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Isolation and Partial Characterization of Rennet-like Proteases from Australian Cardoon (*Cynara cardunculus* L.)

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The yield, protein content, proteolytic activity, and substrate specificity of crude and partially purified extracts from dried and fresh Australian cardoon (*Cynara cardunculus* L.) flowers were determined. Crude water extracts had high yield but low protein content and proteolytic activity, whereas citric acid extracts had low yield but high protein content and proteolytic activity. Fresh flower extracts gave higher yield and proteolytic activity but lower protein content in comparison with dried flower extracts. Purification with ammonium sulfate resulted in significantly increased proteolytic activity for water extracts from both fresh and dried cardoon flowers, whereas the proteolytic activity of citric acid extracts did not change significantly after purification. Irrespective of extraction method, all extracts had higher proteolytic activity against ovine whole and κ-caseins compared to their bovine counterparts, showing optimal activity at 37 °C and pH 6.0. Separation of purified extracts by ion-exchange liquid chromatography yielded three active fractions, each of which when assayed with sodium dodecyl sulfate capillary electrophoresis revealed two subunits with molecular masses of 15.5 and 33.1 kDa, respectively.

KEYWORDS: Cardoon (*Cynara cardunculus* L.); caseinolytic activity; enzyme purification; plant rennet

INTRODUCTION

Cynara cardunculus L. (cardoon) is a variety of thistle, which grows wild and abundantly in uncultivated areas of many Mediterranean regions such as Portugal, North Africa, the Canary Islands and southern Spain. In many of these regions, the dried flowers are used as milk coagulants for the manufacture of traditional cheese varieties (*1*). Incidentally, the cardoon plant (also called wild artichoke), a member of the Compositae family, is a noxious weed found in high concentration in the southern Australian states of Victoria and South Australia. Research has shown that the type and activity of the caseinolytic fractions in the extracts differ, depending on extraction methods, whether dried or fresh flowers were used, the part of the plant extracted, and the location of the plant. For example, Heimgartner et al. (*2*) reported the isolation, under alkaline conditions (pH 8.3), of three aspartic proteases (called cynarases) with different caseinolytic and milk clotting properties from dried cardoon flowers. Furthermore, different electrophoretic profiles from fresh and dried flower extracts were observed. Comparably, two distinct aspartic proteases were isolated by using citric acid (pH 3.0) from fresh cardoon flowers, which were named cardonsins A and B (*3*, *4*). Cardosin A accounted for 75−90% of total enzymes and had chymosin-like specificity, whereas cardosin B (10−25%) had pepsin-like specificity. The two enzymes appeared in the female part (the upper area) of the flowers, whereas in the lower part of the flowers only cardosin B was present (*5*). Extracts isolated from plants collected at different locations have also been shown to possess different proteolytic and milk clotting activities (*6*). Furthermore, Sousa and Malcata (*1*) reported that processing conditions, including pH, salt concentration, and extent of homogenization, affected the properties of the crude extracts from dried flowers. No information is available on the isolation and properties of extracts obtainable from Australian cardoon. Furthermore, there has not been any comprehensive study comparing the effects of extraction media on isolation and properties of crude and purified extracts of dried and fresh flowers from any location.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been the most commonly used method for separating proteins of cardoon extracts (*2*, *4*, *6*). Capillary electrophoresis (CE) is a relatively new method of protein and peptide analysis, but it is gaining increasing popularity due to its reliability and short analysis time. There is no published material available on the use of CE to analyze profiles of cardoon extracts.

This study describes the isolation, purification, fractionation, and properties of proteases from Australian cardoon. Extracts of fresh and dried cardoon flowers were prepared using both water and citric acid solution as extraction medium, purified by fractional precipitation with ammonium sulfate and fractionated by ion-exchange liquid chromatography. The yield, protein content, and caseinolytic activity of the crude and purified extracts on different caseins from ovine and bovine sources were then compared. The purified extracts were also examined by CE, and the molecular masses of the isolated proteases were determined by SDS-CE.

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MATERIALS AND METHODS

Collection of Cardoon Flowers and Leaves. Cardoon flowers and leaves were collected from a single paddock in Werribee, VIC, Australia, during January−February 1998, 1999, and 2000. Both fresh flowers and flowers that had naturally dried on the plant were collected. Fresh flowers had an average moisture content of 63% (w/w) and the dried flowers, 16% (w/w). The flowers and leaves were transported to our laboratory in ice-packed containers and stored in a freezer (−20 °C) until processed. Dry matter content of both fresh and dried flowers and leaves was determined by accurately weighing and drying a representative sample (~10 g) of the plant material to a constant weight in a forced-draft oven set at 100 ± 2 °C.

Chemicals and Reagents. All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich Pty. Ltd., Sydney, Australia, unless otherwise stated. Cellulose dialysis tubing with a molecular mass cutoff at 12000 Da was purchased from the same company and was used for all dialysis studies.

Preparation of Whole and κ-Casein from Ovine and Bovine Milk. Raw bovine milk was collected from the Charles Sturt University dairy farm, located at the university's Wagga Wagga campus, NSW, Australia, and ovine milk (from Awassi breed) was collected from Awassi Australia Pty. Ltd., Cowra, NSW, Australia.

Raw bovine or ovine milk was centrifuged at 2500*g* and 4 °C for 20 min to remove fat. The resultant skim milk was adjusted to pH 4.6 at room temperature with the addition of 1 M HCl to precipitate caseins. The casein precipitate was separated by centrifugation for 10 min at 2500*g* and 25 °C, washed twice with deionized water, and freeze-dried using a Christ Alpha 1-4 freeze-dryer (Biotech International). Bovine κ-casein and ovine κ-casein were prepared from whole casein according to the method of Zittle and Custer (*7*).

