), ed. E. Perlmann & L. Lorand. Academic Press, New York, pp. 226–44.

Aworh, O. C. & Nakai, S. (1986). Extraction of milk clotting enzyme from sodom apple (*Calotropis procera*). *J. Food Sci.*, **51**, 1569–70.

Badia, R. & Canut, E. (1983). Principios básicos de elaboración de quesos autóctonos españoles. AFQA, Barcelona.

Balls, A. K. & Hoover, S. R. (1937). The milk clotting action of papain. *J. Biol. Chem.*, **121**, 737–45.

Barbosa, M., Corradini, C. & Battistotti, B. (1981). Cheese-making experiments carried out on some Italian cheeses with vegetable rennet from cardo (*Cynara cardunculus* L.). *Scienza e tecnica lattiera-casearia*, **34**, 203–21.

Chen, H. C. & Zall, R. R. (1986*a*). Partial purification and characterization of cathepsin B-like and D-like proteases from surf clam viscera. *J. Food Sci.*, **51**, 71–2.

Chen, H. C. & Zall, R. R. (1986*b*). Evaluation of thiol activated proteases from clam viscera as a rennet substitute for cheese-making. *J. Food Sci.*, **51**, 815–20.

Green, M. L. (1972). Assessment of swine, bovine and chicken pepsin as rennet substitutes for cheddar cheese-making. *J. Dairy Res.*, **39**, 261–5.

Iturbe, F. A. & Lopez, A. (1986). Proteolytic enzymes from *Chidoscolus chayamansa* 'Chaya'. *J. Food Sci.*, **51**, 243–4.

Kosikowsky, F. V. (1977). *Cheese and Fermented Foods*, 2nd ed. Edwards Brothers Inc., New York.

Le Jaouen, J. (1982). *La Fabrication de Fromage de Chevre Fermier*. Itovic, Paris.

Ogundiwin, J. O. & Oke, O. L. (1983). Factors affecting the processing of wara—a Nigerian cheese. *Food Chem.*, **11**, 1–13.

Richardson, G. H., Nelson, J. H., Lubnov, R. E. & Schwarberg, R. L. (1967). Rennin-like enzyme from *Mucor pusillus* for cheeses manufacture. *J. Food Sci.*, **50**, 1066–72.

Vanderpoorten, R. & Weckx, M. (1972). Breakdown of casein by rennet and microbial milk-clotting enzymes. *Neth. Milk Dairy J.*, 47–59.

Vieira de Sa, F. & Barbosa, M. (1972). Cheese-making with a vegetable rennet from cardo (*Cynara cardunculus*). *J. Dairy Res.*, **39**, 335–43.

Whitaker, J. R. (1959). Properties of the milk-clotting activity of ficin. *Food Tech.*, **13**, 86–92.

Yamamoto, A. (1975). Proteolytic enzymes. In *Enzymes in Food Processing*, ed. G. Reed. Academic Press, New York, pp. 123–79.

Zingales, B. (1983). Analysis of proteins by sodium dodecyl sulfate-polyacrilamide gel electrophoresis. In *Genes and Antigens of Parasites. A Laboratory Manual*, ed. C. M. Morel. UNDP/WB/WHO, Rio de Janeiro, Brazil.