Quantitative studies on the enzymatic hydrolysis of milk proteins brought about by cardosins precipitated by ammonium sulfate

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Abstract

Hydrolysis of whey proteins may produce peptide mixtures with better functional properties than the original protein mixture, *viz.* higher solubilites and lower allergenic effects. *Cynara cardunculus* is a wild plant that possesses (aspartic) proteases in its flower cells; those enzymes exhibit general proteolytic and specific milk clotting activities, which are rather useful in traditional cheesemaking. This study was thus aimed at characterizing the enzymatic action of crude extracts of said plant after preliminary purification by salting out with ammonium sulfate at two different concentration levels, *viz.* 30% and 70% saturation. The coagulant activity on milk, and the proteolytic activity using casein and azocasein as substrates, of the crude extract and of each precipitated fraction were measured at 37°C and pH 5.2. The profile of hydrolysis of the major whey proteins, *i.e.* α-lactalbumin (α-La), β-lactoglobulin (β-Lg) and bovine serum albumin (BSA) was characterized by gel permeation chromatography and polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate. The 30% and 70% saturation fractions exhibited lower coagulant and proteolytic specific activities than the crude extract. However, the relative ratio of coagulant to proteolytic activity, which is a useful indicator of appropriateness for cheesemaking, was higher for the partially purified fractions. The extents of hydrolysis of whey proteins brought about by the partially purified extracts were above those by their crude counterpart, but qualitative hydrolysis patterns were essentially identical to each other; by 24 h, α-La was substantially depleted, whereas β-Lg was very poorly hydrolyzed and BSA was only slightly hydrolyzed. The native proteins were converted to lower and lower molecular weight peptides.

1. Introduction

Whey, a by-product of the cheese industry, has for long been considered as a waste product and looked upon seriously by environmentalists owing to its high polluting power. The main whey proteins are β-lactoglobulin, β-Lg (*ca.* 50%), α-lactalbumin, α-La (*ca.* 19%), and bovine serum albumin, BSA (*ca.* 5%) (Cayot et al., 1995). Hydrolysis of these proteins produces peptide mixtures with higher solubilities (Chobert et al., 1988), as well as better foaming characteristics, depending on the degree of hydrolysis (Adler et al., 1986); furthermore, the resulting peptides are more easily assimilated by microorganisms (Moneton et al., 1986), which obviously speeds up fermentation processes (Tchorbanov et al., 1988).

Aspartic proteases (EC 3.4.23) make up a family of enzymes that are widely distributed in animals, plants and microorganisms. In particular, the flowers of *Cynara cardunculus* have two aspartic proteases, *viz.* cardosin A and cardosin B; these enzymes are characterized by different amino acid sequences, and are thus probably the result of transcription of two distinct genes; each cardosin consists of two subunits, with apparent molecular weights 31 kDa and 15 kDa for cardosin A, and 34 kDa and 14 kDa for cardosin B (Veríssimo et al., 1995). The relative proportion of these proteinases depends on the cultivar in question, the part of the flower and the degree of maturation; in fresh flowers, the typical amount of cardosin B is *ca.* 25% of the total protein (Veríssimo et al., 1995). Interestingly, the dried flowers of *C. cardunculus* have for ages been successfully employed in Portugal and border regions of Spain as rennet for ewe's milk cheesemaking.

Rational use of these plant enzymes is somewhat difficult: first, the crude extract may contain several other compounds (including non-proteolytic enzymes), that will in principle promote occurrence of extraneous reactions; and

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second, such other enzymes may interfere with normal enzyme assays, thus introducing gross under- or over estimation of activity. Under the first type of problem, the high content of phenolic compounds is worth of notice, because they are easily oxidized (mainly under the influence of endogenous phenoloxidases) to form pigments; these pigments attach to proteins including native enzymes, and some do actually bind covalently thus leading to inactivation of those enzymes. Salting out of proteins is a widely used technique in enzyme purification, which takes advantage of the desolvation effect caused by high concentrations of salts. This phenomenon is appropriate not so much for fractionation of proteins, but mainly concentration of them, and is as well useful for removal of some contaminants.

