Proteolytic Activity of *Lactobacillus bulgaricus* Grown in Milk

ANALIA G. ABRAHAM, GRACIELA L. DE ANTONI, and MARIA C. AÑON
Centro de Investigación y Desarrollo
en Criotecnología de Alimentos (CONICET-UNLP)
47 y 116 (1900) La Plata, Argentina

ABSTRACT

The proteolytic activity of *Lactobacillus bulgaricus* LBB grown in skim milk was determined at 42°C as a function of growth temperature and TCA-soluble N in the growth medium. A new method was used for harvesting bacteria from coagulated milk with the addition of EDTA (pH 12) to reach pH 7.

Maximum specific proteolytic activity was observed with bacteria grown at temperatures between 34 and 38°C in milk with low concentrations of TCA-soluble N. This activity decreased when growth temperature was above 40°C or when TCA-soluble N increased in the growth medium. Specific proteolytic activity did not change in bacteria grown at 42°C when TCA-soluble N varied or when cells were grown in milk with a high concentration of free amino acids at different incubation temperatures. Analysis by SDS-PAGE showed that this strain hydrolyzed α- and β-caseins.
(**Key words**: *Lactobacillus bulgaricus*, proteases, proteolytic activity)

Abbreviation key: **SPA** = specific proteolytic activity, **uPA** = units of proteolytic activity.

INTRODUCTION

Lactobacillus bulgaricus, a lactic acid bacteria with complex growth requirements, is extensively used in the manufacture of cheese and yogurt (11). The pool of free amino acids and peptides present in milk is not enough to ensure optimal bacterial growth (13). The main source of N for this species in milk is provided by the hydrolysis of caseins by the action of *L. bulgaricus* proteases (15, 16).

Received December 18, 1992.
Accepted January 11, 1993.

Proteolytic systems of lactobacilli are complex and are composed of proteinases and peptidases with different subcellular locations. Proteinases of *L. bulgaricus* are associated with the cell wall (1) and are regulated by temperature and growth phase (6).

Results concerning the proteolytic activity of *L. bulgaricus* have been obtained in rich media, such as MRS broth (1), in which bacteria utilized free amino acids present in the broth. In contrast, the proteolytic activity in milk has not been extensively studied. Recently (10), proteases of cells grown in milk and in MRS broth showed identical patterns of hydrolytic products of α- and β-caseins.

The study of the proteolytic activity of *L. bulgaricus* in milk would enhance the knowledge base required for selection of starter cultures. Expression of proteolytic activity is important in relation to symbiotic growth with *Streptococcus thermophilus* during the production of yogurt (7, 14). Objectives of this research were to evaluate the proteolytic activity of *L. bulgaricus* in milk and the influence of TCA-soluble N concentration and growth temperature on this metabolic activity.

MATERIALS AND METHODS

Strains

Lactobacillus bulgaricus LBB was isolated from a yogurt starter and classified by growth temperature and sugar fermentation with the API system (Appareils et Procédés d'Identification, Montalieu Vercieu, France) for lactic acid bacteria. The strain was maintained frozen at −80°C in milk.

Chemicals and Culture Media

Yeast extract and tryptone were obtained from Difco (Detroit, MI). Agar was obtained from Britania (Buenos Aires, Argentina). Lactose, SDS, glycerol, and Na_2-EDTA were obtained from Mallinckrodt Chemical Works

(New York, NY). Folin's reagent and TCA were obtained from Merck (Darmstadt, Germany). Hammarsten quality casein was obtained from Research Organic, Inc. (Cleveland, OH). Reconstituted skim milk (12% wt/vol) was prepared from NDM, sterilized by tyndalisation, i.e., three 30-min treatments at 100°C. This milk had a TCA-soluble N content, expressed as Tyr, of 7.5 mg of Tyr/100 ml. When necessary, casein hydrolysate (Oxoid Ltd., London, England) was added to reach concentrations of 12.5, 15.5, and 22.0 mg of Tyr/100 ml of milk.

