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Modeling the Kinetics of Whey Protein Hydrolysis Brought about by Enzymes from *Cynara cardunculus*

RUI M. BARROS AND F. XAVIER MALCATA*

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, P-4200-072 Porto, Portugal

The purpose of this research work was to study the proteolytic activity of aqueous crude extracts of flowers of the plant *Cynara cardunculus* on the major whey proteins, namely, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La). These extracts, containing a mixture of cardosins A and B (i.e., two distinct aspartic proteases), have been employed for many years in traditional cheese-making in Portugal and Spain. Cow's milk sweet whey was incubated for up to 24 h at various ratios of addition of crude enzyme extract, under controlled pH (5.2 and 6.0) and temperature (55 °C). The samples collected were assayed by gel permeation chromatography and sodium dodecyl sulfate−polyacrylamide gel electrophoresis. A mechanistic model was proposed for the kinetics of the hydrolysis process, which is basically a double-substrate, double-enzyme Michaelis−Menten rate expression; the kinetic parameters were estimated by multiresponse, nonlinear regression analysis. The best estimates obtained for the specificity ratio (i.e., k_{cat}/K_m) of each cardosin within the mixture toward each whey protein indicated that said aspartic proteases possess a higher catalytic efficiency for α-La (0.42−4.2 $mM^{-1}\cdot s^{-1}$) than for β-Lg (0−0.064 $mM^{-1}\cdot s^{-1}$), at least under the experimental conditions used. These ratios are below those previously reported for caseins and a synthetic hexapeptide. Cardosins are more active at pH 5.2 than at pH 6.0 and (as expected) at higher enzyme-to-substrate ratios.

KEYWORDS: Enzymes; dairy products; kinetic mechanism; plant proteases

INTRODUCTION

Increasingly strict economic and environmental issues in the most recent decades pertaining to whey, produced as a byproduct of the cheese-making industry (where it represents ∼90% of the initial milk volume processed), have encouraged technological developments toward its upgrading. Novel applications that may bring about higher added value for whey may be focused either on its protein or on its sugar portion. The most abundant proteins, namely, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La), which together represent ∼70% of the total whey proteins, possess excellent functional performance and nutritional value, owing mainly to their unique contents of Cys, Met, Lys, and Trp residues. These proteins may be recovered by a variety of processes, for example, drying, and may in turn be hydrolyzed enzymatically and later included in the formulation of special diets for enteric nutrition of nursing infants and sick adults.

Aspartic proteinases (EC 3.4.23) make up a class of enzymes that is widely distributed in animals, plants, and microorganisms. Previous publications (*1*−*3*) have shown that it is possible to extract and purify two aspartic proteases, namely, cardosin A and cardosin B, from the flowers of the thistle *Cynara cardunculus*; these enzymes are characterized by considerably different amino acid sequences. The mutual proportion of these proteinases varies among the various cultivars of thistle and depends as well on the stage of maturation at harvest; in fresh flowers, the relative amount of cardosin B is ∼25% of the total protein (*4*). Interestingly, aqueous extracts of the flowers of *C. cardunculus* have for ages been used in Portugal as rennet in cheese-making from ewe's milk. The exact physiological function of those aspartic proteinases is still unknown; however, recent studies (*5*) have indicated that cardosin A may play a primary role in the interaction with pathogens or pollen, and a secondary role in the annual senescence of the flower.

Unlike hydrolysis using short synthetic peptides as model substrates, which are described by simple kinetic expressions, hydrolysis of proteins and protein mixtures leads to quite complex global models; the latter studies will be, nevertheless, of much stronger interest if applications of proteases in whey protein hydrolysis are sought. A study of the former type, carried out at 37 °C and pH 3.1 upon the oxidized form of the insulin B chain (*4*), suggested that cardosin B possesses a broader specificity than cardosin A, although the two enzymes share a similar preference for peptide bonds next to hydrophobic side chains. In terms of specificity, cardosin B is similar to pepsin, whereas cardosin A is similar to chymosin, at least at pH 4.7 and 37 °C (*6*); however, the optimum pH for the proteolytic

* Author to whom correspondence should be addressed (e-mail xmalcata@esb.ucp.pt).

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activity of the crude extract is 5.7 at 37 °C, when casein is selected as substrate.

The aim of the present work was to assess the effect of two easily manipulated processing conditions, namely, pH and enzyme concentration, on protein hydrolysis of plain whey brought about by extracts of *C. cardunculus*. A mechanistic model was proposed for the kinetics of this hydrolysis process. To maximize the information generated pertaining to the action of cardosins on the two major whey proteins, hydrolysates of α-La and β-Lg in whey were assayed by fast protein liquid chromatography (FPLC) and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The kinetic parameters k_{cat} and K_m, as well as their ratio, for the mixture of cardosins A and B were estimated, and compared with those obtained by other researchers.

MATERIALS AND METHODS

Feedstocks and Chemicals. Sweet whey was obtained as a byproduct of rennet-type cheese from a standard cow's milk cheese-making plant and was lyophilized before storage; it contained a β-Lg/α-La ratio of ca. 6:1 (mole/mole). Chemical characterization of said whey was carried out in terms of contents of nitrogen, using the method recommended by IDF (*7*); humidity, by the method recommended by IDF (*8*); lactose, using the method recommended by AOAC (*9*); and total protein, using Bradford's method at 595 nm (*10*).

The crude enzyme feedstock (collected at the end of the flowering period in Serra da Estrela, Portugal) was prepared by macerating 7 g of dry stylets of *C. cardunculus* in 70 mL of a 100 mM aqueous solution of sodium citrate (pH 3.0), as described by Faro (*2*). The crude extract was further characterized by ion exchange chromatography and SDS-PAGE, as described below.

A mixture of molecular weight standards for SDS-PAGE [phosphosylase B (94 kDa), bovine serum albumin (BSA; 67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-La (14.4 kDa)] and a mixture of 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 purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Pure whey proteins of bovine origin (α-La, β-Lg, and BSA) were purchased from Sigma (St. Louis, MO). The chemicals Na_2HPO_4, NaH_2PO_4, $C_6H_8O_7 \cdot H_2O$, $C_6H_5Na_3O_7$, and NaN_3 were purchased from Merck (Darmstadt, Germany). The water used was deionized and distilled. The chemicals employed were all of analytical grade or better and were used without further purification.

Chromatographic Characterization of the Enzyme Mixture. The crude extract prepared at room temperature was centrifuged at 10000*g* for 5 min at 4 °C. The resulting supernatant was subjected to size exclusion gel chromatography (Sephacryl, Bio-Rad) to recover the peak corresponding to cardosins A and B, and to remove the main contaminants present therein. A buffer of 20 mM Tris-HCl (pH 7.6) was used as eluant, and each peak of absorbance (280 nm) was collected as a fraction. For this purpose, two Econo-Column chromatography columns (i.d. 2.5 cm, L 60 cm), operated in parallel, were connected to a UV spectrophotometer and a fraction collector; each column was loaded with 5 mL of crude extract, and the appropriate preparative fraction (termed enzyme extract hereafter) was used to bring about the whey protein hydrolysis, as described below.

Further characterization proceeded with the lyophilized, gel-filtrated fraction containing cardosins A and B, which was reconstituted in a small amount of 20 mM Tris-HCl buffer (pH 7.6); 20 mg of reconstituted extract was then subjected to chromatography using an anion exchange column (DEAE, Bio-Rad). A linear gradient of 0−0.5 M NaCl was employed, using 0.2 M Tris-HCl buffer (pH 7.6) as eluant at a flow rate of 0.2 mL/min.