Preparation of Crude Cardoon Extracts. The crude cardoon extracts were prepared from all plant materials following the method of Faro et al. (*3*) with slight modifications. Leaves, whole flowers, and styles of flowers of cardoon were adjusted to the same dry matter content. Deionized water or 0.1 M citric acid (pH adjusted to 3.5 with 5 M NaOH) was added at a sample to extraction media ratio of 5 g/95 mL for fresh or 2 g/ 98 mL for dried materials, respectively. The mixtures were allowed to stand for 1 h, after which time the samples were homogenized with an Ultra-Turrax T25 homogenizer (Janke and Kunkel). The homogenates were allowed to stand for a further 2 h, after which time they were filtered with glass wool and then centrifuged at 2500*g* for 10 min. The supernatants were filtered through Whatman no. 1 filter paper and dialyzed for 24 h to give crude extracts.

Partial Purification of the Crude Extracts. Aliquots of the crude extracts (95 mL) were partially purified by fractional precipitation at increasing ammonium sulfate concentrations of 30, 50, and 80% (w/v) as described by Campos et al. (*8*). The precipitates at each salt concentration were collected individually, and each resultant supernatant was used for the next precipitation. The precipitates were pooled, dissolved in 95 mL of deionized water, dialyzed for 24 h, and freeze-dried.

Yield of Extractable Matter and Protein Assay. Both the crude and purified extracts were freeze-dried, and the yield of extractable matter was calculated as the dry weight. The protein content of the extracts was determined using the AOAC (*9*) method (no. 997. 09) on a Leco CNS-2000 (FP-2000) system.

Assay of Caseinolytic Activity. The activity of the extracts was assayed following the method described by Sousa and Malcata (*1*) with slight modifications. Aliquots (0.15 mL) of crude and purified extracts, which were adjusted to the same concentration (0.05 mg of dry extract/mL of substrate), were added to 2.85 mL of a 0.2% (w/w) solution of caseins (ovine or bovine whole and κ-caseins) in 0.1 M sodium phosphate buffer (pH 7.0). Each mixture was then incubated at 37 °C in a water bath for 30 min. Samples (0.3 mL) were withdrawn at regular intervals (5, 10,15, 20, 25, and 30 min) and added to 2 mL of *o*-phthaldialdehyde (OPA) stock solution (described below). The absorbance of the mixture was measured immediately at 340 nm in 10 mm quartz cuvettes against a deionized water blank.

The OPA stock solution was prepared on a daily basis by mixing 50 mL of 0.1 M sodium tetraborate with 10 mL of 10% (w/w) sodium dodecyl sulfate (SDS) solution, 80 mg of OPA previously dissolved in 2 mL of methanol, and 0.2 mL of β-mercaptoethanol (β-Me). The mixture was then made up to a final volume of 100 mL with deionized water. A substrate and enzyme control was prepared by adding 0.015 mL of extract to 2 mL of OPA stock solution to terminate the enzyme activity before 0.285 mL of 0.2% casein solution was added. The absorbance of the control was measured, and the corrected absorbance was thus calculated. Caseinolytic activity was expressed as the corrected absorbance at 340 nm. This is the convention used in the published literature (*1*, *10*) and was adopted in this study to facilitate comparison of the results. A unit of enzyme activity was defined as that which caused an increase of one absorbance unit at 340 nm.

To determine the effect of temperature on caseinolytic activity, each cardoon extract was incubated with ovine whole casein in 0.1 M sodium phosphate buffer (pH 7.0) at 25, 27.5, 30, 32.5, 35, 37.5, and 40 °C, respectively, for 30 min. The effect of pH on caseinolytic activity was determined at 37 °C, and the extracts were incubated with ovine whole casein in 0.1 M sodium phosphate buffer for 30 min with pH adjusted to 4.5, 5, 5.5, 6, 6.5, 7, and 7.5. Samples were withdrawn after 30 min and assayed as described earlier.

Separation of Active Fractions from Purified Extracts by Liquid Chromatography (LC). The purified extracts were further fractionated by LC using a DEAE-Sepharose CL-6B ion-exchange column (Pharmacia) according to the method of Heimgartner et al. (*2*) with slight modifications. Elution was achieved using a gradient of 0.1 M NaCl in 0.05 M Tris buffer (pH 8.3). Proteins were eluted with increasing concentrations of NaCl according to the following regimen at a flow rate of 3 mL/min: 0−2 min, 0.00 M; 2−4 min, 0.00−0.20 M; 4−18 min, 0.20 M; 18−23 min, 0.20−0.30 M; 23−38 min, 0.30 M; 38−43 min, 0.30−0.45 M; 43−53 min, 0.45−0.60 M; 53−58 min, 0.60 M; 58−60 min, 0.60−1.00 M; 60−72 min, 1.00 M; 72−90 min, 0.00 M. All peaks were collected and assayed for caseinolytic activity as described earlier. Active fractions were pooled and dialyzed against deionized water for 24 h.

CE of Active Fractions of Cardoon Extracts. CE of crude and partially purified cardoon extracts was performed on a Beckman P/ACE system 5510 capillary electrophoresis unit according to the procedure described by Sheikh and Basha (*11*). Sample extracts were passed through a 0.45 μm filter and pressure injected for 10 s. Separation was carried out in an uncoated fused-silica capillary (75 μm i.d. × 57 cm) at 25 °C using a voltage of 10 kV for 10 min. Peaks were detected at 214 nm and data processed with the P/ACE system 5000 series software. The capillary was rinsed sequentially between successive runs with 0.1 M sodium hydroxide (4 min), 0.1 M HCl (4 min), deionized water (5 min), and 0.3% (w/v) sodium borate buffer containing 6 M urea (pH 8.3) for 2 min.

The molecular mass of the active fractions was determined using the Beckman SDS protein size standard kit (SDS14-200 kit). Orange G dye was used as the reference marker, and filtered (0.45 μm) samples of pooled active fractions from LC analysis were injected for 30 s. Separation was carried out in a coated fused-silica capillary (100 μm i.d. × 47 cm) at 25 °C using a voltage of 14.5 kV, and the detector was set at 214 nm. Data processing was carried out as described earlier, and the capillary was rinsed between successive electrophoretic runs with 1 M HCl solution for 3 min. The molecular masses of separated proteins were calculated by plotting the log of the molecular mass of the protein standards against the reciprocal of the relative migration time (1/RMT).