The objective of the present study was two fold: (i) to optimize the extraction and purification of cardosins using ammonium sulfate as additive; and (ii) to characterize the action of the partially purified enzyme extracts on the profile of hydrolysis of the major whey proteins, *i.e.* α-La, β-Lg and BSA (qualitative action), and on the extent of caseinolysis (quantitative action). In order to maximize the information generated pertaining to the action of these enzymes, high resolution analytical techniques, e.g. Fast Protein Liquid Chromatography (FPLC) and electrophoresis (PAGE), were utilized.

2. Materials and methods

2.1. Substrates

A commercial whey protein concentrate (WPC), with an average protein content of 11% (w/w), was purchased from Sigma (St. Louis MO, USA), and used as standard substrate.

2.2. Chemicals

Azocasein, sodium dodecyl sulfate (SDS), β-mercaptoethanol, ammonium persulfate, TEMED (N′N′N′N-tetramethylethylenediamine), and molecular weight standards for Phast® electrophoresis (BSA-66 kDa, ovalbumin-45 kDa, glyceraldehyde-3-phosphate dehydrogenase-36 kDa, carbonic anhydrase-29 kDa, trypsinogen-24 kDa, trypsin inhibitor-20 kDa and α-La-14.2 kDa) were all purchased from Sigma; trichloroacetic acid (TCA), sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, citric acid monohydrate and extra pure sodium azide were obtained from Merck (Darmstadt, Germany). High density minigels, PhastGel SDS buffer strips, low molecular weight standards for SDS-PAGE (phosphorylase B-94 kDa, BSA-67 kDa, ovalbumin-43 kDa, carbonic anhydrase-30 kDa, soybean trypsin inhibitor-20.1 kDa and α-La-14.4 kDa) and molecular weight standards for FPLC (aldolase-158 kDa, BSA-67 kDa, ovalbumin-43 kDa, β-Lg-36 kDa, α-La-14.4 kDa and ribonuclease-13.7 kDa) were all purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Filter paper (0.22 μm) was purchased from Nalgene (New York NY, USA), whereas nonsterile filters (0.45 μm) were purchased from Nucleopore (Cambridge MA, USA). Tap water was purified in a Milli-Q Plus 185 system (Molsheim, France) to a final conductivity of *ca.* 18.2 $M\Omega.cm^{-1}$. The chemicals used were all analytical grade or better, and were employed without further purification.

2.3. Enzymes

A crude blend of cardosins A and B, as they occur in nature after aqueous extraction from flowers of *C. cardunculus*, was employed as catalyst. The crude enzyme extract was prepared by macerating *ca.* 7 g of stylets and stigmæ of dried flowers of *C. cardunculus* in 70 ml of 100 mM sodium citrate buffer (pH 3.0), as described by Faro (1991). Two enzyme preparations were then obtained from the supernatant via a two-step purification procedure. The supernatant was first precipitated using ammonium sulfate up to 30% saturation, kept at 4°C for 30 min, and then centrifuged (at 16 300 *g* and 4°C, for 10 min) using a Model Refrigerated RC-5C from Sorvall Instruments (Newtown CT, USA). To the new supernatant more ammonium sulfate was then added up to 70% saturation. After a further 30 min, the solution was centrifuged under the same conditions. Both precipitates were redissolved in water up to approximately twice the volume of the pellet, and dialyzed for 48 h at 4°C against a large volume of deionized water (to remove ammonium sulfate and citrate) using dialysis tubing with MW cutoff of 10 kDa (Sigma), and finally lyophilyzed. Determination of the total protein content of the enzyme extract and of the partially purified enzymes took advantage of the Micro Protein determination kit No. A-690 (Sigma).

