APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1993, p. 177–182
0099-2240/93/010177-06$02.00/0

Vol. 59, No. 1

Characterization of a Cell Envelope-Associated Proteinase Activity from *Streptococcus thermophilus* H-Strains

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Received 18 June 1992/Accepted 5 October 1992

The production and biochemical properties of cell envelope-associated proteinases from two strains of *Streptococcus thermophilus* (strains CNRZ 385 and CNRZ 703) were compared. No significant difference in proteinase activity was found for strain CNRZ 385 when cells were grown in skim milk medium and M17 broth. Strain CNRZ 703 exhibited a threefold-higher proteinase activity when cells were grown in low-heat skim milk medium than when grown in M17 broth. Forty-one percent of the total activity of CNRZ 385 was localized on the cell wall. The optimum pH for enzymatic activity at 37°C was around 7.0. Serine proteinase inhibitors, such as phenylmethylsulfonyl fluoride and diisopropylfluorophosphate, inhibited the enzyme activity in both strains. The divalents cations Ca^{2+}, Mg^{2+}, and Mn^{2+} were activators, while Zn^{2+} and Cu^{2+} were inhibitors. β-Casein was hydrolyzed more rapidly than α_{s1}-casein. The results of DNA hybridization and immunoblot studies suggested that the *S. thermophilus* cell wall proteinase and the lactococcal proteinase are not closely related.

The lactic acid bacteria require an exogenous source of amino acids or peptides for optimal growth. In milk the concentration of these low-molecular-weight compounds is not sufficient to ensure growth to a high cell density (16). Thus, the starter bacteria have to utilize caseins, which are hydrolyzed to peptides and amino acids by extracellular proteinases and peptidases (10, 24, 39). On the other hand, the effects of the proteolytic systems of lactic acid bacteria are important to the dairy industry as these systems modify texture and lead to development of flavor during cheese ripening (32).

The cell envelope-associated proteinases of lactococci have been studied extensively (17, 23, 30, 31). These enzymes are released from the cell envelope in a Ca^{2+}-free buffer, and different hypotheses have been proposed to explain the role of Ca^{2+} in the stabilization of the proteinases on the cell wall (9, 29).

Studies of lactococcal proteinases from different strains showed that generally these enzymes degrade β-casein better than they degrade α_{s1}-casein (8, 31, 41). They are inhibited by serine proteinase inhibitors, and the nucleotide sequence of the proteinase gene of *Lactococcus lactis* subsp. *cremoris* Wg2 confirmed that the proteinase of this organism was related to other serine proteases. The mechanisms of proteinase secretion, maturation, and attachment to the cell wall have been studied at the molecular level (14).

Several other lactic acid bacteria, such as *Lactobacillus delbrueckii* subsp. *bulgaricus* (1, 11, 26) and *Lactobacillus helveticus* (42), have been reported to possess a cell envelope-associated proteinase. An extracellular proteinase is also produced by *Lactobacillus murinus* (6).

Less is known about the cell envelope-associated proteinase of *Streptococcus thermophilus*, a thermophilic lactic acid bacterium that is widely used as starter in the production of many dairy products, such as yogurt, hard cooked cheese, and some soft cheeses. *S. thermophilus* exhibits a low level of activity compared with *Lactobacillus delbrueckii* subsp. *bulgaricus* (26, 35) or the mesophilic lactic acid bacteria (28). The occurrence of proteinases in *S. thermophilus* HST which are able to degrade κ-casein more rapidly than they degrade α_s- or β-casein has been reported previously (36); these enzymes have never been characterized as they are produced at low levels.

In a previous paper (34), we reported that among 97 strains of *S. thermophilus* that were tested for their proteolytic activities on fast-slow differential agar (18) or by a biochemical assay in which ^{14}C-methylated whole casein was used as the substrate (7), only 3 strains (designated the H strains) possessed proteinase activities whose levels were similar to the level of proteinase activity in a Prt^+ strain of *Lactococcus lactis* subsp. *lactis* (strain CNRZ 1076). The presence of high proteinase activities allowed these strains to grow and produce acid more rapidly in low-heat skim milk than the 94 other strains (designated the L-strains) could. These H-strains were used as starters in milk with a high protein content. In addition to their role in nitrogen nutrition, the proteinases of H-strains accelerate cheese ripening but sometimes produce bitter peptides and thus should be described more precisely.