CE Analysis of Casein Hydrolysates. Casein hydrolysates were prepared following the method of Sousa and Malcata (*1*) with slight modifications. Solutions (0.8% w/v) of the ovine and bovine whole caseins were prepared in 0.1 M sodium phosphate buffer (pH 6.7). Aliquots (5 mL) of partially purified water extract of fresh cardoon flower (PFW) were added to 105 mL of the solution, and the mixtures were incubated at 37 °C in a water bath for up to 4 h. Samples were withdrawn (30 mL) at 30 min, 2 h, and 4 h intervals, and hydrolysis was terminated by heating samples to 85 °C and holding at this temperature for 10 min. The samples were freeze-dried and analyzed by CE as described below.

CE sample stock solutions were prepared by dissolving 50 mg of the freeze-dried hydrolysates in 5 mL of 0.1 M sodium phosphate buffer (pH 6.7). Before use, the casein hydrolysate solutions were diluted (1:

Table 1. Yield of Crude Extractable Matter from Different Parts of the Cardoon Plant[a]

extract[b]	pH of extraction medium	leaves (g/g of dry matter)	whole flowers (g/g of dry matter)	flower styles (g/g of dry matter)
FW	7.0	0.05 ± 0.01	0.17 ± 0.01	0.36 ± 0.01
FC	3.5	0.04 ± 0.01	0.11 ± 0.01	0.14 ± 0.01
DW	7.0	0.04 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
DC	3.5	0.03 ± 0.01	0.06 ± 0.01	0.08 ± 0.01

[a] Results are mean values of three experiments, with standard deviation. [b] FW, extract of fresh cardoon parts with deionized water; FC, extract of fresh cardoon parts with citric acid solution (0.1 M, pH 3.5); DW, extract of dry cardoon parts with deionized water; DC, extract of dry cardoon parts with citric acid solution (0.1 M, pH 3.5).

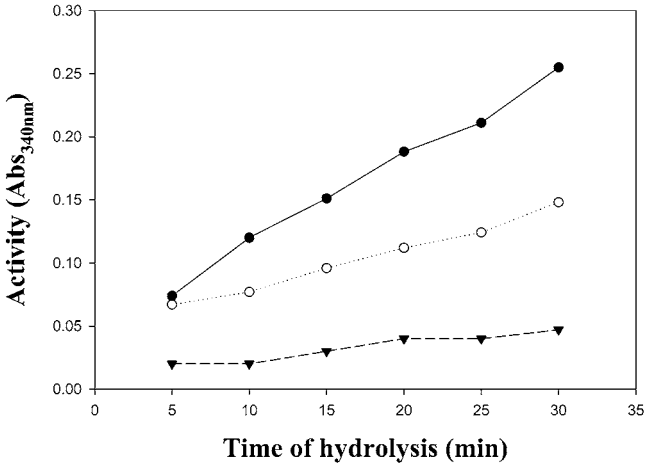

Figure 1. Caseinolytic activity of water extracts from different parts of the fresh cardoon plant (with ovine whole casein used as substrate): (●) styles; (○) whole flowers; (▼) leaves.

1.2) with the sample buffer, consisting of 0.1 M sodium phosphate, 8.4 M urea, and 20 mM dithiothreitol (DTT).

CC of hydrolysates of ovine and bovine whole caseins was performed with the same Beckman P/ACE unit. The method of separation used was as described by Kristiansen et al. (*12*). Separation was carried out in an untreated fused-silica capillary, externally coated with polyimide, with 75 μm i.d., 57 cm total length, and 50 cm to the detector. Samples were injected by pressure for 10 s each and separated at a constant voltage of 14.1 kV. On-line detection was achieved with an ultraviolet detector set at 214 nm. The run buffer consisted of 0.1 M sodium phosphate and 4 M urea, with the pH adjusted to 7.3 with 0.1 M NaOH.

RESULTS AND DISCUSSION

Yield and Caseinolytic Activity of Extracts from Different Parts of Cardoon Plant. To explore the presence of rennet-like proteases in different parts of the cardoon plant, extraction of caseinolytic enzymes was carried out on cardoon leaves, whole flowers, and styles of flowers, and their activities were compared. **Table 1** shows the yield of extractable matter obtained from different parts of the cardoon plant, whereas **Figure 1** shows the caseinolytic activity of the water extracts.

Of the three parts of the cardoon plant investigated, styles of cardoon flowers gave the highest yield followed by whole flowers. The yield from the leaves was the lowest, being generally several times lower than that of the styles and whole flowers. This trend was consistent regardless of the type of plant materials used (fresh or dried) and the pH of the extraction medium.

The yield of extracts was affected by the materials used, with the fresh parts giving higher yields than their dried counterparts. The effect of extraction medium on the yield of the extracts of different parts of cardoon plant was variable. Whereas the effect of extraction buffer on the yield of extracts from cardoon styles and whole flowers was significant ($P < 0.05$), especially for the styles, it was found not to affect the yield of the leaf extract significantly. This is probably because of the generally low yield of leaf extracts. For whole flowers and styles of flowers, significantly ($P < 0.05$) higher yields were obtained when extraction was performed with deionized water (pH 7.0) than with citric acid solution (pH 3.5). These extraction media have been used on cardoon flowers by other investigators (*1*, *3*, *4*, *8*).

Table 2. Yield and Protein Content of Extracts of Cardoon Flowers[a]

cxtract[b]	pH of extraction medium	yield (g/g of dry matter)		protein content (g/g of yield)[d] of	
		crude	purified[c]	crude extracts	purified extracts
FW	7.0	0.36 ± 0.01	0.18 ± 0.01	0.11 ± 0.02	0.37 ± 0.02
FC	3.5	0.14 ± 0.01	0.12 ± 0.01	0.55 ± 0.04	0.46 ± 0.03
DW	7.0	0.11 ± 0.01	0.08 ± 0.01	0.13 ± 0.02	0.33 ± 0.02
DC	3.5	0.08 ± 0.01	0.08 ± 0.01	0.62 ± 0.04	0.59 ± 0.03

[a] Results are mean values of three experiments, with standard deviation. [b] FW, extract of fresh flowers with deionized water; FC, extract of fresh flowers with citric acid solution (0.1 M, pH 3.5); DW, extract of dry flowers with deionized water; DC, extract of dry flowers with citric acid solution (0.1 M, pH 3.5). [c] Crude extracts were partially purified by fractional precipitation with 30, 50, and 80% $(NH_4)_2SO_4$. Precipitates were pooled, freeze-dried, and assayed. [d] Grams of protein per gram of extract.