2.4. Analytical equipment

All spectrophotometric readings were carried out using quartz cuvettes in an UV-VIS spectrophotometer from Shimadzu (Kyoto, Japan). The enzyme aqueous extract was lyophilyzed in a CHRIST ALPHA 1–4 freeze-dryer, from Braun Biotech (Braunschweig, Germany). High density electrophoretic gels were run using a PhastSystem unit (from Pharmacia LKB Biotechnology). SDS-PAGE was performed in a Protean II xi vertical slab-gel unit from Bio-Rad (Watford, UK) coupled with a model 1000/500 power supply also from Bio-Rad, and a refrigerated water bath from Julabo Labortechnik (Seelbach, Germany). All electrophoresis gels were scanned in a GS-700 Imaging Densitometer from Bio-Rad (Hercules CA, USA) using the Molecular Analyst software (Si 1.4) also from Bio-Rad. The FPLC system (Pharmacia LKB Biotechnology) consisted of two P-500 positive displacement pumps, an electrically powered MV-7 motorized valve, a gel filtration column pre-packed with Superose 12 HR 10/30, and an UVII single-path spectrophotometer monitor. Samples were previously

passed through a 0.45 μm filter and then injected through a 100 μl-loop.

2.5. Proteolysis assay

The enzyme-mediated hydrolysis was started with the addition of suitable quantities of enzyme extract so as to get the desired constant ratio of enzyme to substrate (WPC), on a protein weight basis, *viz.* 2/200 (w/w). The reactions were performed batchwise, in 50 ml of 100 mM citrate buffer (pH 5.2, 1 g_{WPC}/mL) at 37°C, in a water bath equipped with an orbital shaker set at 100 rpm. Samples were withdrawn in duplicate at selected time intervals, and duly prepared both for FPLC and SDS-PAGE analyses.

The proteolytic activity was evaluated using two alternative methods. The first was a modification of the method proposed by Tomarelli et al. (1949), which involves digestion of azocasein (obtained from a casein chromophore containing a dinitrogenated arylamine) and quantification of the proteolytic activity based on the peptides released as monitored by absorbance at 440 nm. In this case, the lyophilyzed crude extract and the two fractions precipitated therefrom were dissolved in 0.1 M phosphate buffer (pH 6.0) to produce the enzyme solution. This enzyme solution (0.01 g/mL) was mixed (0.01 ml) with 0.5 ml of 2%(w/v) azocasein and incubated at 25°C for 10 min; the reaction was then quenched by adding 0.5 ml of cold 5%(w/v) TCA. Unhydrolyzed proteins were then removed by centrifugation (using a Model Universal 32-refrigerator from Hettich, Tuttlingen, Germany) at 10 300 *g* and 4°C for 10 min. To intensify the azo-associated color, 1 ml of the supernatant was mixed with 1 ml of 0.5 M NaOH. A blank was prepared in a similar fashion, but with the addition of TCA before addition of enzyme.

The second procedure used a modification of the method by Kunitz (1947), which involves hydrolysis of bovine casein itself. Aliquots (1.9 ml) of 2%(w/v) casein in a 0.1 M phosphate buffer (pH 6.5) were pre-incubated at 30°C for 5 min, in a water bath equipped with an orbital shaker set at 100 rpm. The reaction was initiated by adding 0.1 ml of enzyme solution (0.01 g/mL), prepared as described above, but using 0.1 M phosphate buffer (pH 6.5). Samples (0.45 ml) were withdrawn in triplicate at selected time intervals, and the reaction was quenched by adding 0.9 ml of cold 5%(w/v) TCA. Unhydrolyzed proteins were then removed by centrifugation, at 14 900 *g* and 4°C for 10 min. A blank was prepared in the same manner but with addition of TCA before the enzyme was added. The enzyme activity was based on the amount of peptides released, as monitored by absorbance at 280 nm. For both methods, assays were performed in triplicate.

2.6. Coagulation assay

The clotting activity was determined using the procedure described in IDF-157 (1992). The lyophilyzed crude extract (0.05 g/mL) was dissolved in 10 mM $CaCl_2$ (pH 6.5); the clotting time was measured using 0.2 ml of each enzyme solution, which was mixed with 2 ml of reconstituted milk. One rennet unit (RU) was defined as the amount of crude extract needed to coagulate 10 ml of reconstituted low-heat skim milk powder (NILAC™, from NIZO, Ede, The Netherlands) at 30°C in 100 s (Berridge, 1945). The assay was carried out in triplicate.

2.7. Statistical Analysis

The data were assessed by analysis of variance (ANOVA) using the Excel™ software from Windows 95 v. 7.0; the results were considered significant if the associated *P* value was below 0.05; Student's *t*-tests were employed for comparison of means.