The 1.1.1. broth (4) contained 10, 10, and 10 g/L of tryptone, yeast extract, and lactose, respectively (pH 6.8). The solid media was prepared by the addition of 1.5% agar. The media were sterilized at 121°C for 15 min.

Determination of Bacterial Concentration

The concentration of viable bacteria in cultures and bacterial suspensions was determined by plating serial dilutions in 1 g/L of tryptone on 1.1.1. agar plates. The results were expressed as colony-forming units per milliliter. The pH of the cultures was determined at 25°C using a Cole-Parmer (Chicago, IL) combined glass-calomel microelectrode.

Determination of TCA-Soluble N

Macromolecules were precipitated with TCA at a final concentration of 8.0 g/100 ml, followed by filtration through Whatman number 1 paper (Whatman, Clifton, NJ). The TCA-soluble N was determined in the supernatant with Folin's reagent according to the method of Hull (8) and Citti et al. (3) and expressed as milligrams of Tyr/100 ml. The absorbance at 650 nm was determined in a Shimadzu (Kyoto, Japan) double-beam spectrophotometer.

Growth and Harvest of Bacteria

Frozen cultures were reactivated by growth in milk at 37°C and then subcultured once in milk or broth to obtain an active inoculum. In all experiments, 100 ml of broth or milk with or without the addition of casein hydrolysate were inoculated with 5% active inoculum and incubated to pH 5. Incubation temperature varied from 32 to 48°C depending on the experiment. Bacteria harvested from milk were treated with 2% Na_2-EDTA, pH 12 (12), to obtain a final pH of 6.5 to 7.0 and then centrifuged at 5000 × *g* for 15 min at 10°C. The bacterial pellet was washed twice and resuspended in .1 of the original volume with sterile distilled water to obtain a bacterial suspension. The bacteria grown in broth were harvested by centrifugation without the treatment described.

Determination and Definition of Proteolytic Activity

Casein was used as substrate to measure proteolytic activity. The reaction mixture was .5 ml of bacterial suspension of 1 to 4 × 10^8 cfu/ml and 2 ml of casein (2.5 mg/ml) in .1 *M* K_2HPO_4 buffer (pH 7). The reaction mixture was incubated at 42°C. Colony-forming units neither increased nor decreased during the enzymatic assay. The reaction was stopped by the addition of 5 ml of TCA (12% wt/vol). The precipitate was removed by filtration through Whatman number 1 paper, and TCA-soluble compounds were evaluated as described. In all experiments, the units of proteolytic activity were calculated from the slope of the curve of absorbance at 650 nm versus incubation time. The unit of proteolytic activity (**uPA**) was defined as the amount of enzyme that produced an increase of absorbance at 650 nm of .01 in 1 h at 42°C at pH 7. The specific proteolytic activity (**SPA**) is the uPA per 10^8 cfu, and is expressed as uPA per 10^8 cfu.

Proteinase Activity Against Casein Fractions

Hydrolysis of casein was determined using a reaction mixture containing .5 ml of bacterial suspension and 2 ml of 2.5 mg/ml casein in .1 *M* K_2HPO_4 buffer (pH 7). Aliquots of .5 ml were incubated at 42°C, and the reaction was stopped by freezing at –80°C.

Each sample was subjected to SDS-PAGE by Laemmli's method (9) on gels containing 10 or 12.8% acrylamide in a vertical system (Hoeffer Scientific Instrument, San Francisco, CA). Gel slabs were stained with .1% Coomassie brilliant blue R250 in water:methanol: acetic acid (5:5:2, vol/vol/vol). Samples were diluted 1:1 with pH 7 buffer (.014 *M* Tris, .001 *M* Na_2-EDTA, 1% SDS, 10% glycerol, and

.01% bromophenol blue). The total amount of casein loaded in the gel was 100 μg.