The activities of extracts from the three different parts of the cardoon plant differed significantly ($P < 0.05$) as shown in **Figure 1**. The extract of styles of cardoon flowers exhibited a much higher activity than the extract of whole flowers, whereas the extract of cardoon leaves showed very little activity. Similar trends were found for dry plant parts extracted with either water or citric acid solution and for fresh plant parts extracted with citric acid solution as well (data not shown). This agreed with the finding of Verissimo et al. (*13*), who reported that caseinolytic enzymes accounted for >60% of the total protein present in mature stigmas. This also agreed with Castanheira (*5*), who reported that two enzymes (named cardosins A and B) appeared chiefly in the female part of the flowers of *C. cardunculus* L., that is, in the upper area of the flowers. Because only the extracts of styles showed high caseinolytic activity, subsequent work was focused on them.

Yield and Protein Content of Crude and Partially Purified Extracts of Cardoon Flowers. The effect of raw material, extraction medium, and partial purification on the yield of extractable matter of cardoon flowers and protein content of the extracts was determined, and the results are presented in **Table 2**.

Generally, for both fresh and dried flowers, extraction with water gave a greater yield of crude extract than extraction with citric acid. Specifically though, the yield of the crude extracts of fresh flowers extracted with water (FW) was by far the highest, being 2−4 times higher than that of the other three extracts. This was followed by the citric acid extract of fresh flowers (FC) and the water extract of dried flowers (DW), whereas dried flowers extracted with citric acid (DC) gave the lowest yield. Comparatively, the yield of dry flowers was much lower than that of fresh flowers for both water and citric acid extracts. This was probably due to the fact that extractable materials in the cardoon flowers were gradually degraded and lost during senescence and the natural drying-out process and, consequently, the amount of extractable matter was relatively low in dried flowers. Similar differences in extractable matter between fresh and dried flowers have also been reported (*2*, *13*).

The yields of purified water extracts of dried and fresh cardoon flowers were 0.08 and 0.18 g/g of dry matter,

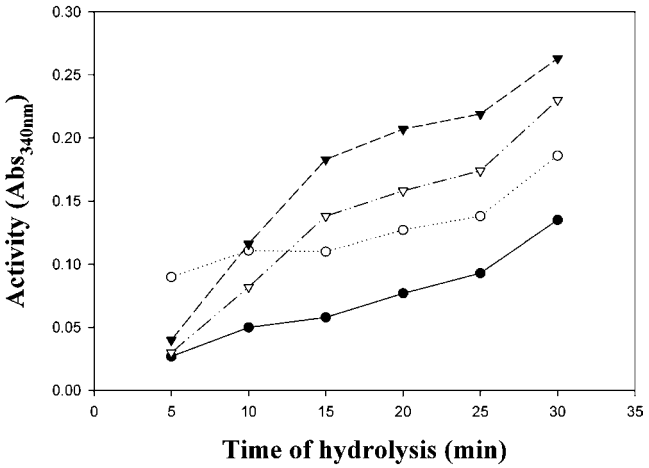

Figure 2. Caseinolytic activity of crude extracts of cardoon flowers with ovine whole casein as substrate: (○) FW; (●) DW; (▼) FC; (▽) DC. Abbreviations as in **Table 2**.

respectively. These were significantly ($P < 0.05$) lower than the yield of crude water extracts of dried and fresh flowers (0.11 and 0.36 g/g of dry matter, respectively). In contrast, there were no significant differences ($P > 0.05$) between the yield of purified citric acid extracts of dried and fresh cardoon flowers at 0.08 and 0.12 g/g of dry matter and that of their crude counterparts at 0.08 and 0.14 g/g of dry matter, respectively.

In contrast to having the lowest yield of extractable matter, the citric acid extract of dried flowers had the highest protein content (0.62 g/g of yield). This was followed by the citric acid extract of fresh flowers (0.55 g/g of yield) and water extract of dried flowers (0.13 g/g of yield), whereas the water extract of fresh flowers had the lowest protein content at 0.11 g/g of yield. Comparatively, the protein content of citric acid extracts (regardless of the cardoon flowers being fresh or dried) was much higher (~5 times) than that of water extracts. This was most likely due to the elimination of nonprotein components at low extraction pH when the citric acid solution was used as the extraction medium. Furthermore, for both water and citric acid extracts, protein content was higher in the extracts of dried flowers than in those of fresh flowers. These trends were exactly the opposite of the trends for the yield of extractable matter (**Table 1**). This was most likely due to the fact that dried flowers were subject to a considerable degree of protein degradation that occurs during the senescence and the natural drying-out process (*2*, *13*). Such degradation could make the protein and the degradation products more soluble (thus extractable).

The partial purification procedure affected the protein content of the extracts differently. The protein contents of partially purified water extracts of fresh and dried flowers were 0.37 and 0.33 g/g of yield, respectively. These were ~3 times higher than that of their crude extract counterparts. In contrast, the protein contents of partially purified citric acid extracts of fresh and dried were at 0.46 and 0.59 g/g of yield, respectively, which were, in general, slightly lower than that of their crude counterparts (**Table 2**). This was again likely due to, and perhaps a further proof of, the citric acid extracts' being purer in that they contained less nonprotein materials than water extracts. As a result, purification by ammonium sulfate precipitation, which is effective mostly on proteins (*8*), would not be as effective on water extracts. The decrease in the protein content was probably due to some of the protein being lost (e.g., due to incomplete precipitation) during the partial purification process.

