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Characterization of "Lettucine", a Serine-like Protease from *Lactuca sativa* Leaves, as a Novel Enzyme for Milk Clotting

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In this work we focused on the characterization of a novel plant rennet purified from lettuce leaves (*Lactuca sativa* L. cv Romana). The lettuce protease, lettucine, showed trypsin-like, SV8-like, and caseinolytic activities. Although the enzyme did not recognize peptides having hydrophobic amino acid residues in the P_1 position of the target bond, it did show milk-clotting activity, suggesting that different bonds rather than the $Phe^{105}-Met^{106}$ of the κ-casein might be cleaved, still inducing milk-clotting. The enzyme exhibited proteolytic activity toward α-casein, β-casein, κ-casein, and milks with different fat contents, with the highest activity observed with partially skimmed milk, total casein, and α- and κ-casein. SDS−PAGE studies showed that lettucine cleaved α-casein, β-casein, and κ-casein. In particular, we showed that α-casein breakdown occurred even though total casein or milks were supplied, suggesting that the lettuce enzyme is able to operate a significant disorganization of the casein's micellar structure. Moreover, the proteolytic activity of the enzyme analyzed under various technological parameters, such as temperature and pH, indicated that the lettuce enzyme is highly consistent with the milk-clotting process.

KEYWORDS: ***Lactuca sativa*; plant rennet; milk clotting; SDS−PAGE; protease**

INTRODUCTION

Coagulation of milk is the basic step in the manufacture of all cheeses. Calf rennet, which contains chymosin as the main enzyme component, has been the most widely used milk-clotting enzyme preparation. Increasing world cheese production, along with a reduced supply of calf rennet, has led to a systematic investigation for new rennet sources. Microbial rennets produced by genetically engineered bacteria have proven suitable substitutes for animal rennet, but increasing attention has been directed toward natural rennet extracted from plants such as *Ananas comosus* (*1*), *Carica papaya* (*2*), and *Calotropis porcera* (*3*), among others. Unfortunately, most of these plant rennets were found unsuitable because they produced extremely bitter cheeses. An exception to this general rule is represented by the aqueous extract of *Cynara cardunculus* flowers containing two aspartic acids-type proteases, named cardosin A and B (*4*), which have been used for years for the manufacture of sheep-milk cheese in several areas of Portugal and Spain (*5*). Studies on the specificity of cardoon protease A showed that it cleaves only the $Phe_{105}-Met_{106}$ bond like chymosin, the proteolytic coefficient being of the same order of magnitude (*4*, *5*). Cardosin B, instead, is similar, in terms of specificity and activity, to pepsin. However, the flowers of *Cynara cardunculus* are not used for the production of cow-milk cheeses as they tend to taste bitter because of the formation of several peptides identifiable in the digests of isolated bovine α_s- and β-casein (*6*). More recent studies comparing the milk-clotting activity of cysteine and serine plant proteases suggested that serine-type plant proteases, although they are not widely distributed among plants, might be suitable for milk-clotting production. Cucumisin, a serine-type plant protease, exhibited the same milk-clotting activity of cysteine proteases such as papain but, in addition, it produced much less bitter testing hydrolysate than those formed by more typical plant cysteine-type proteases (*7*).

Recently, we have purified a novel protease from lettuce leaves (*Lactuca sativa* L.) by combination of $(NH_4)_2SO_4$ fractionation, gel filtration, and anionic exchange chromatography (*8*). The purified enzyme, named lettucine, is made up of a single subunit with an apparent molecular weight of 40,000. The lettuce protease has been classified as serine protease as it showed TLCK and leupeptin sensitive caseinolytic activity. On the basis of these latter features of the lettuce enzyme, the objective of this work was to characterize the proteolytic activity of the lettuce leaves protease with particular observations on cheesemaking technology and cheese ripening. Moreover, since application of enzymes in biotechnological processes is frequently limited by the denaturation of the native protein molecule, we also tested the consistency of the enzyme with several environmental factors such as pH and temperature, which might promote detrimental modifications in the enzyme structure and activity.