Enzyme-Mediated Hydrolysis. Preliminary studies, carried out with whey proteins at 37, 45, and 55 °C, revealed a higher extent and higher hydrolysis rate at 55 °C (data not shown). For this reason, the enzymatic hydrolysis was carried out only at 55 °C in a shaking water bath, at two pH values (5.2 and 6.0). All experimental data are means of two replicates.

For the experiments encompassing variable enzyme concentrations at constant substrate concentration, six aqueous solutions (60 mL) containing 5% (w/v) whey proteins in 100 mM sodium citrate buffer, at pH 5.2 and 6.0, were hydrolyzed for 24 h in a water bath shaken at 100 rpm. Enzymatic hydrolysis was started by the addition of six different amounts of enzyme extract, so as to get six different ratios of enzyme to substrate on a protein weight basis, namely, 1/133, 1/266, 1/532, 1/1800, 1/3600, and 1/7200 (w/w).

For the experiments encompassing variable substrate concentrations at constant enzyme concentration, six aqueous solutions (60 mL) containing different whey protein concentrations in 100 mM sodium citrate buffer, at pH 5.2 and 6.0, were hydrolyzed for 24 h in a water bath shaken at 100 rpm. Enzymatic hydrolysis was started by the addition of identical amounts of enzyme extract, so as to get six different ratios of enzyme to substrate on a protein weight basis, namely, 1/133, 1/266, 1/532, 1/1800, 1/3600, and 1/7200 (w/w).

All experiments had a control carried out at similar running conditions (without enzyme), in order to guarantee that the hydrolysis occurred only in the presence of cardosins. In all experiments, NaN_3 (azide) was added to a final concentration of 0.1% (w/v) to inhibit microbial growth.

The processing conditions set forth for hydrolysis were similar to those described before.

Chromatographic Characterization of the Hydrolysis. Aliquots (1.5 mL) of the incubated solution were taken at 0, 1, 2, 5, 8, 12, and 24 h. The reaction was quenched by immersion in liquid nitrogen so as to inactivate the enzyme; said aliquots were then stored at −20 °C until use. FPLC analysis of the hydrolysates was conducted according to the following protocol: separation was performed after injection of 100 μL of sample in a Superose 12 column HR 10/30 (Pharmacia), with the aid of an injection valve MV-7 and an UV-MII detector (280 nm); the mobile phase was 150 mM NaCl in 0.05 M 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. Prior to chromatographic analysis, the sample was passed through a 0.45 μm filter (Nucleopore, Pleasanton, CA), whereas the buffer was filtered through 0.22 μm paper filter (Nalgene, Rochester, NY). The void volume of the column was determined using blue dextran. The retention times of the peaks obtained were compared with those of a mixture of molecular weight standards: aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), β-Lg (36 kDa), α-La (14.4 kDa), and ribonuclease (13.7 kDa). 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^{-1} β-Lg, 9.10 mg·mL^{-1} α-La, 6.80 mg·mL^{-1} BSA, 0.10 mg·mL^{-1} orotic acid, and 0.020 mg·mL^{-1} uric acid. Each analytical determination was carried out in duplicate.

Electrophoretic Characterization of the Hydrolysis. For the preparation of samples, 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 further added. The vials were subsequently heated at 90 °C for ∼10 min in a boiling water bath, cooled to near room temperature, and centrifuged at 10000 rpm for 10 min. The solution containing SDS, β-mercaptoethanol, sucrose, and bromophenol blue was added to the supernatant of said samples and incubated in a heating block at 90 °C for 5 min. The 15% polyacrylamide gel slabs (pH 8.6), containing 0.1% (w/v) SDS, were prepared and run using the discontinuous buffer system of Laemmli (*11*), at constant voltage (100 V), constant current (70 mA) and constant temperature (19 °C) for 10 h; after this time, the gels were stained with Coomassie blue R-250. Qualitative and quantitative analyses of electrophoretograms were made by densitometric scanning, using a green filter, in a GS-700 imaging densitometer (Bio-Rad, Hercules, CA), and further analyzed using the image analysis software Molecular Analyst.

Theoretical Modeling. The crude extracts from *C. cardunculus* contain two proteases, that is, cardosin A and cardosin B, which catalyze the same reaction and which will hereafter be denoted E_A and E_B. Because whey is composed of essentially two different substrates, that

is, α-La and β-Lg, hereafter denoted S_α and S_β, the following overall chemical scheme should be the simplest one able to describe the reaction phenomena in a logical fashion:

$$S_\alpha + E_A \overset{K_{mA\alpha}}{\rightleftharpoons} E_A S_\alpha \xrightarrow{k_{catA\alpha}} E_A + P_\alpha$$

$$S_\alpha + E_B \overset{K_{mB\alpha}}{\rightleftharpoons} E_B S_\alpha \xrightarrow{k_{catB\alpha}} E_B + P_\alpha$$

$$S_\beta + E_A \overset{K_{mA\beta}}{\rightleftharpoons} E_A S_\beta \xrightarrow{k_{catA\beta}} E_A + P_\beta$$

$$S_\beta + E_B \overset{K_{mB\beta}}{\rightleftharpoons} E_B S_\beta \xrightarrow{k_{catB\beta}} E_B + P_\beta$$

where $K_{m,i,j}$ represents the Michaelis−Menten parameter for enzyme E_i and substrate S_j, and $k_{cat,i,j}$ represents the intrinsic kinetic constant associated with formation of product P_j by enzyme E_i. Assuming that cardosins act preferentially on proteins relative to peptides derived therefrom, and to keep the mathematical modeling tractable, only the native dominant proteins of whey were considered as substrates, and all putative cleavage sites of peptide bonds were lumped together so as to produce only two (generic) types of product. Again for the sake of mathematical simplicity, quasi-equilibrium conditions for the enzyme−substrate complexes were assumed, which correspond to a double-substrate, double-enzyme Michaelis−Menten model. The corresponding equations may thus be written as

$$-\frac{dC_\alpha}{dt} = \left(\frac{k_{catA\alpha} f_A C_{E_t} \frac{C_\alpha}{K_{mA\alpha}}}{1 + \frac{C_\alpha}{K_{mA\alpha}} + \frac{C_\beta}{K_{mA\beta}}} + \frac{k_{catB\alpha} f_B C_{E_t} \frac{C_\alpha}{K_{mB\alpha}}}{1 + \frac{C_\alpha}{K_{mB\alpha}} + \frac{C_\beta}{K_{mB\beta}}} \right) \quad (1)$$

$$-\frac{dC_\beta}{dt} = \left(\frac{k_{catA\beta} f_A C_{E_t} \frac{C_\beta}{K_{mA\beta}}}{1 + \frac{C_\alpha}{K_{mA\alpha}} + \frac{C_\beta}{K_{mA\beta}}} + \frac{k_{catA\beta} f_B C_{E_t} \frac{C_\beta}{K_{mB\beta}}}{1 + \frac{C_\alpha}{K_{mB\alpha}} + \frac{C_\beta}{K_{mB\beta}}} \right) \quad (2)$$