Caseinolytic Activity of Crude and Purified Cardoon Flower Extracts. Figure 2 shows the ovine caseinolytic activities of crude extracts prepared from fresh and dried cardoon flowers using water and citric acid solution as extraction media. The caseinolytic activity of crude extracts of cardoon flowers was affected by both the extraction medium and the raw material used (fresh or dried flowers). When the same raw material (fresh or dry flowers) was used, citric acid extracts gave higher activity than water extracts, whereas with the same extraction medium, extracts of fresh flowers gave higher activity than their dried flower counterparts. Thus, the highest activity was given by FC with an absorbance value of 0.25 after 30 min of hydrolysis, followed by DC at 0.22 and FW at 0.18, whereas the lowest activity was given by DW at 0.14. Similar trends were observed for the caseinolytic activity of crude cardoon extracts using either ovine or bovine whole casein as substrate. This was consistent with the higher protein content of citric acid extracts. It also agreed with the observation of Sousa and Malcata (*1*) that the processing pH (3, 5, or 7) of the aqueous solution utilized in the extraction had the most significant effect on the final specific activity of the extracted enzyme.

Figure 3 compares the ovine caseinolytic activity of crude and partially purified extracts of cardoon flowers. As can be seen, purification had a positive effect on the activity of water extract of fresh flowers, almost doubling the activity of its crude counterpart at the end of the 30 min period of hydrolysis. In contrast, the effect of purification on the activities of citric acid

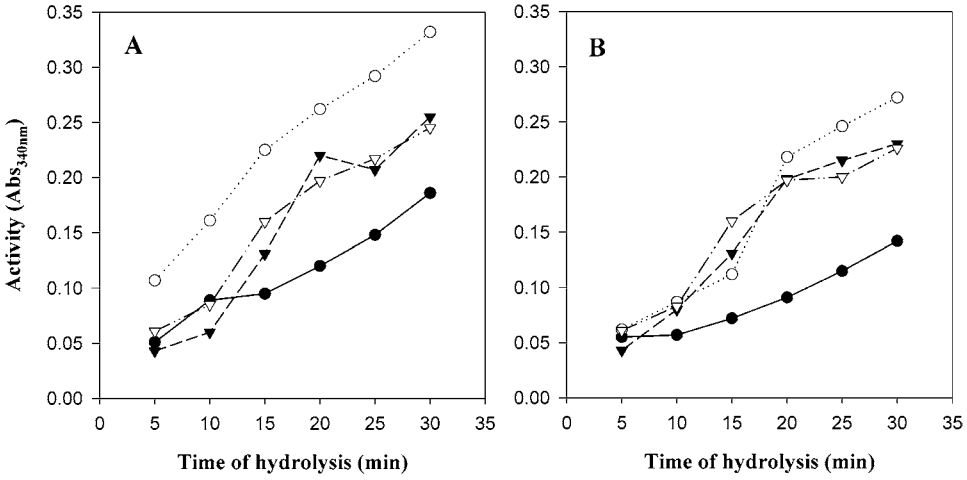

Figure 3. Effect of partial purification on the ovine caseinolytic activity of extracts of fresh (A) and dry (B) cardoon flowers: (●) crude water extracts; (▲) crude citric acid extracts; (○) partially purified water extracts; (△) partially purified citric acid extracts.

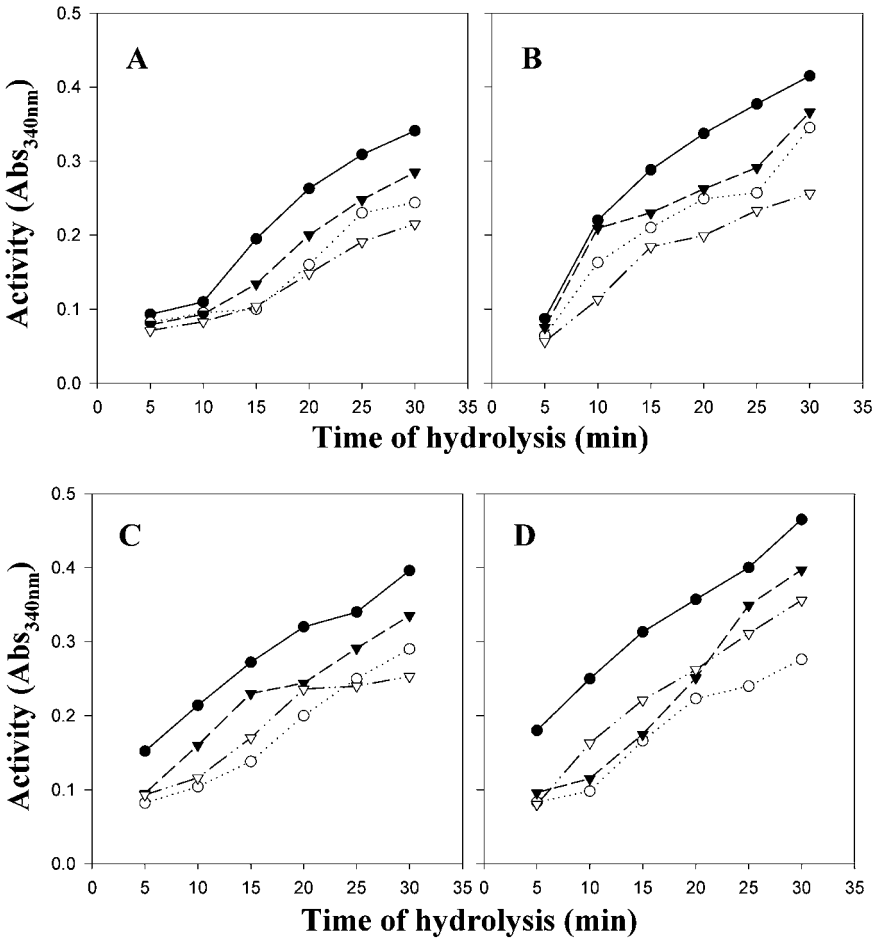

Figure 4. Caseinolytic activity of partially purified cardoon extracts against different substrates [(A) bovine whole casein, (B) bovine κ-casein, (C) ovine whole casein, and (D) ovine κ-casein]: (●) PFW; (▲) PFC; (○) PDW; (△) PDC. Abbreviations as in **Table 3**.