MATERIALS AND METHODS

Chemicals. Synthetic peptides, bovine α-casein, β-casein, κ-casein, and other biochemicals were purchased from Sigma (St. Louis, MO).

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All solutions were prepared with Milli-Q (Millipore, Mississagua, ON) purified water.

Enzyme Preparation. The protease was extracted and purified as described in ref *8*. Briefly, fresh lettuce leaves (*Lactuca sativa* L. cv Romana) obtained from a local market were washed with bidistilled water and homogenized with standard buffer 220 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM cysteine, and 5 mM HEPES−KOH pH 7.5, in a 1:1.25 w/v ratio. The homogenate was filtered and centrifuged at 27,200*g* for 30 min. The resulting surnatant was again centrifuged at 150,000*g* for 60 min, and the surnatant obtained was precipitated with solid $(NH_4)_2SO_4$ at 55% of saturation. The pellet was resuspended in standard buffer and applied to a Sephacryl S-300 column previously equilibrated with 25 mM HEPES−KOH pH 7.5. Samples of 1 mL were collected and assayed for proteolytic activity using casein as substrate; fractions of the second peak with highest proteolytic activity were pooled and applied onto a DEAE-Sepharose CL 6B column equilibrated with 25 mM HEPES−KOH pH 7.5. The column was developed with a linear gradient of 0−2 M NaCl. The active proteolytic fractions were combined and precipitated with solid $(NH_4)_2SO_4$ at 55% of saturation. After centrifugation the pellet was redissolved in HEPES−KOH pH 7.5 and further purified on a Sephacryl S-300 gel filtration column as described above. The active proteolytic peak was used for enzyme characterization.

Proteolytic Activity Assay. Proteolytic activity was assayed at 37 °C using casein, α-casein, *β*-casein, *κ*-casein, and milks at different fat contents as substrates following the method described in ref *9*, except that, after TCA precipitation of the unhydrolyzed substrate, the surnatant was recovered by centrifugation at 10,000 rpm in a benchtop microfuge (Sigma 1-15). Enzyme activity was espressed in units defined as the amount of enzyme that yielded a 0.001 absorbance change per min.

Peptidase Activity Assay. Peptidase activity was estimated by the cleavage of synthetic peptides *N*-Cbz-GGR-*β*NA, *N*-Cbz-LLE-*β*NA, *N*-succinyl-GGF-pNa, *N*-Cbz-VKM-MCA, *N*-succinyl-AAA-pNa, *N*-Cbz-GGL-pNa, *N*-succinyl-GGG-pNa, *N*-succinyl-IIW-MCA, and *N*-succinyl-YLV-pNa. The assay mixture (0.25 mL) contained 100 mM Tris−HCl pH 8.0 and 0.4 mM of synthetic peptide as substrate. After incubation at 37 °C for suitable periods, the reaction was stopped by adding 0.25 mL of 20% (w/v) TCA. Samples were centrifuged at 10,000 rpm for 5 min, and then the released *p*Na, *β*NA, or MCA was measured following the diazotization procedure according to ref *10* as modified in ref *11*.

Milk-Clotting Activity Assay. Milk-clotting activity was measured by the method described in ref *7*. The enzyme (11 nmol) was added to a 10% solution in 67 mM NaH_2PO_4 pH 6.8 of skim-milk powder at 30 °C both in the presence and in the absence of 5 mM $CaCl_2$ in a final volume of 3 mL. The time elapsing between the mixing of reagents and the first appearance of solid material against the background was measured. One rennet unit (R. U.) was defined as the amount of purified enzyme needed to coagulate 10 mL of skim-milk at 30 °C in 100 s.

Hydrolysis of Caseins. Total casein, α-casein, *β*-casein, and *κ*-casein were dissolved in 67 mM NaH_2PO_4 pH 6.8 to a final concentration of 1.5 mg/mL. The enzyme (30 pmol) was then added, and the experimental solutions (final volume 0.3 mL) were incubated for 3 h at 37 °C in a thermostated water bath. Samples obtained as described above were prepared for SDS−PAGE by adding an equal volume of double-concentrated sample loading buffer.