Gels were scanned in a Shimadzu dual wavelength TLC scanner CS-910 using a sample wavelength of 570 nm and reference wavelength of 395 nm. Amount of protein was determined by reading the area covered by each band using an integrator (Morphomat 30 Zeiss, Oberkochen, Germany).

RESULTS

Assay of Proteolytic Activity

Figure 1 shows the hydrolysis of casein by *L. bulgaricus* LBB at 42°C. The assay was performed with a bacterial suspension obtained from a culture in milk at 37°C. The increase of TCA-soluble products was linear with time for 120 min using 2.0 mg/ml of casein. The assay performed with 1 × 10^8 cfu showed a proteolytic activity of 4 uPA. A sample of milk, acidified with HCl and treated further as in preparing the bacterial suspension, was tested as a control; TCA-soluble products did not increase after 24 h of incubation, indicating that the proteolytic activity detected was due to the bacterial proteases. Hydrolysis rate was maximal when casein concentrations ranged from 2.0 to 8.0 mg/ml. Therefore, all experiments were performed using casein concentration of 2.0 mg/ml to avoid solubility problems that can occur at higher casein concentrations.

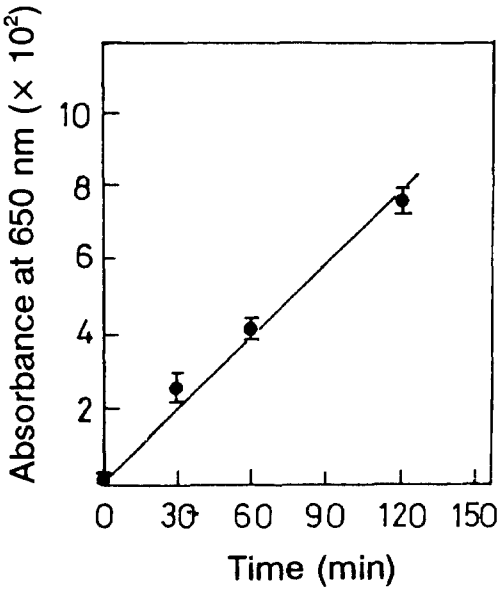

Figure 1. Kinetics of proteolytic activity. The assay was performed at 42°C with a single culture of *Lactobacillus bulgaricus* LBB. The bacterial suspension (1.8 × 10^8 cfu/ml) was obtained from cells grown in milk at 37°C. Units of proteolytic activity were calculated from the slope of the curve and defined as the amount of enzyme necessary to produce an increment of absorbance of .01 in 60 min. The error bars are standard deviations obtained with three replicates (r = .988).

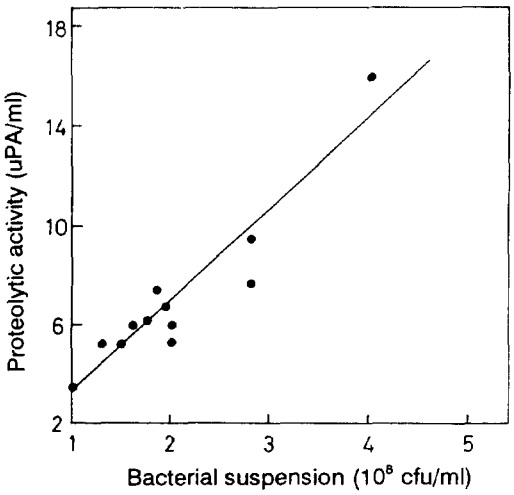

Figure 2. Relation of proteolytic activity to number of cells in the assay for 12 different trials with *Lactobacillus bulgaricus* LBB grown in milk at 37°C. Units of proteolytic activity (uPA) were defined as the amount of enzyme necessary to produce an increment of absorbance of .01 in 1 h. Each point was from independently isolated cultures of strain LBB (r = .911).