where

$$C_{E_t} = C_{E_{A0}} + C_{E_{B0}} \quad (3)$$

$$f_A = \frac{C_{E_{A0}}}{C_{E_t}} \quad (4)$$

$$f_B = \frac{C_{E_{B0}}}{C_{E_t}} = 1 - f_A \quad (5)$$

and where subscript t denotes total and subscript 0 denotes initial conditions. The classical lumped constant, $v_{max,i,j}$, will thus be defined as

$$v_{maxA,j} = k_{catA,j} f_A C_{E_t} \quad (6)$$

$$v_{maxB,j} = k_{catB,j} f_B C_{E_t} \quad (7)$$

The first term in the right-hand side of eqs 1 and 2 accounts for the action of cardosin A on α-La and β-Lg, respectively, whereas the second term accounts for the action of cardosin B on α-La and β-Lg, respectively. The mathematical model thus proposed (with nine parameters) consubstantiates an attempt to unravel the particular double-enzyme, double-substrate Michaelis−Menten reaction in more detail. A numerical method based on finite differences was employed to obtain the integration of eqs 1 and 2 throughout reaction time. To obtain the best possible estimates of the kinetic parameters in this model, a multiresponse nonlinear regression analysis was done to the experimental data using the General REGression package, GREG (*12*); this package performs nonlinear regression analysis to multiresponse data, using small differences as approximants of the derivatives of the objective function with respect to each parameter. This model was fitted simultaneously to all data produced encompassing six enzyme/substrate ratios, independently generated via changing initial enzyme concentration or initial substrate concentration, at either of two different pH values (but at the same temperature).

Although it may be claimed that a rather sophisticated treatment was made for a (crude) enzyme extract, note that only a few proteins exist in said extract following size exclusion (preparative) chromatography, and only two of those are active (i.e. cardosins A and B); furthermore, if the breakdown of whey was to be carried out by *C. cardunculus*, the crude aqueous mixture or, at most, its fraction containing cardosins previously purified by size exclusion chromatography would be employed, and not the pure cardosins separated therefrom, owing to obviously high processing costs of the latter that would render the whole process economically unfeasible.

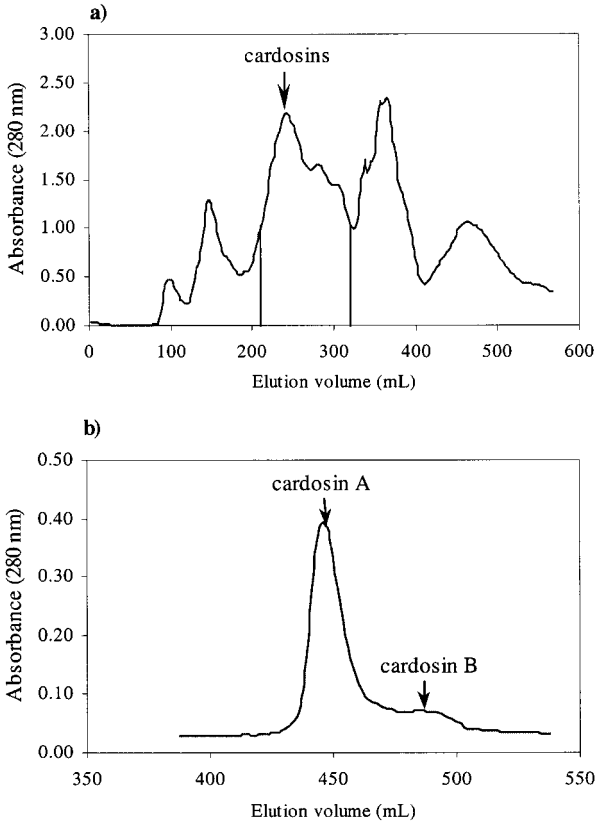

Figure 1. Elution profiles by (a) gel permeation chromatography of the crude aqueous extract with Tris-HCl buffer (pH 7.6) as eluant, and by (b) weak anion exchange chromatography (DEAE) of the enzyme extract obtained in (a) using a linear gradient of NaCl in Tris-HCl buffer (pH 7.6) as eluant.

RESULTS AND DISCUSSION

Enzyme Characterization. The size exclusion gel chromatographic pattern of crude extract on Sephacryl is shown in **Figure 1a**. The third peak is accounted for by cardosins, whereas the other peaks are accounted for by contaminants. After collection, each peak was characterized by electrophoresis (Phastgel high-density gels). The results revealed that only the third peak contained protein matter (cardosins). Furthermore, each peak was tested for coagulant activity for 2 h, and only the cardosin peak proved positive. Proteolytic activity was determined according to the procedure described by Tommarelli (*13*). The results showed once more that only the cardosin peak had proteolytic activity. All hydrolyses were carried out with this