Optimum pH and Temperature. Optimum pH values were determined by monitoring the peptidase activity of the enzyme toward synthetic peptides *N*-Cbz-GGR-*β*NA and *N*-Cbz-LLE-*β*NA at pH values between 5.0 and 9.0. The buffers used were 50 mM MES pHs 5.0, 5.5, and 6.0, 50 mM bis-tris-propane pH 6.5, 50 mM HEPES pHs 7.0 and 7.5, 50 mM Tris pHs 8.0, 8.5, and 9.0. The optimum temperature for proteolytic activity was determined by carrying out standard assays at temperatures ranging from 5 to 50 °C at pH 6.8.

Enzyme Stability. Enzyme stability was determined under standard conditions by assaying the remaining caseinolytic activity after preincubation for 4 h at different temperatures, and, after preincubation at 10 °C for 4 h at different pH values.

Gel Electrophoresis and Protein Assay. SDS−PAGE (12.5% slab gels) was performed according to ref *12*. Gels were stained with Coomassie Blue R-250 and destained by repeated washing in a methanol/acetic acid/water (2:1:10) solution. Protein content was routinely measured by the method of Bradford (*13*). The Lowry's method was used to determine milk's protein content (*14*). Bovine serum albumin was used as standard.

Table 1. Substrates Specificity of the Purified Lettuce Leaves Protease

substrate	relative activity (%)
N-cbz-Gly-Gly-Arg-*β*NA[a]	100
N-cbz-Leu-Leu-Glu-*β*NA	13
N-cbz-Gly-Gly-Leu-pNa	0
N-succinyl-Gly-Gly-Phe-pNa	0
N-succinyl-Ala-Ala-Ala-pNa	0
N-cbz-Val-Lys-Met-MCA	0
N-succinyl-Gly-Gly-Gly pNa	0

[a] The specific activity was 83 pkcat/mg.

Table 2. Hydrolysis of Both Casein's Fractions and Milks by the Lettuce Leaves Protease

substrate	specific activity (U/mg)
casein	0.22
α-casein	0.21
β-casein	0.07
κ-casein	0.21
partially skimmed milk	0.24
skimmed milk	0.19
whole milk	0.13

RESULTS

Substrates Specificity and Caseins Degradation. The purified enzyme was tested for substrates specificity using different synthetic peptides. **Table 1** showed that the lettuce protease hydrolyzed *N*-cbz-GGR-*β*NA, which is a model substrate for trypsin-like activity (*15*), more efficiently than the other substrates examined. It did not exhibit activity toward *N*-succinyl-GGF-pNa, *N*-Cbz-VKM-MCA, *N*-succinyl-AAA-pNa, *N*-Cbz-GGL-pNa, *N*-succinyl-GGG-pNa, *N*-succinyl-IIW-MCA, and *N*-succinyl-YLV-pNa, whereas it was slightly active against *N*-cbz-LLE-*β*NA, a model substrate for peptidyl glutamyl hydrolase like activity (SV8-like activity) (*15*). The proteolytic activity of the protease from lettuce leaves assayed toward caseins and milks at different fat contents is shown in **Table 2**. The enzyme exhibited the highest degrees of proteolysis on partially skimmed milk, total casein, α-casein, and *κ*-casein. The tendency to hydrolyze strongly decreased when whole milk was used as substrate, as well as when proteolysis was measured against *β*-casein or α-lactalbumin.

Hydrolysis of Casein's Fractions and Milks by the Lettuce Leaves Protease. Figure 1 shows the SDS−PAGE patterns of the single fractions of casein along with their respective breakdown products. The electrophoretogram of untreated total casein revealed the presence of two main bands corresponding to α- and *β*-casein. The estimated molecular weights were, respectively, 32,500 and 29,600 (**Figure 1**, lane D). The total casein digestion by the lettuce protease yielded two additional bands whose molecular weights were calculated at around 22,200 and 14,500 (**Figure 1**, lane E). The treatment of α-casein alone with lettucine (**Figure 1**, lane G) suggested that these latter bands might have arisen from hydrolysis of α-casein. The hydrolysis of *β*-casein formed several fragments of different molecular weights (**Figure 1**, lane I). The major hydrolysis product of *β*-casein had an apparent molecular weight of 20,200 (**Figure 1**, lane I) and it was the unique fragment of *β*-casein also detectable among the hydrolysis products of the total casein