The SPA was determined using 12 different *L. bulgaricus* cultures of strain LBB grown in milk at 37°C and varying the bacterial concentration in the enzymatic assay from 1 to 4 × 10^8 cfu/ml (Figure 2). The uPA increased with the number of cells in the bacterial suspension over the concentration range used. The SPA of strain LBB was 3.6 uPA/10^8 cfu. These results demonstrate that proteolytic activity depends only on the concentration of cells and that the methodology for harvesting cells makes repeatable the results obtained from different cultures.

Proteolytic Activity of Strain LBB at Different Temperatures and Concentrations of Free Peptides

The SPA were obtained from bacterial suspensions of cultures grown in plain milk at 37 and 42°C. Table 1 shows the results from four different bacterial suspensions of strain LBB obtained from independent cultures. Cells grown at 37°C presented higher SPA than those grown at 42°C, and the difference between conditions was 1.7 to 2 uPA/10^8 cfu.

The SPA was determined with bacteria grown at both temperatures in milk with increasing free amino acids and peptides provided by the addition of casein hydrolyzate (Figure 3, A and B). At 37°C (Figure 3A), SPA decreased sharply as the concentration of Tyr increased from 7.5 to 15.5 mg of Tyr/100 ml. This minimal activity continued at concentrations up to 22 mg of Tyr/100 ml. However, bacteria grown at 42°C showed no significant change ($P > .05$) in the SPA when TCA-soluble Tyr increased in milk (Figure 3B); a minimal value, similar to that obtained at 37°C, was found at all concentrations of free N compounds. The same minimal value also was obtained for strain LBB grown in 1.1.1. broth (Figure 4) in which the concentration of free Tyr was 31 mg/100 ml.

The low SPA of bacteria grown in media with high concentrations of free amino acids and peptides could not be attributed to an inhibition of the proteases activity because the proteolytic assays performed with and without casein hydrolysate (Table 2) showed no change in SPA at either growth temperature.

The contrast between the proteolytic responses at low (7.5 mg of Tyr/100 ml) and high (15.5 mg of Tyr/100 ml) concentrations of TCA-soluble N compounds (Figure 3) was studied at temperatures of growth varying from 32 to 48°C (Figure 5, A and B). The SPA was highest and constant for bacteria grown in milk with low concentrations of Tyr at temperatures below 37°C. The SPA decreased sharply between 38 and 40°C, reaching a minimum four to five times lower than that observed at 37°C. This low SPA was constant between 40 and 48°C. In contrast, bacteria grown in milk with a higher (15.5 mg Tyr/100 ml) concentration of TCA-soluble compounds had depressed SPA at all growth temperatures between 32 and 48°C. The decrease in SPA could be attributed to the effect of the growth temperature on synthesis or on enzyme activity. To determine how temperature affected enzyme ac-

TABLE 1. Specific proteolytic activity (SPA) in units of proteolytic activity (uPA) per 10^8 cfu of cells grown in milk[1] at 37 and 42°C.

Experiment[2]	SPA 37°C	SPA 42°C	Change in SPA[3]
	(uPA/10^8 cfu)		
1	3.7	1.7	2.0
2	4.8	3.2	1.7
3	3.1	1.2	1.9
4	2.9	1.2	1.7

[1]Milk had a TCA-soluble N content, expressed as Tyr, of 7.5 mg of Tyr/100 ml.

[2]Experiments 1, 3, and 4 were carried out with the same batch of milk. Experiment 2 was carried out with milk of different origin.

[3]Difference between SPA of cells grown at 37 and 42°C.