In this study, we characterized the cell envelope-associated proteinases of *S. thermophilus* CNRZ 385 and CNRZ 703 obtained from purified cell wall fractions.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. thermophilus* CNRZ 385 and CNRZ 703, which were isolated from a Japanese yogurt (Yakult Company) and from tarag (Mongolian generic name of yogurt) respectively, were obtained from the collection of the Institut National de la Recherche Agronomique Research Center, Jouy-en-Josas, France. The following two culture media were used: M17 broth (37) and low-heat skim milk powder (Nilac; NIZO, Ede, The Netherlands) reconstituted to a concentration of 10% (wt/vol) with sterile 75 mM β-glycerophosphate buffer. Each medium was inoculated with an overnight culture (2%, vol/vol), and the preparations were incubated at 42°C. The growth of each

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culture was followed by measuring pH and absorbance (A_{480} for the milk medium [40] and A_{650} for the M17 medium).

Preparation of cell fractions. Cell fractions were prepared as described by Gruber et al. (13), with the modifications described below. Cells were harvested from M17 broth at the end of the exponential phase, washed, and disrupted 10 times (1.96 tons/cm^2) with an X-Press apparatus (type X25; AB Biox, Nacka, Sweden). Unbroken cells were removed by centrifugation at 500 × *g* for 15 min at 4°C. The cell wall and membrane fractions were pelleted by a second centrifugation (12,000 × *g*, 10 min, 4°C). The cytoplasmic membrane was dissolved by treatment with Triton X-100 (0.05%, final concentration) for 30 min at room temperature, and the cell wall fraction was collected by centrifugation at 8,000 × *g* for 10 min at 4°C. The cell walls were washed 10 times and suspended in 50 mM sodium phosphate buffer (pH 7.0) to a concentration of 40 μg of protein per ml. The amount of proteinase released from the cell walls was assayed by treating the cell walls with lytic enzymes, such as lysozyme (5 mg/ml) and mutanolysin (20 μg/ml) (1 h at 37°C in 50 mM Tris HCl [pH 7.0]), or with other agents known to release proteins from bacterial walls. These other agents included urea (2 and 8 M), NaOH (0.01 N), Triton X-100 (0.5, 1, 5, and 10% [wt/vol]), Tween 80 (0.5, 1, 5, and 10% [wt/vol]), LiCl (2 and 6 M), and *N*-lauroylsarcosine (0.2, 1, and 5% [wt/vol]) (2, 19, 27, 33). The cell wall fraction was suspended in sodium phosphate buffer (pH 7.0) containing the agent, and the preparation was incubated at 4°C for 2 h. The agent was then removed by dialysis against distilled water at 4°C for 2 h.

The rigorous criteria established by Thomas and Mills (38) were used to determine possible contamination of the cell wall preparation by cytoplasmic or membrane fractions. No significant amount of membrane was detected by electron microscopy. The presence of cytoplasmic enzymes was determined by measuring the β-galactosidase and lactate dehydrogenase activities. β-Galactosidase activity was not detected, and the lactate dehydrogenase activity was less than 0.04% of the total activity in each cell extract.

Assay of proteinase activity. Proteinase activity was measured as described by Monnet et al. (30) by following the release of 6% trichloroacetic acid-soluble products from 0.1% ^{14}C-methylated whole casein prepared by reductive methylation of the lysine residues of the casein with KBH_4 and $H^{14}CHO$ (specific activity, 7.3 MBq/g) (7). To 100 μl of casein, 100 μl of cells (10 mg, wet weight) or 100 μl of a cell wall fraction (4 μg of protein) was added, and the preparation was incubated for 1 h at 37 or 45°C. The reaction was stopped by adding 200 μl of 12% trichloroacetic acid; 1 U of activity was defined as the amount of enzyme that hydrolyzed 1 μg of ^{14}C-methylated whole casein in 10 min. The effects of the proteinase inhibitors diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, *para*-chloromercuribenzoate, and EDTA on proteinase activity were tested at a concentration of 1 mM. The effects of mono- and divalent ions on proteinase activity were tested at a concentration of 5 mM. Whole casein and α_{s1}- and β-casein were purified in our laboratory (30).

Protein quantification. Proteins were estimated by the method of Bradford (3), with crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) used as the standard.