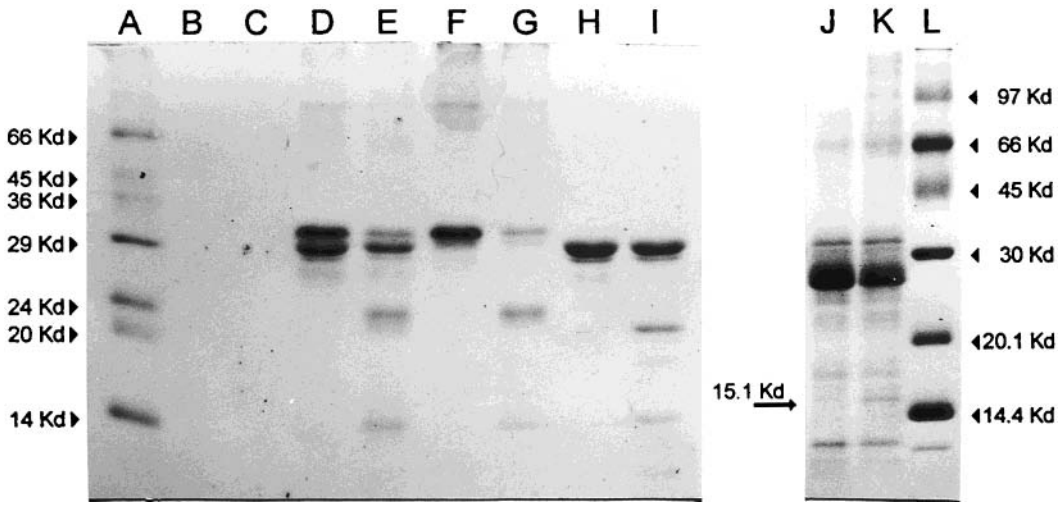

Figure 1. SDS–PAGE pattern of casein's hydrolysis products. Total casein and casein's single fractions were incubated with lettuce protease (30 pmol) as described in Materials and Methods; then, aliquots (10 μg of caseins) were loaded onto a SDS gel (12.5% slab gel) and analyzed. Lanes A and L, molecular weight standards; lane B, lettuce leaves enzyme; lane C, sample buffer; lane D, control casein; lane E, hydrolyzed casein; lane F, control α-casein; lane G, hydrolyzed α-casein; lane H, control β-casein; lane I, hydrolyzed β-casein; lane J, control κ-casein; and lane K, hydrolyzed κ-casein. Lane B showed that the amount of lettuce enzyme contained in each sample was not detectable on the gel.