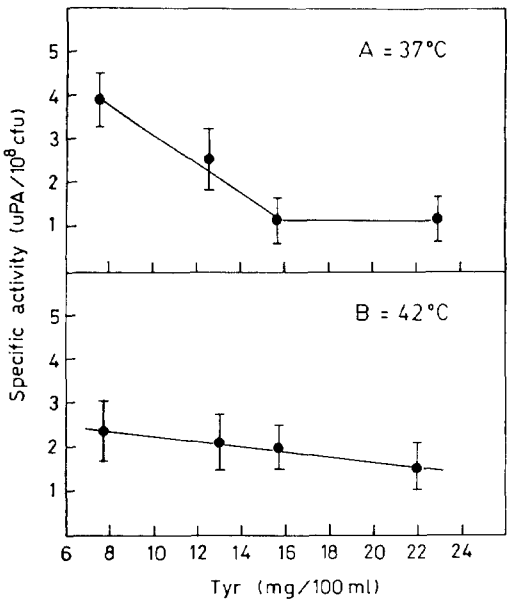

Figure 3. Influence of TCA-soluble N (milligrams of Tyr/100 ml) in the milk growth medium on the specific proteolytic activity of *Lactobacillus bulgaricus* LBB at two growth temperatures, A) 37°C and B) 42°C. Each point is the mean of at least four experiments. Least significant difference = 1.1 ($P = .05$). Error bars are standard deviations.

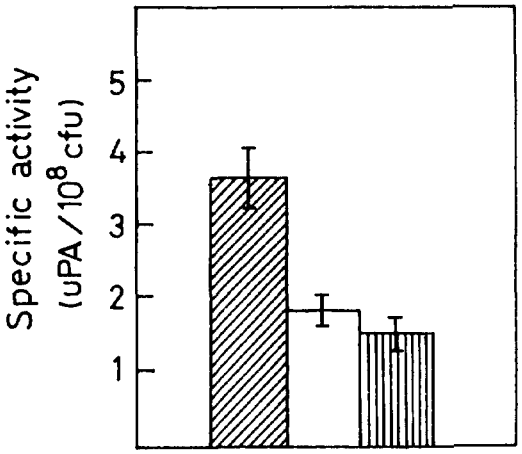

Figure 4. Specific proteolytic activity of *Lactobacillus bulgaricus* LBB cell suspensions obtained from cultures grown in milk containing 7.5 mg of TCA-soluble N as Tyr/100 ml (diagonal hatched bar), milk with casein hydrolysate containing 15.5 mg of TCA-soluble N as Tyr/100 ml (open bar), and 1.1.1. broth containing 31 mg of TCA-soluble N as Tyr/100 ml (vertical hatched bar). Results are means of at least four experiments. Error bars are standard deviations.

tivity, the proteolytic assay was performed at four different temperatures. The reaction rate increased with temperature from 30 to 48°C as observed in the Arrhenius plot (Figure 6). Therefore, the low proteolytic activity in milk at growth temperatures of 40 to 48°C (Figure 5A) could not be attributed to decreased protease activity.

TABLE 2. Influence of TCA-soluble N in the proteolytic enzyme assay on the specific proteolytic activity in units of proteolytic activity (uPA) per 10^8 cfu.

Growth temperature	Composition of substrate	
	Casein[1]	Casein + casein hydrolysate[2]
(°C)	(uPA/10^8 cfu)	
37	3.20	3.14
42	.83	.87

[1]2 mg of Tyr/100 ml.

[2]15 mg of Tyr/100 ml.

Proteolytic Activity at Different Stages of Growth

Growth, acidification, and SPA of *L. bulgaricus* LBB were evaluated in skim milk without casein hydrolyzate at 37 and 42°C (Figure 7, A and B). Bacteria grown in 1.1.1. broth at 37°C were harvested, assayed for their proteolytic activity, and used to inoculate the skim milk. Harvested bacteria had an SPA of 1.2 uPA/10^8 cfu. During the growth in milk, the lag phase was approximately 3 h at 37°C, but no lag phase was observed at 42°C (Figure 7A). The initial rate of acidification was similar at both temperatures, but, after 3 h of incubation, acid production increased for the culture grown at 42°C, which reached pH 5 with 2×10^8 cfu/ml after 5 h of incubation. The same results were observed for the culture at 37°C after 6 h of incubation. The SPA (Figure 7B) of culture grown at 37°C increased from 1.2 to 3.0 uPA/10^8 cfu during the lag