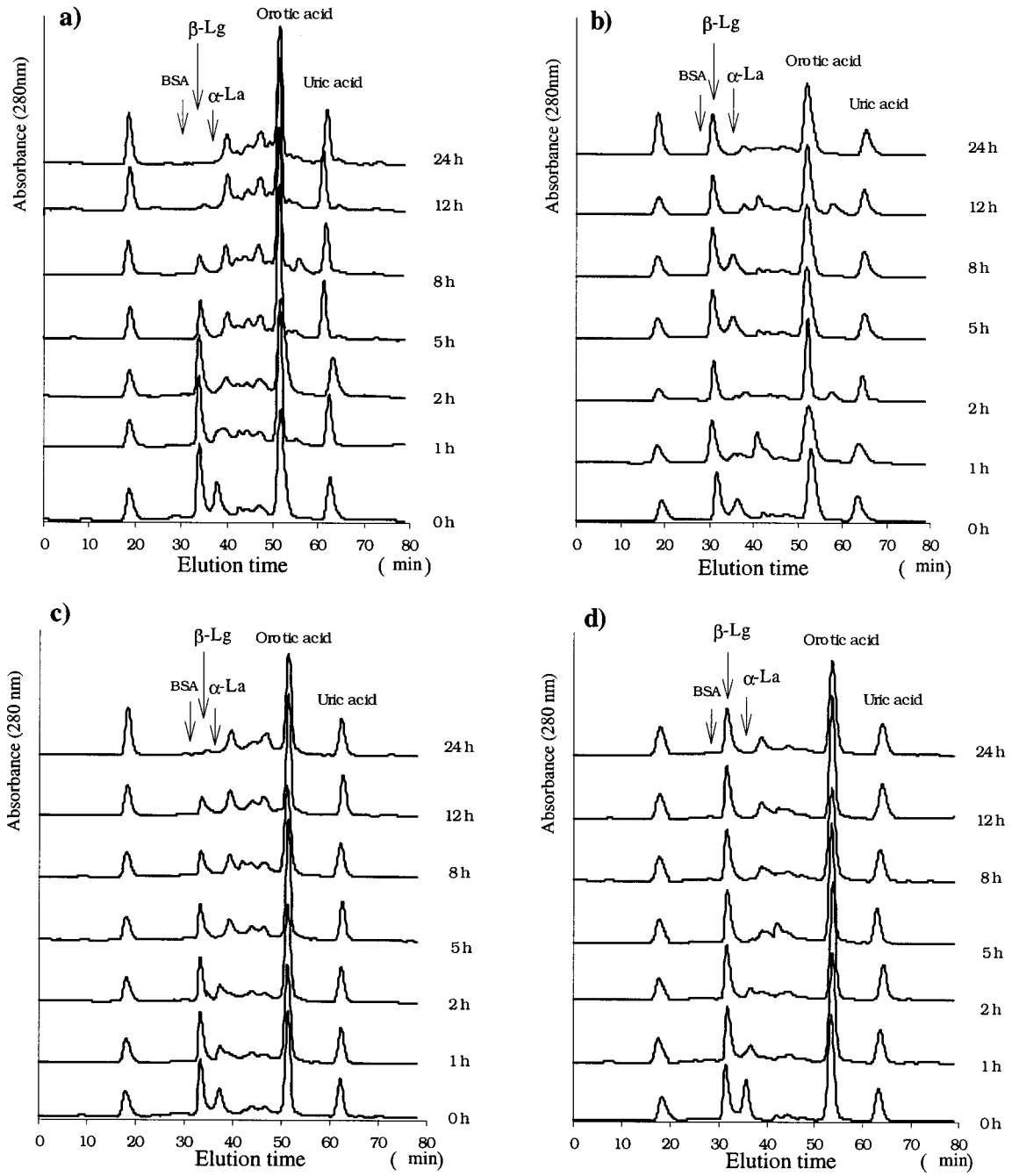

Figure 2. Elution profile of whey by gel permeation chromatography, after hydrolysis at 55 °C by enzyme extract for 0, 1, 2, 5, 8, 12, and 24 h, at the E/S ratios (a) 1/133 at pH 5.2, (b) 1/532 at pH 5.2, (c) 1/133 at pH 6.0, and (d) 1/532 at pH 6.0.

crude enzyme extract; the contents in cardosins A and B were confirmed by SDS-PAGE (see **Figures 3** and **4**). Both cardosins A and B appeared as double bands because, in the presence of the reducing agent β-mercaptoethanol in the sample buffer, the interpeptide disulfide bonds are reduced, thus releasing two monomers (*1*, *4*). It is possible to detect the two cardosins (A and B) in lane 10, which correspond to bands with approximate molecular weights of 30 and 15 kDa and 35 and 14 kDa, respectively. This piece of experimental evidence supports the model assumptions that only two proteolytic enzymes were present in the crude enzyme mixture.

To provide extra evidence that the enzyme extract obtained by size exclusion chromatography contained only cardosins A and B, they were further separated by ion exchange chromatography; as shown in **Figure 1b**, the profile revealed only two peaks, the first (and major) corresponding to cardosin A and the second corresponding to cardosin B. The relative amount of cardosin A to cardosin B was determined by gel filtration chromatographic peak area integration, and revealed that the crude extract fraction contains about three-fourths cardosin A and one-fourth cardosin B.

Analysis of FPLC Elution Profiles. The elution profile of the main proteins present in cow's milk whey, following hydrolysis at 55 °C, is shown in **Figure 2a**,**b** at pH 5.2 and in **Figure 2c**,**d** at pH 6.0. When the hydrolysates were analyzed by FPLC, the three major whey proteins (i.e., BSA, β-Lg, and α-La) were eluted at approximate retention times of 28, 31, and 35 min. Control samples in the absence of enzyme showed negligible hydrolysis. The elution patterns of the hydrolysates at pH 5.2 were quite similar to those obtained at pH 6.0. When

the same E/S ratio was considered, the FPLC profiles revealed a slightly higher hydrolysis rate at pH 5.2 than at 6.0, mainly for α-La and β-Lg. The FPLC profiles also exhibited a rapid decrease in the concentration of α-La, mainly at higher E/S ratios (see **Figure 2a**,**c**). In general, it was possible to detect β-Lg for a long time, whereas α-La disappeared after a few hours of hydrolysis. From inspection of **Figure 2**, it is possible to conclude that the behaviors of β-Lg at the lowest and highest E/S ratios are quite different: β-Lg remained almost unaltered at the lowest E/S ratio (1/532) by 24 h, whereas β-Lg vanished by 12 h at the highest E/S ratio (1/133). In view of the above, it seems that the appearance of low molecular weight peptides is more a result of hydrolysis of α-La than of β-Lg.

Analysis of SDS-PAGE Electrophoretograms. Figures 3 and **4** show the time course of proteolysis of the whey proteins and the concomitant appearance of new fragments, at two pH levels and at various E/S ratios, using SDS-PAGE. Our results indicate that the whey proteins are hydrolyzed to a higher degree at higher enzyme concentrations, as expected, in the whole pH range studied. When crude extract was added to whey, substantial hydrolysis of α-La was noticed; α-La seems indeed to be the preferential substrate for proteases and actually vanishes from the electrophoretograms rapidly, whereas the other proteins seem to remain essentially unaltered. Inspection of **Figures 3** and **4** indicates that β-Lg is hydrolyzed more gradually. Different hydrolysis patterns for BSA are observed at pH 5.2 and 6.0 (see **Figures 3** and **4**): at pH 5.2, BSA vanishes by 5 h when the lower enzyme concentration is used; however, it remains almost intact at pH 6.0 irrespective of the enzyme concentration.

Cardosins, as aspartic proteases, are known to cleave peptide bonds between amino acid residues with large hydrophobic side chains, such as Phe and Leu (*14*). Furthermore, recent studies indicated that cardosin B possesses a broader specificity than cardosin A, although the two enzymes share a similar preference for peptide bonds next to hydrophobic side chains (*4*). The higher order topology of β-Lg indicates a high degree of organization and compactation, via nine β sheets and two intramolecular disulfide bridges; this likely makes β-Lg resistant to enzymatic proteolysis (*15*). On the other hand, the three-dimensional structure of α-La (*16*, *17*) encompasses a large hydrophobic area on the surface of the protein, which apparently possesses a high affinity for the enzymes. For these reasons, it seems reasonable to consider that conformational differences should explain, at least partially, the behavior of cardosins in terms of the different hydrolysis extent and rate of whey proteins.

Variable Enzyme Concentration. The time course of the enzyme-mediated hydrolysis of whey proteins, for the six different concentrations of enzyme extract, is plotted in **Figure 5**. A steady tendency toward increasing conversions can be observed as the ratio of enzyme rises, as anticipated. However, the results point to a different behavior of the enzyme extract with regard to the two most important whey proteins: for α-La, the reaction rates were higher at initial times (1−2 h), and the hydrolysis rates of β-Lg were significantly lower than those recorded for α-La. During incubation at higher enzyme/substrate ratios, α-La was completely broken down by 2 h (**Figure 5a**,**c**), whereas a large portion of β-Lg remained intact (**Figure 5b**,**d**). Concertation of the action of the two enzymes and competition of the two substrates for their active sites certainly account for these observations.

The major variation observed in the hydrolysis profiles when going from pH 5.2 to 6.0 was a slight decrease in the reaction rate for both proteins; nevertheless, β-Lg was poorly hydrolyzed, whereas α-La was virtually hydrolyzed in full (except at very low enzyme/substrate ratios)—the differences in structure of these proteins (and the effect of pH thereon) might account for these observations. Note that the optimum pH for the proteolytic activity of the enzyme extract is 5.7 at 37 °C, using casein as substrate (*6*).