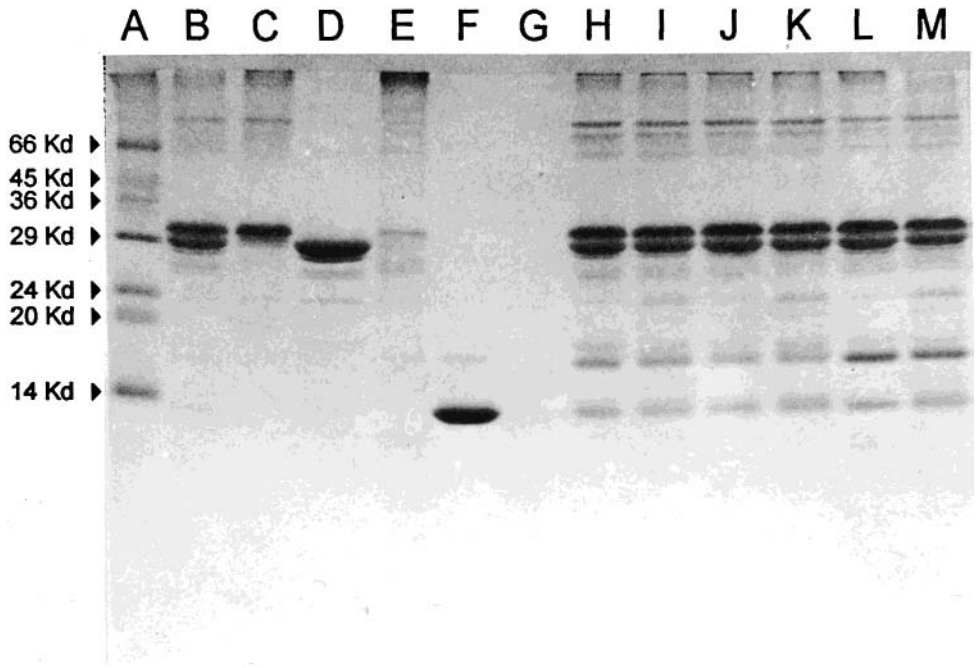

Figure 2. SDS–PAGE pattern of milks subjected to lettucine treatment. Milks were incubated with lettuce protease (30 pmol) as described in Materials and Methods. Successively, samples corresponding to 10 μg of milk protein fraction were loaded onto a 12.5% slab gel and analyzed. Lane A, molecular weight standard; lane B, control casein; lane C, control α-casein; lane D, control β-casein; lane E, control κ-casein; lane F, control α-lactalbumin; lane G, lettuce protease; lane H, control skimmed milk; lane I, treated skimmed milk; lane J, control partially skimmed milk; lane K, treated partially skimmed milk; lane L, control whole milk; lane M, treated whole milk. In lane G it is shown that the amount of lettuce enzyme contained in each sample was not detectable on the gel.

(**Figure 1**, lane E). The electrophoretic pattern of commercially available κ-casein showed a main band of 26,500 along with small quantities of contaminant bands of different molecular weights (**Figure 1**, lane J). The 26,500 band probably corresponds to κ-casein as reported in ref *16*, which estimated κ-casein's molecular weight from migration in SDS−PAGE rather than from primary structure studies. The treatment of κ-casein with lettucine induced an extensive degradation of κ-casein and yielded an additional band of 15,100 that was not noticeable in the control κ-casein (**Figure 1**, lane K).

The SDS−PAGE pattern of milks at different fat contents and their hydrolysate is shown in **Figure 2**. The major digestion products detectable in all samples were two main bands of 22,500 and 14,500 (**Figure 2**, lanes I, K, and M) which, as mentioned above, could correspond to α-casein hydrolysates.

Milk-Clotting Activity. The lettuce protease was tested for milk clotting activity as described in Materials and Methods. As shown in **Table 3** the enzyme exhibited milk clotting activity which was correlated with protease concentration. No apparent effect on milk clotting was observed when the reaction medium was supplemented with 5 mM $CaCl_2$.

Effect of $CaCl_2$ on Proteolytic Activity of the Lettuce Protease. As the presence of calcium ions represents a crucial element during the cheesemaking process, we evaluated the

Table 3. Milk-Clotting Activity of the Lettuce Leaves Protease

enzyme concentration (μM)	milk-clotting activity (R. U.)	
	− $CaCl_2$	+ $CaCl_2$
1.9	20	20
3.8	40	40

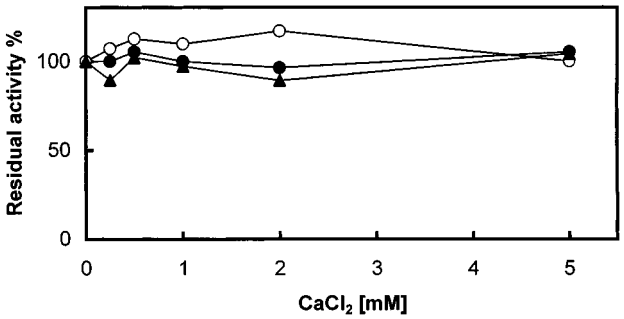

Figure 3. Effect of $CaCl_2$ on the caseinolytic (●), trypsin-like (○), and SV8-like (▲) activities of the lettuce protease. The activities of the enzyme (9 μg) were measured in standard buffers supplemented with increasing concentrations of calcium chloride as described in Materials and Methods.