SDS-PAGE. (i) Sample preparation. To prepare samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cell wall samples (100 μl; 1.83 U of proteinase activity) were added to 10-μl portions of solutions

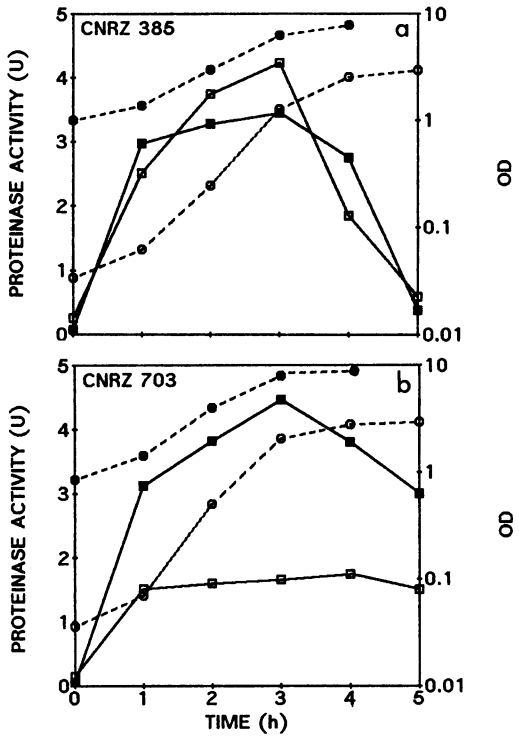

FIG. 1. Proteinase activities (■ and □) and optical densities (OD) (● and ○) of *S. thermophilus* CNRZ 385 (a) and CNRZ 703 (b) cultures grown in low-heat skim milk medium (■ and ●) or in M17 medium (□ and ○).

(1%, wt/vol) of whole casein, α_{s1}-casein, or β-casein in 50 mM sodium phosphate buffer (pH 7.0), and the preparations were incubated for 1, 2, 4, 8, and 24 h at 42°C. At the appropriate time, 30 μl of stopping buffer was added (15). The samples were then incubated for 10 min at 80°C and centrifuged at 24,000 × *g* for 5 min at 4°C. To 85 μl of supernatant, 15 μl of loading buffer (15) was added. The samples were stored frozen at −30°C until they were used.

(ii) SDS-PAGE analysis. SDS-PAGE was performed as described previously (25) by using a vertical gel electrophoresis unit (Mini-Protean II; Bio-Rad Laboratories, Richmond, Calif.) and a running gel containing 14% acrylamide combined with a 5% stacking gel. The casein hydrolysates prepared previously were heated for 5 min at 100°C, and 10-μl samples were applied to the gel. After electrophoresis at 30 mA for 90 min, the gel was stained with Coomassie blue R-250 (0.1% [wt/vol] solution in 50% ethanol and 7% [vol/vol] acetic acid) at room temperature for 30 min and then destained in 15% ethanol–7% acetic acid. The molecular masses of the protein bands were estimated by using low-molecular-weight reference proteins (Bio-Rad).

RESULTS

Influence of culture medium on proteinase activity. The proteinase activities of *S. thermophilus* CNRZ 385 and CNRZ 703 grown in M17 broth (a medium containing an immediately available nitrogen source) and in low-heat skim milk medium were compared (Fig. 1). The culture medium had no significant effect on the proteolytic activity of strain CNRZ 385. The activity was highest at the end of the exponential phase and decreased rapidly at the beginning of

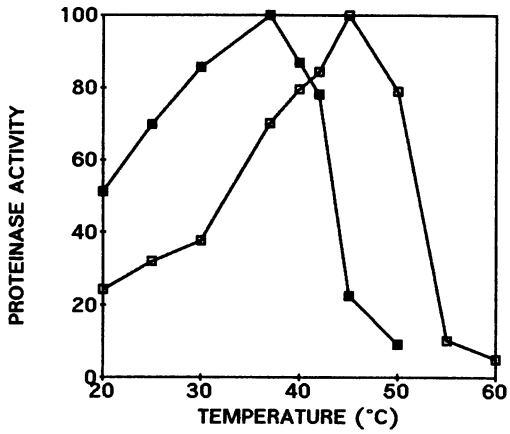

FIG. 2. Effects of temperature on the proteinase activities of *S. thermophilus* CNRZ 385 (■) and CNRZ 703 (□) expressed as percentages of the proteinase activity at the optimum temperature.

the stationary phase. Strain CNRZ 703 grown in low-heat skim milk medium exhibited threefold-higher proteolytic activity than the same strain grown in M17 broth exhibited. The activity was maximal at the end of the exponential phase and remained stable during the stationary phase.