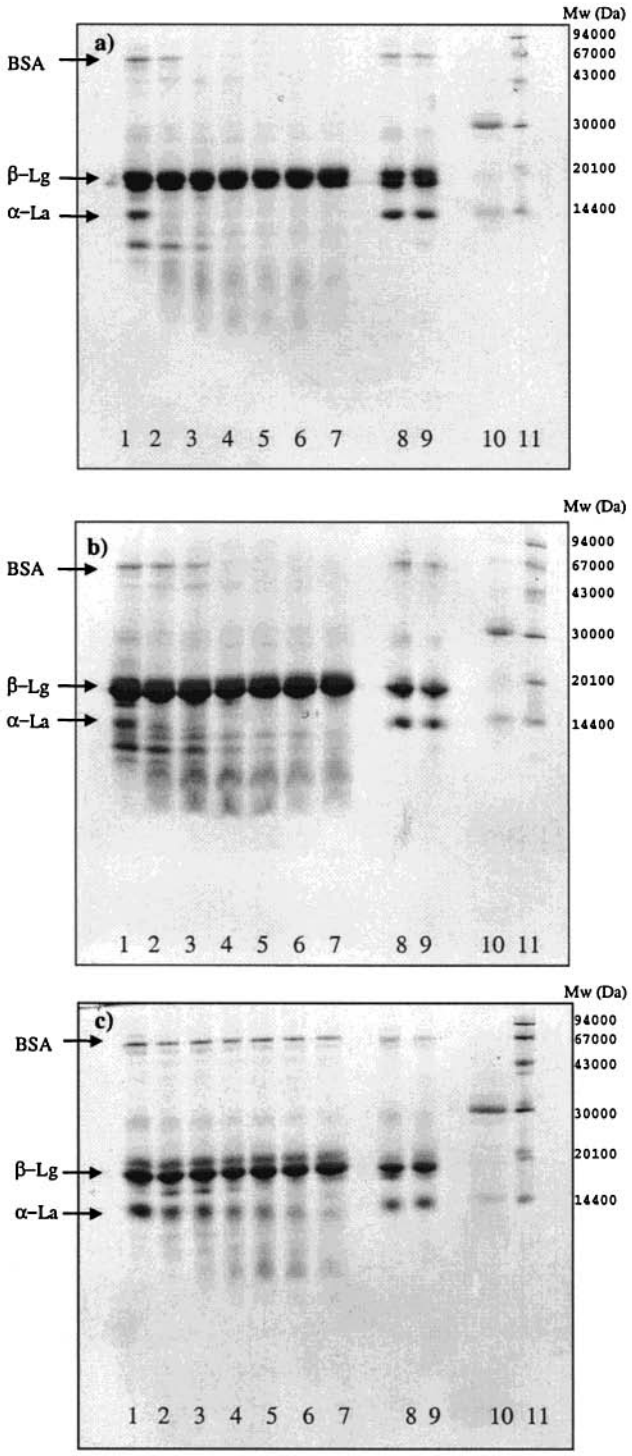

Figure 3. Electrophoresis patterns of whey after hydrolysis at 55 °C and pH 5.2 by enzyme extract, at the E/S ratios (a) 1/133, (b) 1/266, and (c) 1/532, for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 5 h (lane 4), 8 h (lane 5), 12 h (lane 6), and 24 h (lane 7); controls at 0 h (lane 8) and by 24 h (lane 9) of reaction; crude extract (lane 10); and low molecular weight markers (lane 11).

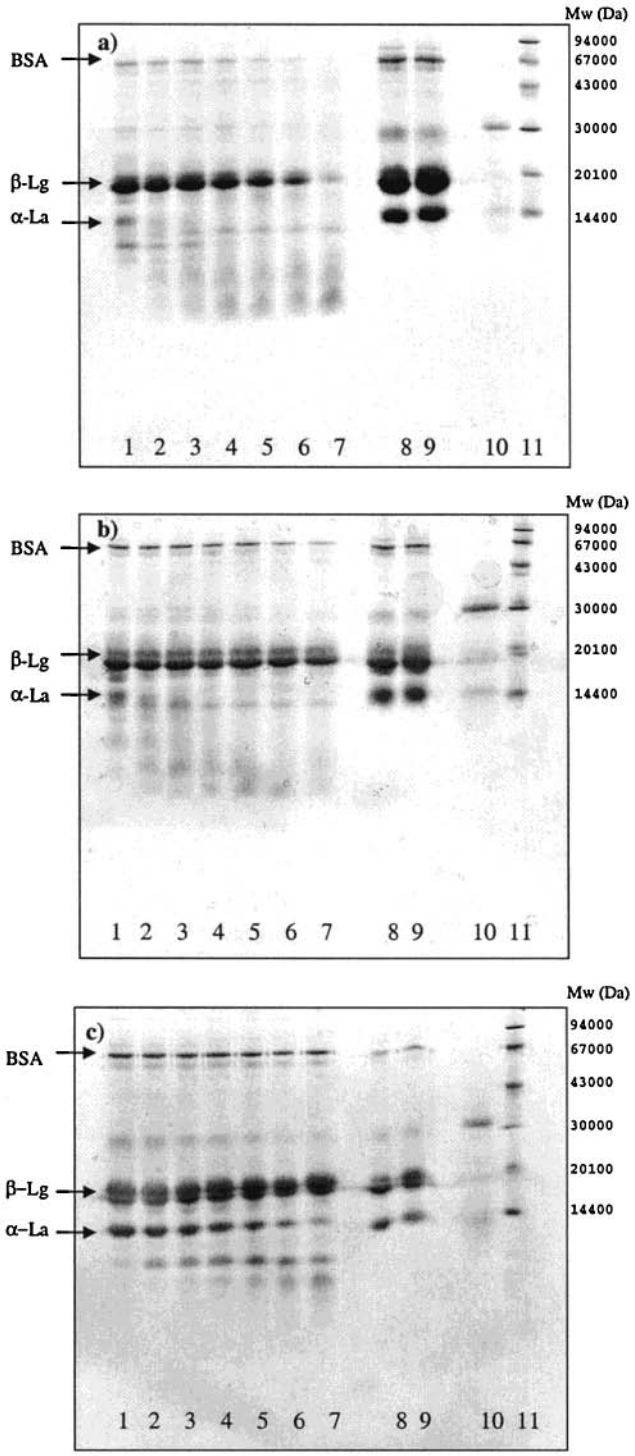

Figure 4. Electrophoresis patterns of whey after hydrolysis at 55 °C and pH 6.0 by enzyme extract, at the E/S ratios (a) 1/133, (b) 1/266, and (c) 1/532, for 0 h (lane 1), 1 h (lane 2), 2 h (lane 3), 5 h (lane 4), 8 h (lane 5), 12 h (lane 6), and 24 h (lane 7); controls at 0 h (lane 8) and by 24 h (lane 9) of reaction; crude extract (lane 10); and low molecular weight markers (lane 11).

Variable Substrate Concentration. The time course of the enzyme-mediated hydrolysis of whey proteins for six different concentrations of substrate is plotted in **Figure 6**. During incubation, α-La was completely broken down to peptides (**Figure 6a**,**c**), whereas a large portion of *β*-Lg remained almost intact (**Figure 6b**,**d**). When the enzyme/substrate ratio was increased, the rate of enzymatic hydrolysis of α-La increased considerably, whereas that of *β*-Lg underwent no significant change; for a very low enzyme/substrate ratio, hydrolysis of *β*-Lg did virtually not occur by 24 h. Inspection of **Figure 6** also indicates that the hydrolysis profile of each of the two major whey proteins is almost independent of the initial concentrations of either substrate. On the other hand, the rates of hydrolysis of both substrates were slightly lower at pH 6.0 than at pH 5.2.