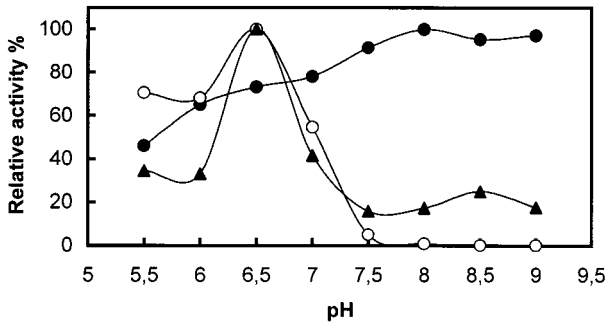

Figure 4. Effect of pH on the peptidase activities and stability of the lettuce leaves protease. Aliquots of the purified enzyme (7 μg) were used to measure peptidase activities on *N*-cbz-GGR-βNA (●) or N-cbz-LLE-βNA (○) at different pHs as described in Material and Methods. The pH stability of the protease was determined by monitoring the caseinolytic activity of the enzyme after 4 h incubation at different pHs (▲).

effect of increasing concentrations of $CaCl_2$ upon the lettuce protease reaction media. As shown in **Figure 3** $CaCl_2$ did not affect the catalytic activity of the enzyme measured both against casein and synthetic peptides.

Effect of pH on Peptidase Activity and Stability of the Purified Enzyme. As casein is substantially insoluble at pH values below 6.0, the pH-dependence of lettucine activity was evaluated by monitoring the peptidase activities against *N*-Cbz-GGR-βNA (trypsin-like activity) and *N*-Cbz-LLE-βNA (SV8-like activity) at various pH values. As shown in **Figure 4**, the trypsin-like activity of the enzyme was expressed in a wide range of pH values exhibiting maximal activity at alkaline pH. Qualitatively different results were obtained using *N*-Cbz-LLE-βNA as substrate. The enzyme activity was in fact higher at acid pH values with optimal activity occurring at 6.5, whereas above pH 7.0 it was completely lost. The enzyme from lettuce leaves was stable in a narrow pH range at 10 °C for 4 h. The pH stability was in fact maximal at 6.5, whereas below and above this pH value the activity abruptly decreased (**Figure 4**).

Effect of Temperature on Proteolytic Activity and Stability of the Protease from Lettuce Leaves. Figure 5 shows the temperature dependence of the lettucine proteolytic activity determined toward total casein, α-casein, β-casein, and κ-casein

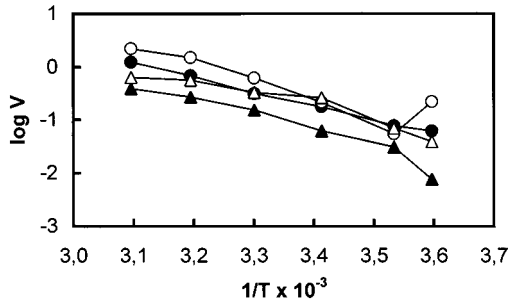

Figure 5. Temperature dependence of the lettuce leaves protease. The proteolytic activity of the enzyme (7 μg) on total casein (●), α-casein (○), β-casein (▲), and κ-casein (△) was assayed at different temperatures in standard buffer as described in the Materials and Methods section. Data are plotted as Arrhenius graphs.