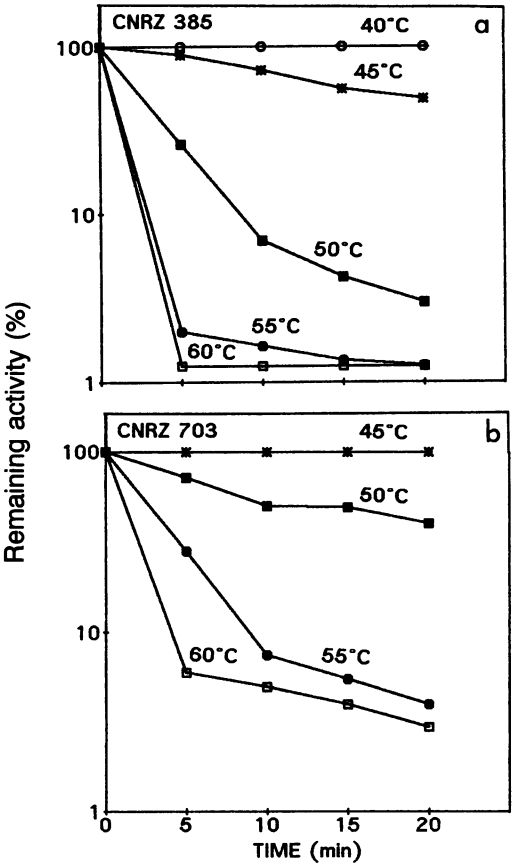

FIG. 3. Comparison of the heat stabilities of the proteinase activities of *S. thermophilus* CNRZ 385 (a) and CNRZ 703 (b), expressed as percentages of activity remaining after heat treatment (on a logarithmic scale).

TABLE 1. Effects of potential proteinase inhibitors and cations on the proteinase activities of *S. thermophilus* CNRZ 385 and CNRZ 703

Prepn	Relative activity (%)	
	Strain CNRZ 385	Strain CNRZ 703
Control (without ions)	100	100
Inhibitors[a]		
EDTA	99	94
p-Chloromercuribenzoate	70	85
Phenylmethylsulfonyl fluoride	3	18
Diisopropylfluorophosphate	5	23
Ions[b]		
Ca^{2+}	646	568
Mn^{2+}	508	249
Mg^{2+}	418	267
Cu^{2+}	22	27
Zn^{2+}	2	28
K^{+}	128	139
Na^{+}	107	109

[a] Tested at a final concentration of 1 mM.
[b] Tested at a final concentration of 5 mM.

Location of the proteinase activity. Strain CNRZ 385 was harvested from M17 broth during the exponential phase of growth (2 h) and disrupted in the X-press apparatus, and the proteinase activities in the different cell fractions were determined; 41% of the activity was associated with the cell wall fraction, 22% was associated with the cytoplasmic membrane, and 37% was associated with the cytoplasm. No proteinase activity was detected in the cell-free medium.

Characterization of the cell envelope-associated proteinase. No significant proteinase activity was released from the cell wall after treatment in a Ca^{2+}-free buffer or after the cell envelope was treated with lytic enzymes, such as lysozyme or mutanolysin. Treatments with other agents, such as urea, NaOH, Triton X-100, Tween 80, LiCl, and *N*-lauroylsarcosine, which are known to stimulate the release of protein from bacterial walls, did not give reproducible results, especially in large-scale purification experiments (data not shown). Thus, the properties of the cell envelope-associated proteinase were determined by using the purified cell wall fraction.

Effect of pH. The optimum pH of the enzyme in 50 mM phosphate buffer was around 7.0 for both strains. The activity was higher in sodium phosphate buffer than in morpholineethanesulfonic acid (MES) or Tris HCl buffer.

Effect of temperature. The enzymatic activities were determined at pH 7.0 at various temperatures. The highest activities were found at 37 and 45°C for strains CNRZ 385 and CNRZ 703, respectively (Fig. 2). The thermosensitivity of the enzyme was also determined. The proteinase activity of strain CNRZ 385 was only 10% of the maximum activity at 50°C, whereas the proteinase of strain CNRZ 703 retained 80% of its activity at the same temperature. The thermal denaturation curves confirmed the thermosensitivity of CNRZ 385 proteinase. The curves have two distinct slopes at temperature above 50°C (Fig. 3).