2.8. Electrophoretic characterization

High density gel electrophoresis was performed with a PhastSystem unit and appropriate minigels (50 mm high x 43 mm wide $\times$ 0.45 mm thick). For the preparation of samples, pre-determined amounts (*ca.* 0.01 g) of each (partly purified) cardosin extract were placed in separate eppendorf vials, and 100 μl of 10%(w/v) SDS was added (to eliminate shape and charge effects). The vials were subsequently heated at 90°C for *ca.* 10 min in a heating block, and then cooled to near room temperature. Approximately 4 μl of solution of denatured sample (or markers, as appropriate) were applied onto the gel surface. After electrical resolution of the sample proteins, the gels were transferred to the development section of the PhastSystem unit, and duly stained with Coomassie Blue R 250.

For the preparation of samples for SDS-PAGE, 500 μl of the reaction medium (withdrawn at regular time intervals) was poured into eppendorf vials, into which 100 μl of 10%(w/v) SDS was latter added. The vials were subsequently heated at 90°C for *ca.* 10 min in a boiling water bath, cooled to near room temperature and centrifuged at 10 300 *g* and 4°C for 10 min. A solution containing SDS, β-mercaptoethanol, sucrose and bromophenol blue was added to the supernatant of said samples, which were incubated in a heating block at 90°C for *ca.* 5 min. The 15%(w/v) polyacrylamide gel slabs (20 cm high x 16 cm wide x 0.75 mm thick), at pH 8.6 and containing 0.1%(w/v) SDS, were prepared and run for 10 h using the discontinuous buffer system of Laemmli (1970) at constant voltage (100 V), constant current (70 mA) and constant temperature (19°C); after this time, the gels were stained with Coomassie Blue R 250. Qualitative and quantitative analyses of the electrophoretograms produced were by densitometric scanning, using a green filter in a GS-700 imaging densitometer; the data were later subject to the Molecular Analyst™ software for image analysis. Each line was scanned twice.

2.9. Chromatographic characterization

The reaction samples were assayed by gel permation in an FPLC system, according to the procedure described by Pintado and Malcata (1996). Aliquots (1.5 ml) of the incubated solution were taken at 0, 0.5, 1, 2, 5, 8, 12 and 24 h, and the reaction therein was quenched by immersion in liquid nitrogen; said aliquots were stored at −20°C until use. Prior to chromatographic analyses, each sample was passed through a 0.45 μm filter (Nucleopore CA, USA), whereas the mobile phase was filtered through 0.22 μm paper filter (Nalgene). Aliquots (100 μl) were injected into the FPLC equipped with a Superose 12 column HR 10/30 (Pharmacia) and an UV-MII-detector (280 nm); the mobile phase was 150 mM NaCl in a 50 mM aqueous phosphate buffer (pH 7.0), containing 0.2 g L^{-1} NaN_3 as preservative, at a flow rate of 0.4 ml min^{-1} for 80 min. The retention times of the peaks obtained were compared with those of a mixture of molecular weight standards. Quantitative calibration of the column, in terms of bovine whey proteins, was performed using various dilutions of an aqueous solution containing 4.10 mg/mL of α-Lg, 9.10 mg/mL of α-La, 6.80 mg/mL of BSA, 0.10 mg/mL of orotic acid and 0.020 mg/mL of uric acid. The void volume of the column was previously determined using blue dextran (the retention time was *ca.* 19.5 min). All analyses were carried out in duplicate.

3. Results and discussion

3.1. Enzyme purification

The initial step in purification of the proteases present in the crude aqueous extract of *C. cardunculus* was based on salting out of proteins at two different levels of ammonium sulfate. The protein concentration obtained was 92, 410 and 197 $mg_{protein}$ g^{-1} in the lyophilyzed crude extract, and in the 30% and 70% saturation fractions, respectively. The associated SDS-PAGE electrophoretograms (Fig. 1) indicate that both precipitates were qualitatively similar, and in particular contained polypeptides with molecular weights of *ca.* 30 and 15, and of *ca.* 35 and 14 kDa, which correspond to the two subunits of cardosin A and B, respectively. The intensity of the band that is accounted for by cardosin A was higher in the 30% fraction than in the 70% one, whereas the other bands with similar mobilities in both fractions showed similar intensity as well. Furthermore, the fraction precipitated at 30% saturation exhibited two extra bands, characterized by molecular weights 20–26 kDa.