Kinetic Parameters. The fits of the model to the data pertaining to the catalytic action of the proteases extracted from *C. cardunculus*, when present together, on the two major whey proteins, also present together (represented by solid lines in **Figures 5** and **6**), generated the estimates of kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) given in **Table 1**. The set of starting parameter estimates affected considerably convergence of GREG to the final estimates: if poor starting estimates were used, GREG would sometimes satisfy a convergence criterion (minor change in the residual sum of squares between successive iterations), but the final parameter estimates would still be poor, and the associated residual sum of squares would sometimes be orders of magnitude greater than the estimates themselves; in other cases, the final parameter estimates would produce reasonable residuals, yet they would be physically unrealistic. For the sake of easy comparison, the kinetic parameters k_{cat}, K_m, and k_{cat}/K_m of cardosins A and B obtained by other authors, when using a synthetic hexapeptide and *κ*-casein as substrates, are also presented in **Table 1**. Although the chemical nature of the substrate, the pH, and the temperature selected for our experiments were different from those used by those researchers, such data are the only available that pertain to cardosins.

Assuming that the estimates for k_{cat} and K_m are essentially uncorrelated with one another, and assuming that those parameters reflect the catalytic efficiency and the affinity, respectively, of the enzyme for the substrate (*18*), our results suggest that the efficiency and the affinity of cardosins A and B differ from each other to a great extent. The values of K_m found for cardosins A and B, when whey proteins were used as substrates, were closer to those generated using the synthetic hexapeptide (*4*) than with *κ*-casein (*19*): the values of K_m found for cardosins A and B (i.e., 0.02−0.73 mM), which were of the same order of magnitude as those found for the same enzymes acting on Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe as substrate (i.e., 0.08−1.08 mM), were at least 50-fold higher than those of proteases (at a concentration of 0.16 *μ*M) when *κ*-casein was taken as substrate (*4*, *19*); however, note that these experiments had been carried out at lower temperatures and at a distinct pH value. This observation is consistent with the selection of said proteases as rennet for the cheese-making process, where they exhibit a higher affinity to milk insoluble proteins (i.e., caseins) than to milk soluble proteins (i.e., whey proteins). The values of K_m found for cardosins A and B toward α-La are remarkably lower than those obtained toward *β*-Lg, thus suggesting a substantially higher affinity of these enzymes for α-La than for *β*-Lg. In addition, the affinities of both cardosins for α-La and *β*-Lg (at a given pH) are quite similar; that is, $K_{mA\alpha}$ and $K_{mB\alpha}$ are essentially identical. However, at pH 5.2 they are slightly higher than at pH 6.0. For *β*-Lg, the K_m value obtained for cardosin A is remarkably lower at pH 5.2 than at pH 6.0, thus reflecting a considerably higher affinity of this enzyme for that substrate at pH 5.2; unlike cardosin A, cardosin B exhibits a higher value for K_m at pH 5.2 than at pH 6.0.

Inspection of **Table 1** indicates that the order of magnitude of the values of k_{cat} for cardosins A and B (i.e., 0.002−0.102 s^{-1}) is rather different from that previously reported by Macedo (*19*) and Veríssimo (*4*): the activity of said cardosins upon whey proteins was $\sim 1/10$ that determined upon *κ*-casein (*19*); when

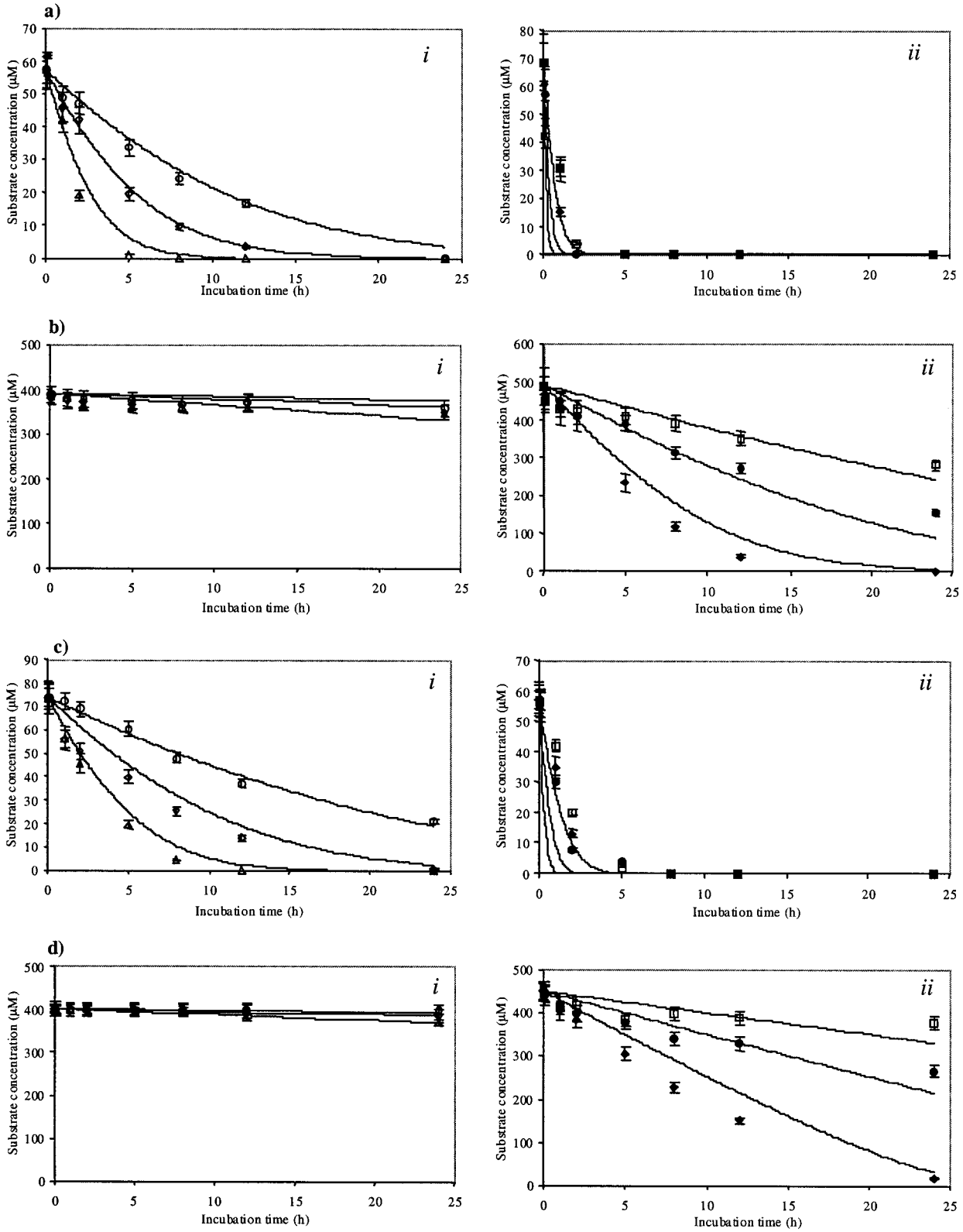

Figure 5. Variation, with incubation time, of the concentration (and associated standard error) of substrate proteins α-La (a, c) and β-Lg (b, d) at various initial enzyme concentrations, at pH 5.2 (a, b) or 6.0 (c, d). E/S ratios 1/7200 (○), 1/3600 (◇), and 1/1800 (△) are shown in (*i*), whereas E/S ratios 1/532 (□), 1/266 (●), and 1/133 (▲) are shown in (*ii*). Experimental data are represented as discrete points, and theoretical values as solid lines (—).