Effects of proteinase inhibitors and cations. The effects of various inhibitors and cations on proteinase activity were investigated (Table 1). The proteinase activities of the two strains were inhibited by the serine enzyme inhibitors, such as diisopropylfluorophosphate and phenylmethylsulfonyl fluoride. They were also partially inhibited by the thiol

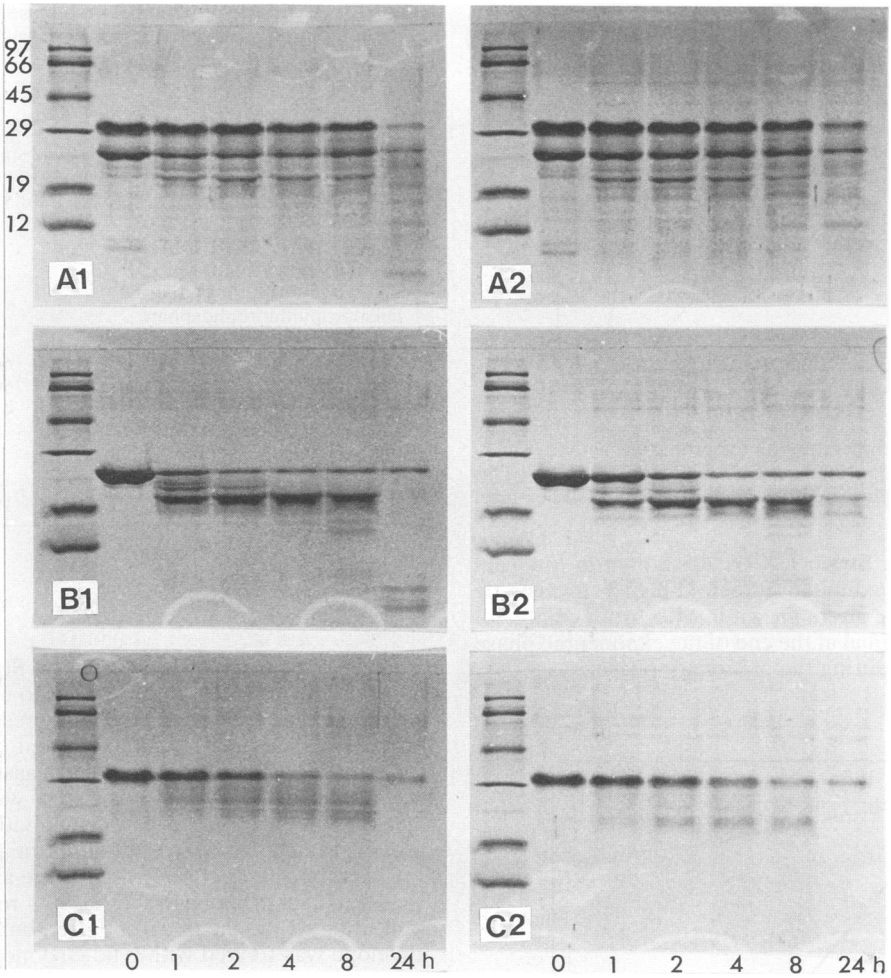

FIG. 4. SDS-PAGE patterns showing the action of the proteinases (1.83 U) from *S. thermophilus* CNRZ 385 (A1, B1, and C1) and CNRZ 703 (A2, B2, and C2) on 1% (wt/vol) bovine whole casein (A1 and A2), 1% β-casein (B1 and B2), and 1% α_{s1}-casein (C1 and C2). The lengths of the incubation times (in hours) are indicated at the bottom. On each gel the left lane contained reference proteins, including myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (19 kDa), and lysozyme (12 kDa).

enzyme inhibitor *para*-chloromercuribenzoate. There was no significant change in activity when the proteinase was treated with chelating agents, such as EDTA.

Proteinase activity was enhanced in the presence of the divalent cations Ca^{2+}, Mn^{2+}, Mg^{2+} and to a lesser extent by the monovalent cations K^+ and Na^+. The cations Zn^{2+} and Cu^{2+} strongly inhibited proteinase activity.

Degradation of caseins. Purified α_{s1}- and β-caseins and whole casein were tested as substrates for the cell envelope-associated proteinase. β-Casein was degraded faster than α_{s1}-casein (Fig. 4). Hydrolysis of β-casein was observed after 1 h of incubation, while α_{s1}-casein required 4 h of incubation for hydrolysis. The degradation of both types of casein was complete after 24 h. There were no apparent differences in the patterns of degradation of caseins by the proteinases from the two strains, as determined by SDS-PAGE.