Table 3. Caseinolytic Activity of Cardoon Flower Extracts against Different Substrates[a]

	casein substrate			
extract[b]	ovine κ-casein	ovine whole	bovine κ-casein	bovine whole
FW	0.26 ± 0.02	0.21 ± 0.03	0.22 ± 0.01	0.20 ± 0.02
FC	0.41 ± 0.02	0.30 ± 0.05	0.32 ± 0.05	0.29 ± 0.02
DW	0.18 ± 0.01	0.13 ± 0.04	0.14 ± 0.02	0.12 ± 0.02
DC	0.34 ± 0.02	0.26 ± 0.01	0.27 ± 0.01	0.25 ± 0.02
PFW	0.47 ± 0.02	0.40 ± 0.02	0.42 ± 0.04	0.34 ± 0.03
PFC	0.40 ± 0.03	0.34 ± 0.02	0.35 ± 0.06	0.29 ± 0.03
PDW	0.36 ± 0.03	0.29 ± 0.04	0.37 ± 0.01	0.24 ± 0.05
PDC	0.28 ± 0.01	0.25 ± 0.03	0.26 ± 0.02	0.22 ± 0.03

[a] Results are mean values of three experiments, with standard deviation. The caseinolytic activities were recorded after 30 min of hydrolysis. [b] Crude extract abbreviations as in **Table 1**. The prefix "P" denotes partially purified counterparts of the crude extracts using ammonium sulfate precipitation as defined in **Table 2**.

extracts of fresh flowers was insignificant, and the crude and purified extracts gave very similar caseinolytic activities, especially at the later stages of the reaction. The effect of purification on the activities of dried flower extracts showed a similar trend; that is, a much higher activity was observed for purified water extracts than for crude water extracts, whereas there were no significant changes in the activity of citric acid extracts of dried flowers after purification.

Substrate Specificity of Crude and Partially Purified Cardoon Extracts. To investigate the specificity of these extracts, their activity against whole and κ-caseins from ovine and bovine milk was assayed. Results of the activities after 30 min of hydrolysis with both crude and partially purified extracts are shown in **Table 3**, whereas **Figure 4** shows the effect of the partially purified extract of cardoon flowers (PFW) on the four substrates. It was apparent that the extracts exhibited generally higher activities against the κ-caseins compared to the whole caseins. This was further supported by CE analysis of the hydrolysates, which showed that both the bovine and ovine κ-casein peaks had already disappeared after 30 min of hydrolysis but α- and β-caseins were broken down much less extensively (electrophoretograms not shown). This is in very close agreement with Faro et al. (*3*) and Verissimo et al. (*4*). Cordeiro et al. (*6*) also reported that κ-casein was more susceptible to hydrolysis by cardoon rennet than either α_s- or β-casein. The lower activity of cardoon extracts against whole casein was likely due to its relatively low concentration of the more susceptible κ-casein substrate.

The results also indicated that the extracts, especially the partially purified ones, exhibited significantly ($P < 0.05$) higher activities on ovine whole casein than on bovine whole casein (**Table 3**). Swaisgood (*14*) and Kalantzopoulos (*15*) reported that the proportion of κ-casein was higher in ovine (11−12%) than in bovine whole casein (9%). According to Anifantakis (*16*), α_{s1}-, α_{s2}-, β-, and κ-caseins are present roughly in 4:1:4:1 ratios in bovine casein and 1.8:1.8:5.0:1.4 ratios in ovine casein. As the cardoon rennet showed a higher activity toward κ-casein compared to the α- and β-caseins (*17*), the higher proportion of κ-casein in ovine whole casein would thus explain the higher activity displayed by cardoon extracts toward ovine whole casein compared to its bovine counterpart.

Within the first 30 min of hydrolysis, CE analysis of both bovine and ovine whole casein showed that κ-casein was preferentially hydrolyzed compared to α_s- and β-caseins. In fact, electrophoterograms (not shown) indicated that at the end of 30 min of hydrolysis, the κ-casein peak had completely disappeared, whereas the α_s- and β-casein peaks remained significantly unchanged. Interestingly, however, when the whole casein solutions were hydrolyzed for >30 min, CE analysis showed that the cardoon extracts were more active toward bovine α_s- and β-caseins than their ovine casein counterparts. For example, after 2 and 4 h of hydrolysis, there were still large amounts of ovine α_s- and β-caseins left intact, whereas bovine α_s- and β-caseins were significantly hydrolyzed. From the electrophoretograms (not shown) the total area counts of ovine α_s- and β-casein peaks changed from 32000 to 16000, compared with bovine α_s- and β-casein peaks that changed from 42600 to 1678, after 4 h of hydrolysis. This suggests that most of the activity reported for cardoon extract, especially when ovine whole casein was used as substrate (**Figure 4**), would be attributable to the hydrolysis of κ-casein. It also confirms the findings of previous workers (*18*) about the nonsuitability of cardoon rennet for cheese-making with bovine milk due to excessive proteolysis. Although the behavior of rennet has been shown to be different in casein solutions from that in milk systems (*19*), it appears from this study that this excessive proteolysis in bovine milk caseins would occur especially after clotting has been completed.

Although the substrate preference of cardoon flower extracts was not affected by the purification process, there were significant differences between the activities of crude and purified extracts. For example, partially purified water extracts showed much higher caseinolytic activities against all substrates compared to their crude counterparts. In comparison, partially purified citric acid extracts showed only slight increases in caseinolytic activities over the crude extracts, and in some cases, the activities even decreased. This is very much in agreement with the data presented in earlier sections on caseinolytic activity and the protein content of the extracts.