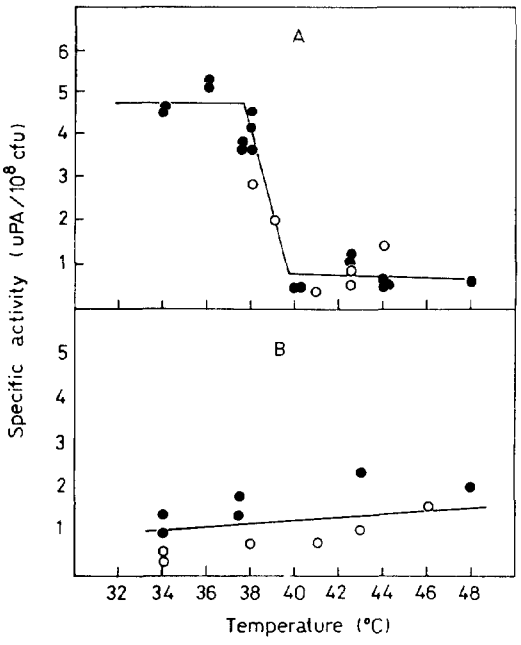

Figure 5. Influence of growth temperature on specific proteolytic activity in units of proteolytic activity (uPA) per 10^8 cfu of *Lactobacillus bulgaricus* LBB grown in milk containing 7.5 (A) and 15.5 (B) mg of TCA-soluble N as Tyr/100 ml. o,• Different batches of milk. Least significant difference = .7 (P = .05).

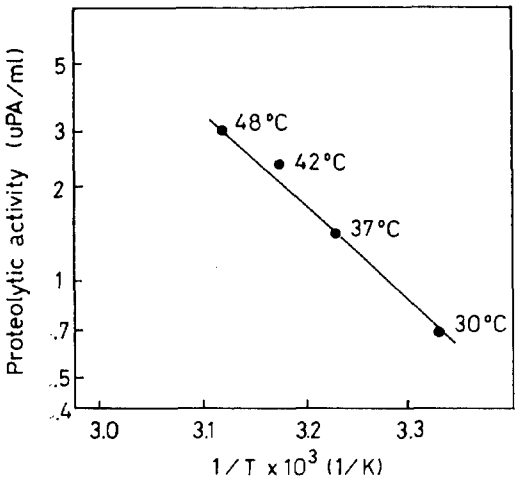

Figure 6. Arrhenius plot for proteolytic activity in units of proteolytic activity (uPA) of *Lactobacillus bulgaricus* LBB grown at 37°C in plain milk with 7.5 mg of TCA-soluble N as Tyr/100 ml (r = .990). T = Temperature in kelvin (K).

phase and maintained that value during the logarithmic and stationary phases. However, bacteria propagated at 42°C showed low and constant SPA during all growth phases (Figure 7B). In stationary phase cultures with 1×10^8 cfu/ml, values of TCA-soluble compounds were similar after growth in milk at 37 and 42°C, demonstrating that the degree of proteolysis was the same under either condition (data not shown). These results suggest that bacteria with low SPA synthesized proteases at 37°C to achieve a concentration that allowed growth in milk. At 42°C, growth was possible at the basal level of proteases. This interpretation is in agreement with results of Figure 6, which showed that SPA of proteases at 42°C was 1.6 times higher than at 37°C. Therefore, to produce the same amount of TCA-soluble N from casein, the concentration of proteases per bacteria must be twice that obtained when the growth temperature is 37°C.