3.2. Quantitative activity

The coagulation activity in bovine milk, and the proteolytic activity on casein and azocasein of the two partially purified enzyme fractions are shown in Table 1. Although a model system more representative of the whey proteins might be preferable, no data are available in the literature pertaining to cardosins acting on whey proteins, so critical discussion based on comparison with other works would be impossible. The clotting activity is related to the enzyme's capacity to cleave κ-casein at the Phe105-Met106 peptide bond, which is the starting point of enzymatic cheesemaking. According to Veríssimo (1995), cardosin B has a broader specificity than cardosin A, although they share the same preference for peptide bonds with hydrophobic side chains. The statistical analysis (ANOVA) show that there were significant differences between the coagulant activity obtained for the crude extract and for the two other fractions ($P < 0.005$). The data also show that precipitation produced a slight decrease on the coagulant activity in the first fractionation step (*ca.* 13%), but a higher decrease in the second fractionation step (*ca.* 28%). The results also indicate that coagulation activity of the fractions at 30 and 70% saturation were significantly different ($P < 0.001$). This difference is confirmed by the lower intensity of the band corresponding to cardosin A in the 70% saturation fraction (Fig 1, lane 4) than in the 30% saturation one (Fig. 1, lane 3).

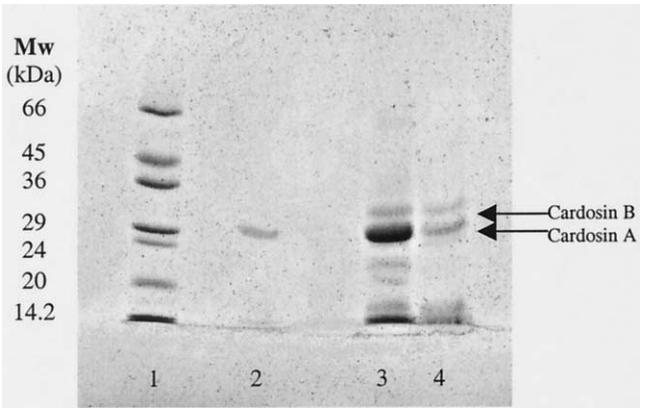

Fig. 1. High density electrophoretogram of the fractions obtained upon precipitation with ammonium sulfate. Lane 1: molecular weight (Mw) markers; lane 2: supernatant at 70% saturation; lane 3: fraction precipitated at 30% saturation; and lane 4: fraction precipitated at 70% saturation.

The data tabulated in Table 1 show that fractionation produces a decrease in the proteolytic activity relative to that of the crude extract. The higher decrease on proteolytic activity, *ca.* 88 and 84%, occurred for fractionation at 30% saturation, when tested with casein and azocasein, respectively. In both cases, the percent variation was much higher for specific proteolytic activity than for specific coagulant activity. The lower values for the specific activity at 30% when compared with those at 70% saturation fractions are concomitant with the presence of two extra bands, coupled with an apparently higher relative amount of cardosin A than cardosin B (Fig. 1). The differences between the three cases studied for proteolytic activity, either tested with casein or azocasein, were significant ($P < 0.0001$).

The analysis of the relative ratio of clotting activity to

Table 1
Enzymatic activity of ammonium sulfate-precipitated fractions, as compared with crude extract from *C. cardunculus* (thistle).

Specific activity*					
Thistle	Coagulant (c) (U/g)	Proteolytic (p)		Ratio c/p	
		Casein ($\Delta A_{280}/g/min$)	*Azocasein* ($\Delta A_{440}/g/min$)	*Casein* ($U*min/\Delta A_{280}$)	*Azocasein* ($U*min/\Delta A_{440}$)
Crude extract	103.6 ± 4.1	16.4 ± 1.3	1.53 ± 0.10	5.34 ± 0.72	68 ± 10
30% sat. fraction	90.6 ± 2.0	2.00 ± 0.14	0.242 ± 0.011	45.3 ± 6.6	378 ± 35
70% sat. fraction	74.6 ± 2.7	4.85 ± 0.21	0.791 ± 0.042	15.3 ± 1.7	94 ± 12