the hexapeptide was used as model substrate (*4*), cardosin A performed at least 200-fold worse and cardosin B 1000-fold worse. The highest activity (i.e., 0.102 s^{-1}) was recorded for cardosin A acting on α-La at pH 5.2; however, if one compared the product $k_{cat}f$ (i.e., the product of the specific activity of each enzyme, k_{catA} and k_{catB}, by its corresponding enzyme fraction, f_A or f_B) for β-Lg rather than k_{cat} itself, there are no significant differences at either pH studied. For α-La the situation was different: the values of $k_{cat}f$ obtained for each enzyme were higher than those obtained for β-Lg at the same pH. The highest activity was recorded with cardosin A (i.e., 0.045 s^{-1}) at pH 5.2 and with cardosin B at pH 6.0 (i.e., 0.041 s^{-1}). Note that

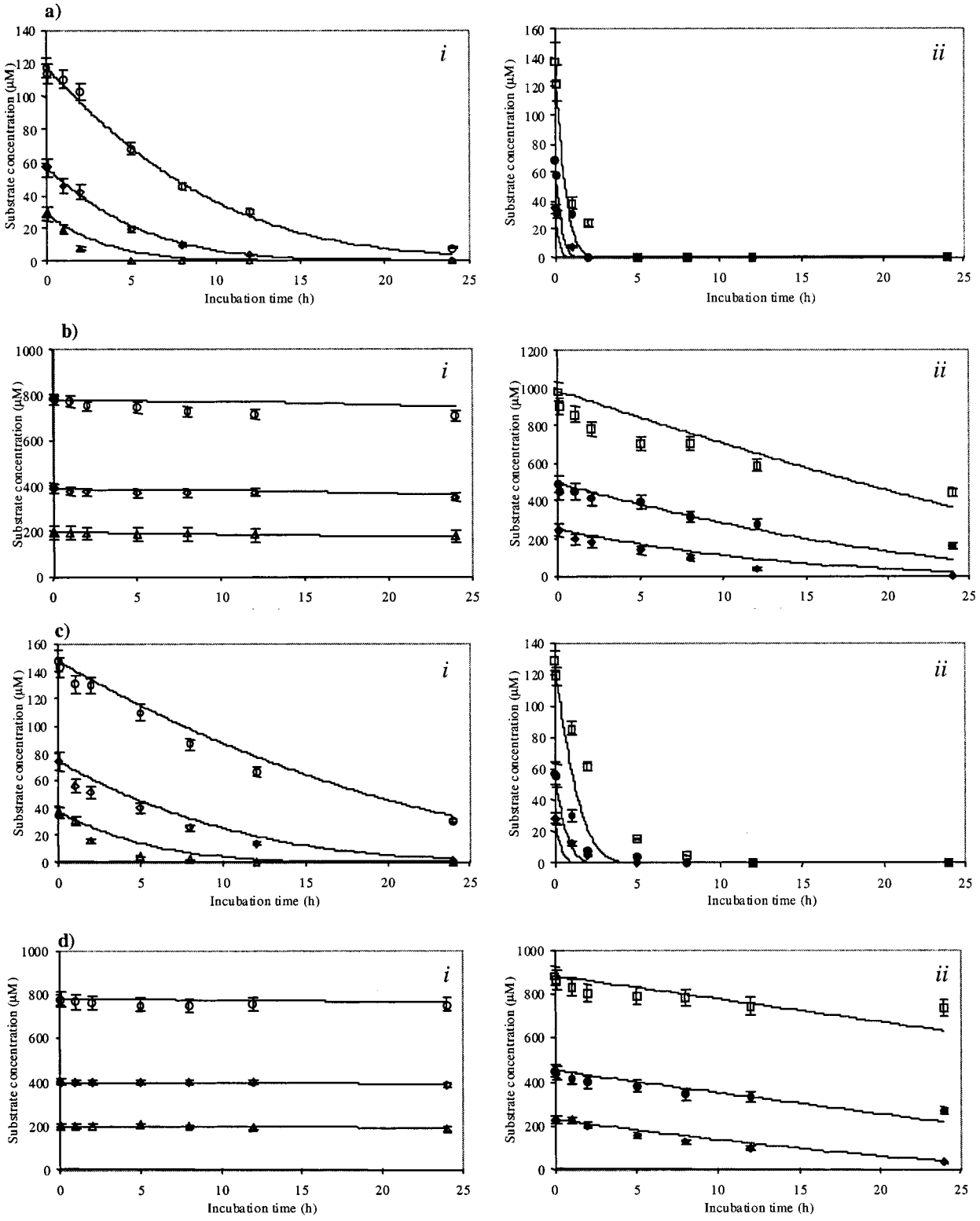

Figure 6. Variation, with incubation time, of the concentration (and associated standard error) of substrate proteins α-La (a, c) and β-Lg (b, d) at various initial substrate concentrations, at pH 5.2 (a, b) or 6.0 (c, d). E/S ratios 1/7200 (○), 1/3600 (◇), and 1/1800 (△) are shown in (*i*), whereas E/S ratios 1/532 (□), 1/266 (●), and 1/133 (◆) are shown in (*ii*). Experimental data are represented as discrete points, and theoretical values as solid lines (—).

the purpose of this work was to characterize the action on whey of the enzymes in the actual crude mixture, so specific use of κ-casein or the synthetic hexapeptide, although permitting a better comparison with published data, would be out of scope and somewhat meaningless.

It is also apparent from inspection of **Table 1** that the range of values for the ratio k_{cat}/K_m with regard to α-La (i.e., 0.42−4.2 $mM^{-1}\cdot s^{-1}$) is considerably wider and higher than that with regard to β-Lg (i.e., 0−0.064 $mM^{-1}\cdot s^{-1}$). This realization suggests that cardosins have a higher affinity for α-La than for β-Lg, at least under the experimental conditions tested. When these values are compared with others found hitherto for κ-casein (*19*) and for the hexapeptide (*4*), one easily grasps that they are not of the same order of magnitude; for either whey protein,

Table 1. Kinetic Parameters That Characterize the Catalytic Action of Proteases Extracted from *C. cardunculus* upon Several Substrates

substrate	enzyme	T (°C)	pH	K_m (mM)	k_{cat} (s^{-1})		k_{cat}/K_m (mM^{-1} s^{-1})	ref
synthetic peptide	cardosin A	37	4.7	1.08	23.6		21.87	*4*
[Leu-Ser-Phe(NO_2)-Nle-Ala-Leu-oMe]	cardosin B	37	4.7	0.080	85.5		1057	*4*
κ-casein	cardosins A + B	30	6.4	0.00016	1.04		6500	*19*
					k_{cat} (s^{-1})	$k_{cat}f$ (s^{-1})		
α-lactalbumin	cardosin A	55	5.2	0.039	0.10	0.045	2.6	this study
α-lactalbumin	cardosin A	55	6.0	0.0080	0.0060	0.0030	0.75	this study
α-lactalbumin	cardosin B	55	5.2	0.038	0.016	0.0090	0.42	this study
α-lactalbumin	cardosin B	55	6.0	0.020	0.084	0.041	4.2	this study
β-lactoglobulin	cardosin A	55	5.2	0.31	0.0020	0.0010	0.0060	this study
β-lactoglobulin	cardosin A	55	6.0	0.73	0.0000	0.0000	0.0000	this study
β-lactoglobulin	cardosin B	55	5.2	0.31	0.0070	0.0040	0.023	this study
β-lactoglobulin	cardosin B	55	6.0	0.047	0.0030	0.0020	0.064	this study