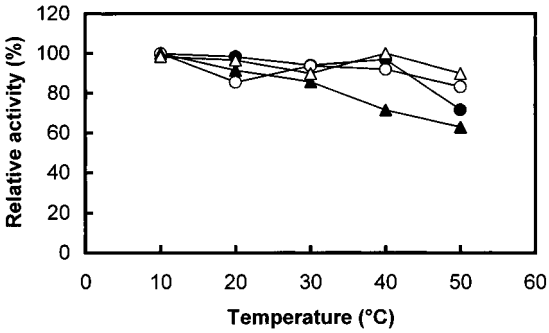

Figure 6. Thermal stability profiles of the lettuce leaves protease. The enzyme was incubated for 4 h at different temperatures, then aliquots (8 μg) were used to assay the residual activity toward total casein (●), α-casein (○), β-casein (▲), and κ-casein (△) as detailed in Materials and Methods.

at pH 6.8. The proteolytic activity of the enzyme increased along with temperature and reached an optimum at 50 °C. After 4 h incubation at pH 6.8, the proteinase was totally stable up to 40 °C, although when β-casein was used as substrate the enzyme showed 70% of remaining activity (**Figure 6**). However, incubation at 50 °C did not greatly affect the enzyme, as it retained the proteolytic activity against κ-casein and α-casein at values higher than 80% of the original activity.

DISCUSSION

The results presented above show that the lettuce leaves protease exhibited trypsin-like, SV8-like, and caseinolytic activities (**Table 1**). The enzyme did not hydrolyze synthetic peptides with hydrophobic residues, such as Phe, in the P_1 position of the susceptible bond. The comparison of lettucine specificity with other calf rennet substitutes revealed that most of them behave like chymosin cleaving the Phe^{105}−Met^{106} bond, although it has been reported that the *Endothia parasitica* protease hydrolyzed the preceding bond (Ser^{104}−Phe^{105}) without affecting the clotting (*5*). Considering, as shown in **Table 2**, the lettuce enzyme does have high milk-clotting activity, this probably could be due to the cleavage of different peptide bonds in the region close to the Phe^{105}−Met^{106} bond. Analysis of the κ-casein primary structure suggested selection of the Arg^{97}−His^{98}, Lys^{111}−Lys^{112}, or Lys^{112}−Lys^{113} as putative target sites. With respect to proteolytic activity assayed toward caseins, the lettuce leaves enzyme digested preferentially α-casein and κ-casein (**Table 2**). Moreover, it was shown that α-casein's hydrolysis products could be obtained either when α-casein alone was given as substrate (**Figure 1**, lane G), or in the case

that total casein (**Figure 1**, lane E) and milks (**Figure 2**, lanes I, K and M) were used as substrate. As α-casein is localized in the internal portion of the casein's micellar structure maintained in solution by the κ-casein hydrophilic domain, the breakdown of milk's α-casein could account for a disorganization of the micellar structure provided by the lettuce protease. Among the milks tested, the lettuce protease showed the highest proteolytic activity on partially skimmed milk, whereas whole milk was cleaved to a lesser extent (**Table 2**). This finding suggested that milk's fat content might affect the lettucine structure or that it might act by hiding susceptible bonds. However, different degrees of proteolysis varying upon milks used as substrates represent a desirable effect which might lead to the production of novel cheeses characterized by their unique flavor and taste along with different fat contents (*17*−*19*).

Furthermore, the analysis of the lettucine consistency with respect to the cheesemaking process revealed that the enzyme will be available for milk-clotting by setting up the optimum conditions. In fact, either the proteolytic activity or milk-clotting activity of the enzyme were not affected by $CaCl_2$, which is usually added to facilitate milk-clotting. As regards temperature, no denaturation of the enzyme molecules occurred even when the activity was performed at 50 °C (**Figure 5**). In addition, at that temperature the enzyme retained most of the proteolytic activity on α-casein and κ-casein for 4 h (**Figure 6**). As shown in **Figure 4**, the pH of the reaction medium is critical either for enzyme stability or proteolytic activity; pH 6.5 could represent an optimum value to perform clotting, as both the peptidase activities of the enzyme were totally expressed and the enzyme stability was the highest registered.

ABBREVIATIONS USED

N-cbz-, *N*-carbobenzyloxy; EGTA, ethylene glycol-bis-(β-aminoethyl ether) N,N,N′,N′,-tetracetic acid); βNA, β-naphthylamide; pNa, *p*-nitroanilide; MCA, 7-amino-4methylcoumarylamide; TCA, trichloroacetic acid; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone.

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