* Average values ±95% confidence interval.
Note: ΔA_{280} - variation of absorbance at 280 nm.
ΔA_{440} - variation of absorbance at 440 nm.

proteolytic activity (c/p), which is an useful indicator of protease adequacy for use as coagulant in cheesemaking, is underlaid in Table 1. When all enzyme preparations were compared which each other, ANOVA revealed that there are significant differences ($P < 0.0001$) in said ratio. Ammonium sulfate fractionation produced an increase of this ratio relative to that in the crude extract: the highest increase occurred at 30% saturation, which produced a 7-fold increase when casein was used as substrate ($P < 0.005$) and a 6-fold increase when azocasein was used instead ($P < 0.001$). At 70% saturation, the difference was also significant ($P < 0.005$) when comparison was made with the crude extract, but the rise of values for the c/p ratio was slightly lower, *viz.* 1.4- and 2.4-fold increase for casein and azocasein as substrate, respectively. Finally, when the c/p ratios obtained at 30% and 70% saturation were compared, the differences were again significant ($P < 0.005$). According to these results, the enzyme fractionate obtained at 30% saturation possesses the best c/p ratio for use as coagulant in cheesemaking; however, use in actual cheesemaking practice is worthy extra work for full validation of this statement.

3.3 Qualitative activity

The elution profile of the major whey proteins as incubation time elapses is shown in Fig. 2. The elution patterns are virtually identical, irrespective of preliminary precipitation of the crude extract with ammonium sulfate. These profiles reveal a decrease in concentration of α-La, whereas the concentration of β-Lg remains unaltered with incubation time.

The time course of the enzyme-mediated hydrolysis of whey proteins, brought about by the two different fractions and by the crude extract, as assessed by SDS-PAGE is available in Figs. 3 and 4. The action of enzymes precipitated at 30% or 70% saturation on the main whey proteins was rather similar to that of the crude extract. In all cases studied, α-La was never hydrolyzed in full, but it was possible to observe that the hydrolysis rate of α-La was slightly lower by the 30% than by the 70% saturation fraction (Figs. 3a, 4a). This situation was also apparent