Casein Degradation by SDS-PAGE

Casein degradation was evaluated by SDS-PAGE at different incubation times with bacterial suspensions obtained from cultures grown in skim milk at 37°C. Figure 8 shows densitograms obtained from gels corresponding to incubations of 0, 2, 4, and 6 h. The α- and β-casein fractions, at 36 and 32 kDa, respectively, were degraded after 2 h of incubation, and a new fraction was observed at 18 kDa. After 4 h, the peptides released by the proteases were also degraded, and a fraction was observed at 14.5 kDa; after 6 h, peptides of even lower molecular weight were observed.

DISCUSSION

Most work on proteolytic activity of lactobacilli has been conducted with strains grown in culture media in which the concentration of free amino acids was high enough to sustain growth without proteolytic enzymes. In the manufacture of dairy products, milk without addition of free amino acids is used. Therefore, it is important to study the response of lactobacilli grown in such milk. In addition, in starter production for yogurt, the bulk starter used should contain sufficient proteases to sus-

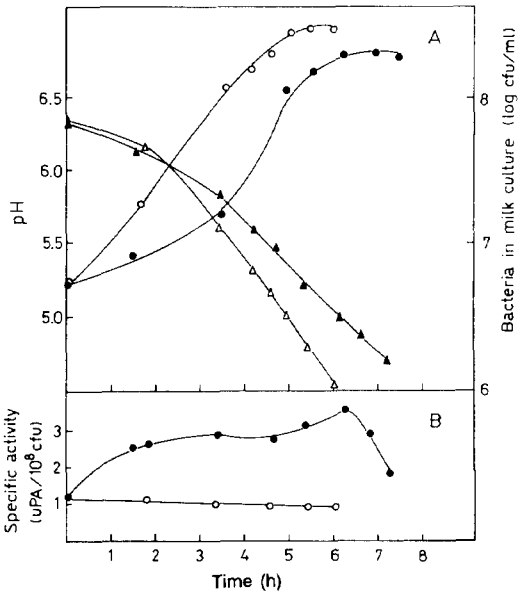

Figure 7. Kinetics of growth and acidification (A) and specific proteolytic activity in units of proteolytic activity (uPA) per 10^8 cfu (B) of *Lactobacillus bulgaricus* LBB at 37 and 42°C. Least significant difference = .7 (P = .05). Acidity at 37°C (▲) and 42°C (△); bacterial growth at 37°C (●) and 42°C (○); specific proteolytic activity at 37°C (●) and 42°C (○).

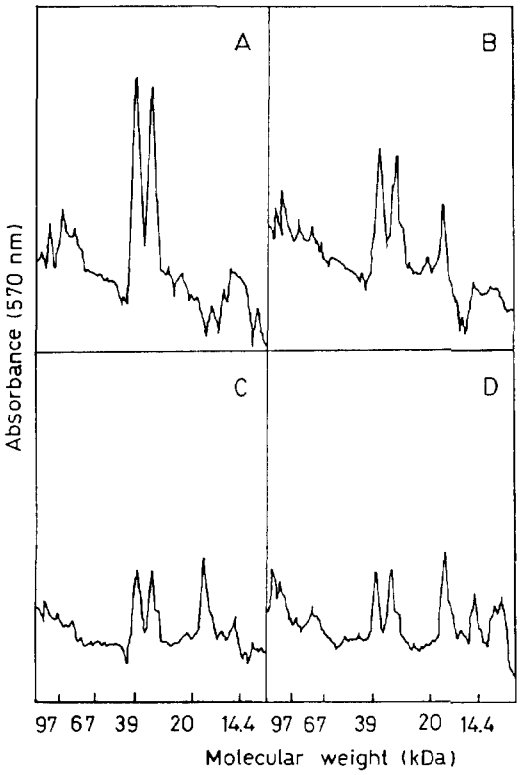

Figure 8. Densitograms of SDS-PAGE of caseins after incubation with *Lactobacillus bulgaricus* LBB A) 0 h, B) 2 h, C) 4 h, and D) 6 h at 42°C.

tain the growth of *S. thermophilus* during the fermentation process.