Effect of Temperature and pH on Caseinolytic Activity of Cardoon Extracts. The effects of temperature and pH on the caseinolytic activity of cardoon extracts are shown in parts A and B, respectively, of **Figure 5**. The caseinolytic activities of cardoon extracts increased with temperature up to 37 °C, at which point the activities peaked. Thereafter, activities declined sharply with further increases in temperature, indicating that the optimal temperature for all cardoon extracts was ~37 °C. The effect of temperature on the caseinolytic activity of cardoon extracts followed a typical activity−temperature relationship of enzymes.

The effect of pH on the caseinolytic activity of cardoon extracts showed that maximal activity of all cardoon extracts occurred at pH 6.0 (**Figure 5B**). At pH values >6.0, the activities declined almost linearly up to pH 7.5. With pH <6.0, the activities decreased in roughly an exponential fashion with the largest decline occurring between pH 5.0 and pH 5.5. The pH dependence is a typical behavior of enzymes, and cardoon proteases were found to be no exception. The maximal specific activity of the Australian cardoon extracts at pH 6.0 was in reasonable agreement with the optimal pH values of 5.7 (*8*), 6.0 (*3*), and 5.9 (*1*) reported for the proteases isolated from the same plant species grown in Portugal, Chile, and Spain, respectively. It was significantly different, however, from the value of pH 5.1 reported for dry extracts from Portuguese cardoon flowers (*2*).

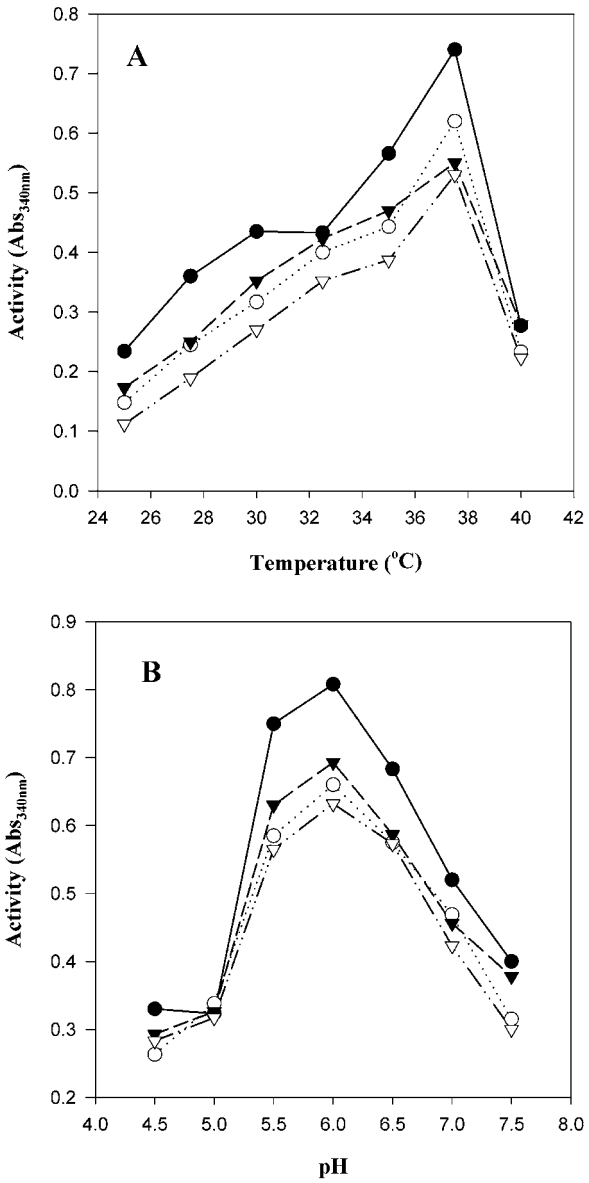

Figure 5. Effect of temperature (A) and pH (B) on caseinolytic activity (after 30 min of hydrolysis) of partially purified water extracts of cardoon flowers: **(●)** PFW; **(○)** PDW; **(▲)** PFC; (△) PDC. Abbreviations as in **Table 3**.

CE Profiles of Cardoon Flower Extracts. Figure 6 shows the CE profiles of crude and partially purified extracts of fresh cardoon flowers. The electrophoretogram of the crude water extract of fresh flowers (**Figure 5A**) contained a significantly greater number of peaks that were larger in size compared to those of the crude citric acid extract of fresh flowers (**Figure 6C**). After partial purification by ammonium sulfate precipitation, a number of the peaks in the water extract of fresh flowers disappeared (the number of significant peaks decreased from >10 in the crude extract to ~4 in the purified extract), and the remaining peaks had much smaller areas (**Figure 6B**). However, the CE profile of the crude citric acid extract of fresh flowers (**Figure 6C**) did not differ markedly from that of its purified counterpart (**Figure 6D**). Furthermore, the peaks in purified water extract (**Figure 6B**) were greater in size than those of the purified citric acid extract (**Figure 6D**).

Similar trends were observed for extracts from dry flowers, except that the CE profile of the purified citric acid extract of dry flowers was similar to that of the purified water extract.