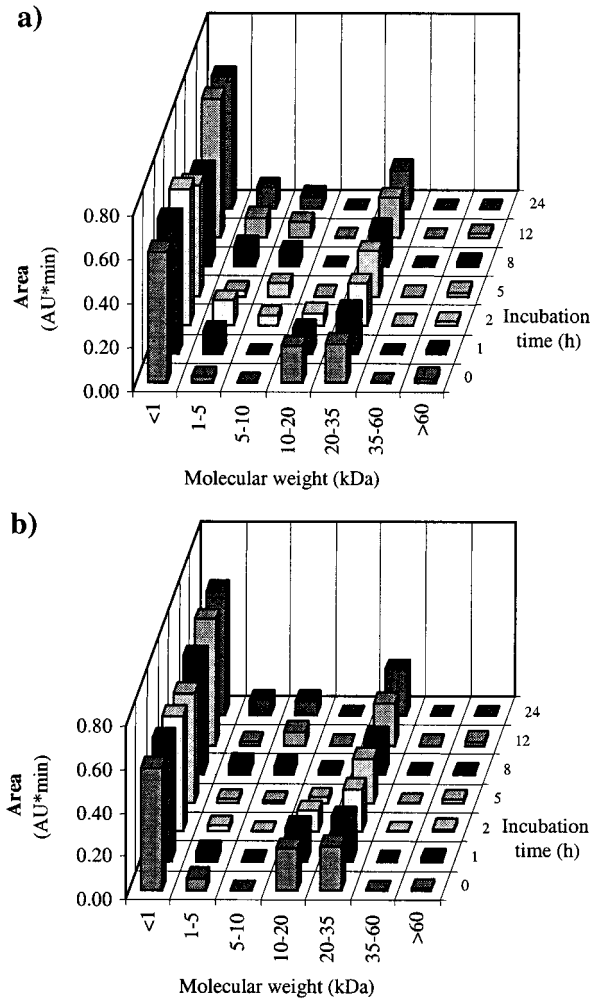

Figure 7. Molecular weight distribution of peptides in whey hydrolyzed at 55 °C by enzyme extract, at E/S ratio 1/7200 and at (a) pH 5.2 and (b) pH 6.0 (AU = absorbance unit).

the ratio k_{cat}/K_m was even lower than for the other substrates (see **Table 1**). The catalytic efficiency (k_{cat}/K_m) for cardosin A toward α-La is higher at pH 5.2 than at pH 6.0, whereas the opposite holds for cardosin B (**Table 1**). The experimental data produced pertaining to the reaction products (see **Figures 2**−**4** and **7**) indicate that there are in general multiple hydrolyses of α-La and β-Lg; in other words, there are multiple binding and cleavage sites in either protein. However, one objective of this piece of research was to keep the modeling complexity to a minimum, so all hydrolysis sites were lumped together, and thus only two substrates (α-La or β-Lg, as appropriate) were considered; this also implies that the k_{cat} and K_m values are average values for all bonds in stake. Despite this limitation, qualitative information can be gleaned from the chromatograms and electrophoretograms of the hydrolysates, as emphasized below.

Finally, it should be noted that, once a mechanistic model for a process is established, all parameters therein should be fitted simultaneously; if some parameters are estimated from fitting to independent data and introduced into the full model, this will constrain the nonlinear fitting procedure and make the results and inferences thereof statistically invalid. Therefore, even at the expense of a lower efficiency of the fitting procedure, f was fitted concomitantly with all remaining kinetic parameters. Note that the estimates of f_A provided by the model fit (0.45−0.65) do compare reasonably well with those determined experimentally (0.75), on a gel filtration chromatographic peak area.

Peptide Characterization. The hydrolysates were analyzed by FPLC (**Figures 2** and **7**) and SDS-PAGE (**Figures 3** and **4**). Hydrolyses carried out at pH 5.2 and 6.0 did not yield significant differences in the resulting peptide profile. However, when crude extract was added to whey at increasing ratios, increases in intensity of each band (or peak area, as appropriate) were noticed as expected. The best substrate for the crude extract was α-La, as shown in the electrophoretograms. Analysis of the hydrolysates allowed detection of a primary breakdown product, corresponding to two (peptide) bands with molecular masses of 3−6 kDa (see **Figure 3b**). These fragments were further hydrolyzed during prolonged incubation and eventually disappeared from the gel (**Figures 3** and **4**). In general, FPLC analysis (**Figure 7a**,**b**) revealed hydrolysate patterns that are similar to those obtained by SDS-PAGE, in that they show a gradual decrease in their average chain length.

Conclusions. This research effort has demonstrated that a solution containing two (protein) substrates—as in plain whey—and two (aspartic) proteases—as in an enzyme extract of the thistle flower—can be modeled by a double-substrate, double-enzyme Michaelis−Menten model. The major whey proteins, α-La and β-Lg, are differently acted upon by cardosins A and B: these enzymes hydrolyze α-La at higher rates and to higher extents (for the same incubation time) than β-Lg. The enzyme activity on either substrate protein is, as expected, dependent on the amount of enzyme added, but this effect is more perceptible on α-La than β-Lg. Both cardosins are less active on whey proteins than on κ-casein or a synthetic hexapeptide. For both enzymes and substrate proteins, the extent of hydrolysis is slightly higher at pH 5.2 than at pH 6.0. Finally, whey proteins (mainly α-La) can be hydrolyzed in whole whey with the

enzyme crude extract, thus resulting in peptide mixtures that may eventually be used for various commercial food applications, namely, in the soft drink, bakery, and dairy industries, or as complementary sources of nitrogen in fermentation processes.

Notation

C_α	concentration of α-lactalbumin (mM)
C_β	concentration of β-lactoglobulin (mM)
C_{E_t}	total concentration of enzyme (mM)
$C_{E_{A0}}$	total concentration of cardosin A (mM)
$C_{E_{B0}}$	total concentration of cardosin B (mM)
$k_{catA\alpha}$	intrinsic first-order kinetic constant associated with cardosin A acting on α-lactalbumin (s^{-1})
$k_{catB\alpha}$	intrinsic first-order kinetic constant associated with cardosin B acting on α-lactalbumin (s^{-1})
$k_{catA\beta}$	intrinsic first-order kinetic constant associated with cardosin A acting on β-lactoglobulin (s^{-1})
$k_{catB\beta}$	intrinsic first-order kinetic constant associated with cardosin B acting on β-lactoglobulin (s^{-1})
$K_{mA\alpha}$	Michaelis−Menten constant associated with cardosin A acting on α-lactalbumin (mM)
$K_{mB\alpha}$	Michaelis−Menten constant associated with cardosin B acting on α-lactalbumin (mM)
$K_{mA\beta}$	Michaelis−Menten constant associated with cardosin A acting on β-lactoglobulin (mM)
$K_{mB\beta}$	Michaelis−Menten constant associated with cardosin B acting on β-lactoglobulin (mM)
t	time (s)
f_A	fraction of cardosin A (−)
f_B	fraction of cardosin B (−)

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