Proteolytic activity in lactic acid bacteria grown in milk has been evaluated in *Streptococcus cremoris* (5) and *L. bulgaricus* by isolation of cells after addition of NaOH and citrate to the milk (2). The method of dissolving the casein precipitate with Na_2-EDTA, pH 12, has been used to determine absorbance of milk cultures but not to extract cells from milk cultures. These results, obtained with *L. bulgaricus* LBB, demonstrate that this method is applicable for extraction of bacteria from milk when the proteolytic activity is evaluated, and correlation between proteolytic activity and colony-forming units was good in 12 different experiments. Treatment with Na_2-EDTA did not affect proteolytic activity, because cells grown in 1.1.1 broth presented SPA of .75 and .90 uPA/10^8 cfu with and without Na_2-EDTA treatment, respectively (data not shown).

Under assay conditions, *L. bulgaricus* LBB produced 3.6 uPA/10^8 cfu. However, that value depended on milk composition. Milk of different origins yielded cultures with different SPA. Proteolytic activity for strain LBB was twice as high for cells grown in milk than for those grown in 1.1.1 broth (Figure 4). These results are in agreement with those obtained in Figure 3. When strain LBB was grown at 37°C, the SPA decreased with addition of casein hydrolysate. A high availability of peptides and amino acids can be postulated to repress synthesis of proteolytic enzymes, to affect the transformation of the enzyme to an active form, or to inhibit its activity. An enzymatic assay performed with casein and casein hydrolysate as substrate yielded the same SPA (Table 2). Therefore, the inhibition of enzyme activity can be eliminated. These results are in agreement with those of Laloi et al. (10), in which casein hydrolysis by *L. bulgaricus* grown in MRS broth was significantly lower than that observed with bacteria grown in milk.

Repression of proteolytic activity was also observed with increased growth temperature (Figure 5). The SPA was high and constant at low concentrations of amino acids and peptides in milk growth media and at low temperatures, between 34 and 38°C; SPA decreased sharply when cells were grown between 38 and 40°C. This low value was maintained at temperatures higher than 40°C. The effect of repression by temperature was not due to a modification of the enzyme activity, because the SPA increased with temperature, as shown by Arrhenius plot (Figure 6).

CONCLUSIONS

Proteolytic activity of strain LBB may be regulated by amino acid and peptide contents of the media and by the growth temperature. This regulation was not due to a modification of the enzymatic activity but was related to an increase or decrease in the synthesis of proteases or to a modification that transforms the enzyme to an active or inactive form.

A possible interpretation of these results is that a basal level of SPA, 1 uPA/10^8 cfu, is present in strain LBB. The activity of proteases increases with temperatures from 32 to 48°C. When bacteria are grown in milk at high tem-

perature, the basal level of proteases is sufficient to sustain bacterial growth because the enzyme is more active than at 37°C. Consequently, the concentration of free Tyr of milk increased from 7.5 to 10 mg/100 ml (data not shown) by the proteolytic activity of 10^8 cfu/ml. The concentrations of free Tyr and colony-forming units are the same in milk at 37°C, but, in this case, SPA is twice as high as that observed when the growth is developed at 42°C, indicating the synthesis of a higher concentration of proteases at 37°C.

The proteases produced by strain LBB had proteolytic activity that degraded α- and β-caseins at the same rate, in contrast with other proteases that hydrolyze β-casein more rapidly (16).

These results suggest that bulk starters might be prepared at lower temperatures when a higher concentration of protease is desired. However, cells grown in 1.1.1 broth with low content of proteases were able to increase SPA after brief incubation in milk at 37°C.

ACKNOWLEDGMENTS

The authors are grateful to E. A. Disalvo for revising the manuscript, L. A. Brandi for technical assistance, T. E. Quattrini for typing the manuscript, and Unión Gandarense SACIA, Buenos Aires, Argentina for supplying the NDM. This work was supported by Comision de Investigaciones Científicas de la Provincia de Buenos Aires (CIE) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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