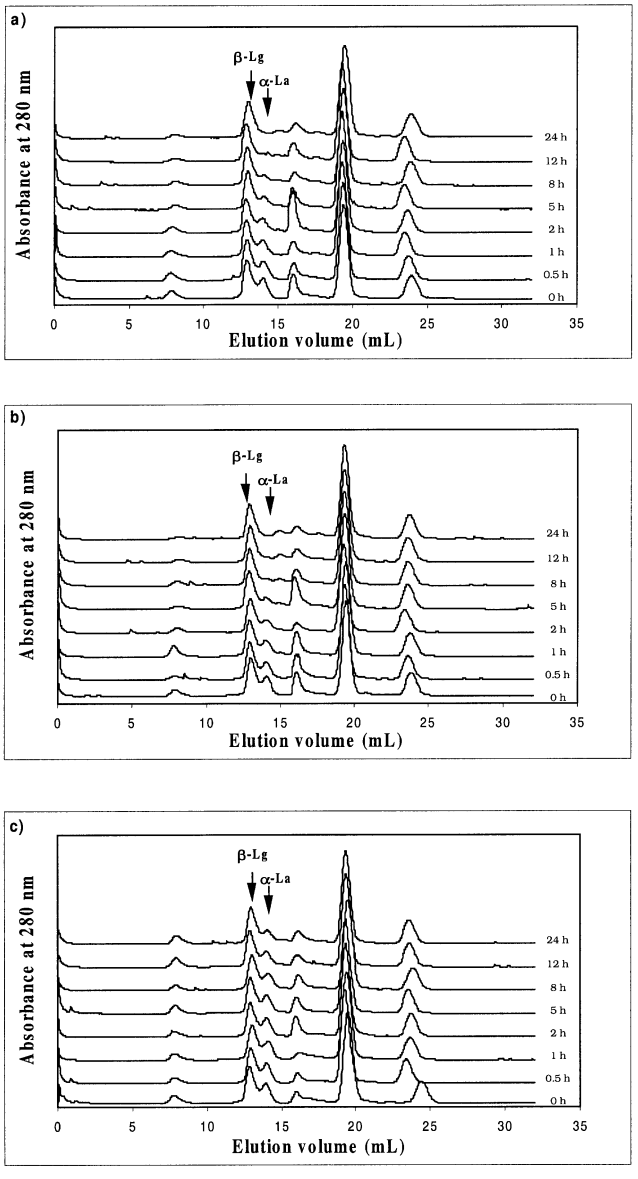

Fig. 2. FPLC profile of whey proteins after hydrolysis for 0, 0.5, 1, 2, 5, 8, 12 and 24 h, effected by the fraction of crude extract precipitated at (a) 30% and (b) 70% saturation with ammonium sulfate, and by (c) the crude extract.

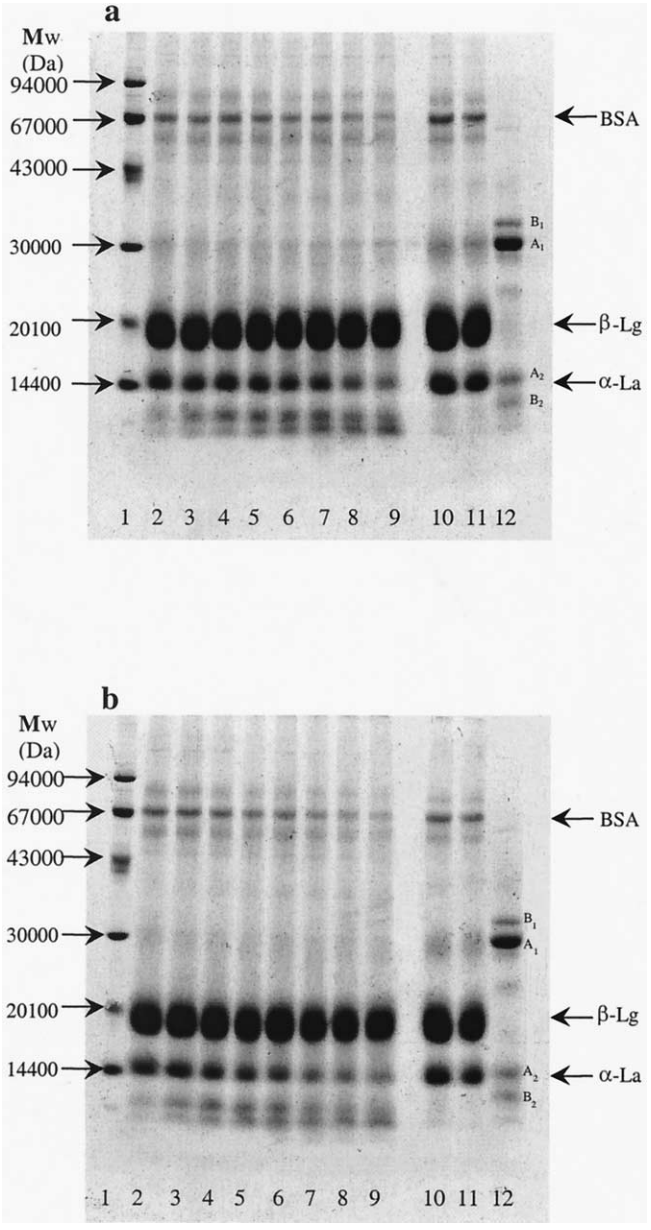

Fig. 3. SDS-PAGE electrophoretograms of the hydrolyzates produced by the fraction precipitated at 30% (a) and 70% (b) saturation with ammonium sulfate. Lane 1: molecular weight (Mw) markers; lane 2–9: samples withdrawn at 0, 0.5, 1, 2, 5, 8, 12, and 24 h of incubation, respectively; lanes 10–11: control without enzyme, at 0 and 24 h, respectively; and lane 12: crude extract: cardosin A (subunits A_1 and A_2) and cardosin B (subunits B_1 and B_2).