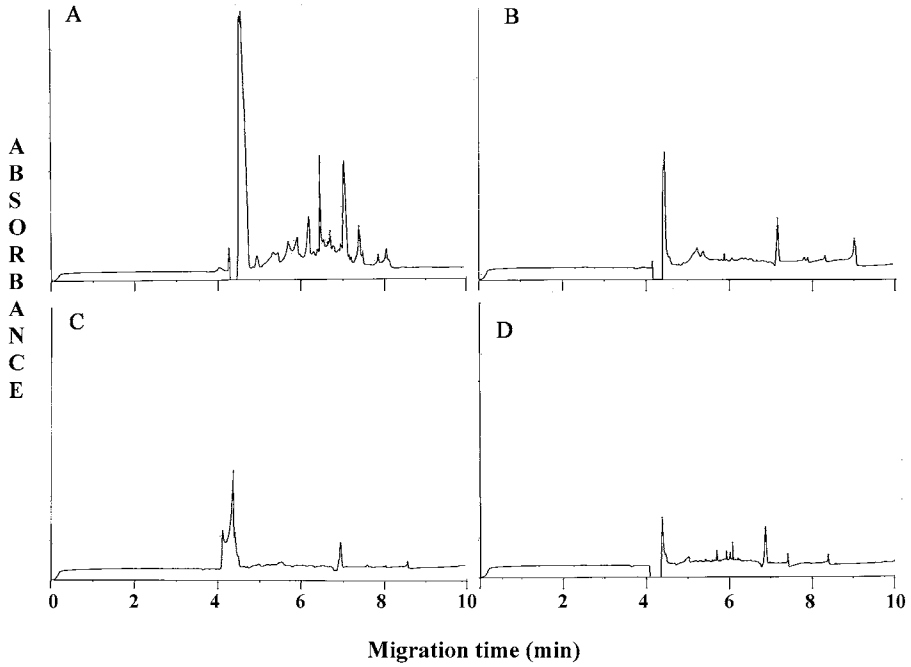

Figure 6. Effect of purification on the capillary electrophoretic profiles of extracts of fresh cardoon flowers: (A) crude water extracts; (B) partially purified water extracts; (C) crude citric acid extracts; (D) partially purified citric acid extracts. Absorbance (arbitrary units) was measured at 214 nm.

Furthermore, extracts of fresh flowers contained more and bigger peaks than their dried flower counterparts. These trends were in close agreement with the results on the yield of the various extracts reported in earlier sections.

LC of Cardoon Extracts. After partial purification by fractional precipitation with increasing concentrations of ammonium sulfate and subsequent dialysis, the extracts were further fractionated on a DEAE-Sepharose CL-6B ion-exchange column. Elution was achieved with a series of linear gradients of sodium chloride. Fractions under each peak were collected separately, and the activities of the fractions were analyzed.

Considerably more peaks were obtained from the extracts of fresh flowers than from those of dried flowers (chromatograms not shown), a trend that agreed with the yield data described earlier. However, three fractions with caseinolytic activity were identified and isolated from the citric acid or water extracts of both fresh and dried cardoon flowers. Similar results were observed for citric acid extracts of both fresh and dried flowers (chromatograms not shown).

CC Profiles of the Active Fractions of Cardoon Extracts. The active fractions were further analyzed by CE to examine their profiles and by SDS-CE to determine their molecular masses. **Figure 7** shows the CE profile of the active fractions of cardoon extracts. Three definite peaks were common to all extracts. These peaks were identified at 4.9, 6.5, and 8.4 min on the electrophoretograms.

Figure 8 shows the SDS-CE profile of the three pooled active fractions from the water extract of fresh flowers. Two peaks (peaks 1 and 2) with apparent molecular masses of 15.5 and 33.1 kDa, respectively, were revealed. SDS-CE was also performed on other extracts, and the same CE profile was obtained (electrophoretograms not shown). Heimgartner et al. (*2*), using SDS-PAGE, showed that three proteases were isolated from cardoon flowers and were each composed of two different subunits with molecular masses similar to those obtained in the present study. The cardoon proteases reported by Faro et al. (*20*) and Verissimo et al. (*4*) also consisted of two subunits each, with molecular masses of 31 and 15 kDa for cardosin A and 34.3 and 14 kDa for cardosin B, respectively. Results obtained

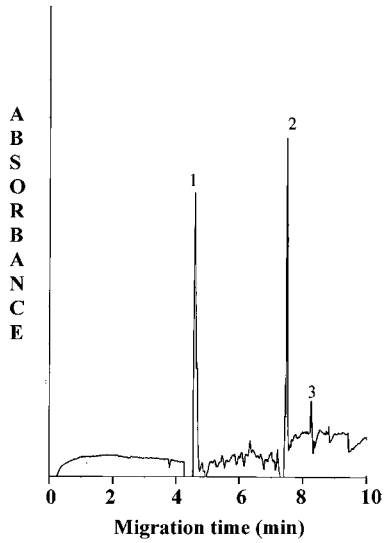

Figure 7. CE profile of active fractions (numbered) of fresh cardoon flower water extracts. Absorbance (arbitrary units) was measured at 214 nm.

from this study suggest that all active peaks are essentially the same but eluted at different times, probably because they possess different degrees of glycosylation. This is not unexpected when conjugated proteins are subjected to analysis.

It has been shown in this study that Australian cardoon exhibited properties similar to those of its European and South American counterparts when subjected to similar conditions of extraction, purification, and fractionation. The caseinolytic enzymes extracted also seemed to be similar in their molecular masses. As far as extraction of caseinolytic enzymes from Australian cardoon is concerned, the best source was found to be the styles of the fresh flowers, and the best procedure was extraction with water followed by purification by fractional precipitation with ammonium sulfate. However, if citric acid solution was used for extraction, the ammonium sulfate purification procedure would not be necessary. Finally, the cardoon rennet was confirmed to be more specific toward

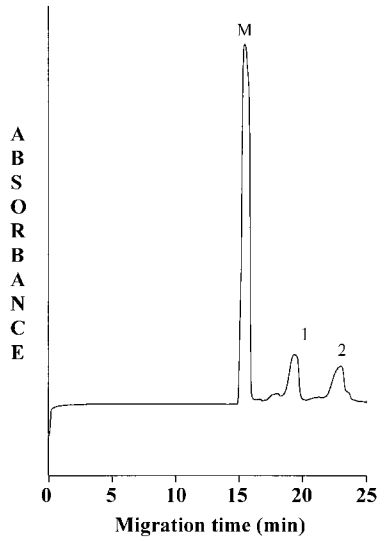

Figure 8. SDS-CE profile of the caseinolytic fractions of fresh cardoon flower extracts. The first peak (M) was orange G marker. The molecular masses of peaks 1 and 2 were calculated to be 15.5 and 33.1 kDa, respectively. Absorbance (arbitrary units) was measured at 214 nm.

κ-casein compared to whole caseins from either bovine or ovine milk. Research on the milk-clotting properties of the cardoon extracts in both bovine and ovine milk systems has also been carried out and will be reported in a subsequent paper.

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