when the 30% and 70% saturation fractions were tested with casein and azocasein as substrates (see Table 1). Conversely, BSA was poorly hydrolyzed during the same time frame; finally, β-Lg remained essentially intact by 24 h of hydrolysis. In general, α-La was hydrolyzed up to 90% within 24 h, whereas BSA was hydrolyzed only to 65–70% (see Figs. 4a, 4c). These observations are a clue to the higher affinity of cardosins A and B for α-La and, to a lesser extent, BSA. Cardosins are indeed known to cleave peptide bonds between amino acid residues with large hydrophobic

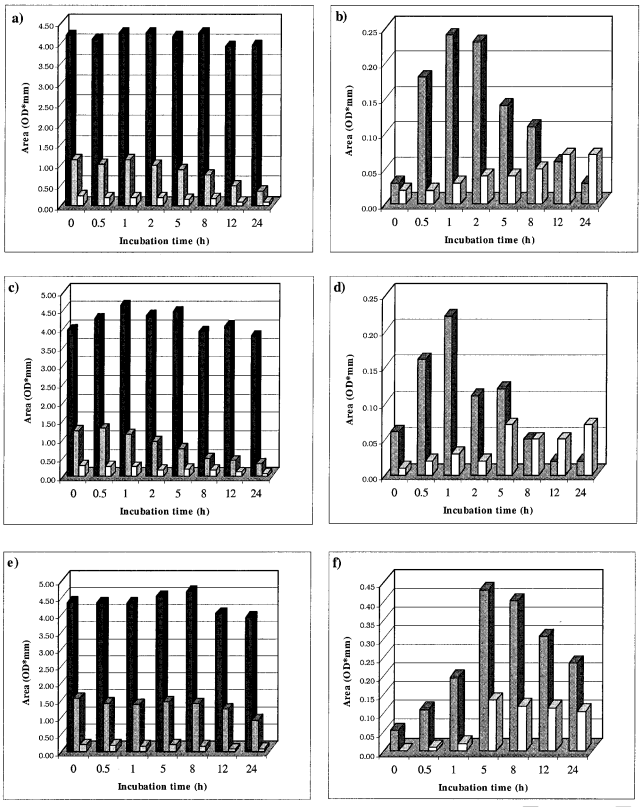

Fig. 4. SDS-PAGE evolution of the concentration of β-Lg (■), α-La (▨) and BSA(□) with incubation time, when acted upon by the fraction precipitated at (a) 30% saturation and at (c) 70% saturation with ammonium sulfate, and by (e) the crude extract; the two main peptides formed, with molecular weights 6 kDa (▩) and 3 kDa (□), by the action of the fraction precipitated at (b) 30% saturation and at (d) 70% saturation, and by (f) the crude extract are also shown.

side chains, such as Phe and Leu (Webb, 1991); the three-dimensional structure of native α-La (Warme et al., 1974; Acharya et al., 1990) encompasses a large hydrophobic area on the surface of the protein.

The electrophoresis patterns of the enzyme-mediated hydrolysis of whey proteins produced via the action of the two distinct fractions were similar to each other (Fig. 3); the enzymes present in either fraction acted mainly on α-La (as already discussed), which led to generation of medium- and low-molecular weight peptides (Fig. 4). The primary breakdown peptides accounted for two bands, with molecular weights between 3 and 6 kDa; as hydrolysis time elapsed, these peptides were gradually broken down to smaller ones (Fig. 3). In Figs. 4b and 4d, the band associated with the 6 kDa-fragment became thicker by 1 h and then gradually disappeared; conversely, the band associated with the 3 kDa-fragment increased in intensity as hydrolysis time elapsed.

4. Conclusions

Precipitation with ammonium sulfate is an effective way to produce substantial amounts of active proteases from the

flowers of *C. cardunculus.* Said precipitation produces a decrease of coagulant and proteolytic specific activities when azocasein or casein are used as substrate, but contributes to higher relative ratios of coagulant to proteolytic activities, which improves clotting capacity for actual cheesemaking. The casein and azocasein substrates correlate well with one another, so they can interchangeably be used in monitoring proteolytic activity. The major whey proteins were differently acted upon by the enzymes under consideration: α-La was hydrolyzed to a higher extent than BSA, and to a much higher extent than β-Lg for the same incubation time.

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