DISCUSSION

Previously (34), we have shown that the proteinase activity present in H-strains CNRZ 385 and CNRZ 703 plays a crucial role in stimulating the growth of cells in low-heat skim milk. Furthermore, the results of a study of a proteinase-negative mutant of strain CNRZ 385 suggested that this proteinase activity plays a role in nitrogen metabolism similar to that of the cell wall-associated proteinase of lactococci. Both enzymes initiated the degradation of native caseins, while the peptidases operated at subsequent steps of hydrolysis to further break down the casein protein to smaller peptides and free amino acids, which could then be taken up by the cell.

As in lactococci, the production of a cell envelope-associated proteinase may be influenced by the composition of the culture medium. The presence of an available nitrogen source affects the overall proteinase activity of strain CNRZ 703 cells but not the overall proteinase activity of strain CNRZ 385 cells. Similar observations have been made for proteinase production in different lactococcal strains. The cell wall proteinases of *Lactococcus lactis* subsp. *cremoris* E8 and AM1 and *Lactococcus lactis* subsp. *lactis* NCDO 763 are inducible (8, 9, 31), whereas this enzyme is constitutive in *Lactococcus lactis* subsp. *cremoris* Wg2 (17).

The proteinases from *S. thermophilus* and from lactococci share a number of properties, including pH optimum, temperature optimum, activators, and inhibitors. Both proteinases belong to the serine type of proteinases as they are inhibited by phenylmethylsulfonyl fluoride and diisopropylfluorophosphate but not by EDTA and other inhibitors. The most relevant difference is that no release of proteinase activity occurred in $CaCl_2$-free buffer. This suggests that the *S. thermophilus* proteinase is tightly attached to the cell wall or the cell membrane, as the C5a peptidase from *Streptococcus pyogenes* is (4). Alternatively, the *S. thermophilus* proteinase could be rapidly denatured or self-digested when it is extracted.

The proteinase from strain CNRZ 385 differs from the proteinase from strain CNRZ 703 in thermosensitivity. A thermodenaturation study showed that the curves had two slopes at temperatures above 50°C. This may mean either that there is another proteolytic enzyme in the purified cell wall extract or that the enzyme is partially protected against heat by the cell wall. Complete purification of the proteinase from the cell wall would help explain this property.

There was no significant difference in the patterns of degradation of caseins digested with proteinase obtained from the cell walls of strains CNRZ 385 and CNRZ 703, as determined by SDS-PAGE. Both α_{s1}- and β-caseins are hydrolyzed, but β-casein is the preferred substrate. Such a degradation pattern is also found with the type AM1 lactococcal proteinase (41). Further analyses of casein hydrolysates by high-performance liquid chromatography and identification of the peptide products could determine the specificity of this proteinase.

As strains CNRZ 385 and CNRZ 703 are plasmid free, the gene encoding the *S. thermophilus* proteinase is not plasmid encoded, as it is in lactococci (12, 23); it is located on the chromosome, as is the proteinase gene of *Lactobacillus casei* HN14, which is homologous to the lactococcal gene (20). The biochemical properties of the cell envelope-associated proteinases of *Lactobacillus plantarum* and *Lactobacillus acidophilus* were similar to those of lactococci, suggesting that this family of serine proteinases is widely distributed in dairy microorganisms (21, 42). However, a hybridization study of the chromosomal DNA from *S. thermophilus* with the lactococcal proteinase probe pGKV552 (22), as well as the immuno-cross-reaction between polyclonal antibodies specific to the *Lactococcus lactis* subsp. *cremoris* Wg2 purified proteinase and the *S. thermophilus* proteinase, did not give any positive signal (data not shown). This suggests that even if the *S. thermophilus* proteinase shares a number of biochemical properties with the proteinases of lactococci, it is genetically different and shares no recent common ancestor.

High levels of proteinase activity were found in only 3 of the 97 strains of *S. thermophilus* tested (34), suggesting that this characteristic is not common in this species. Nevertheless, it is probable that these strains acquired the proteinase independently as (i) the three strains were isolated from traditional products of three geographically isolated countries (India, Mongolia, and Japan), (ii) the restriction fragment length polymorphisms as determined with the pNST21 probe (5) are different in all three strains (data not shown), and (iii) the strain CNRZ 385 and CNRZ 703 proteinases differ in both regulation and thermosensitivity. The origin of this activity in a limited number of strains remains unknown. It may have appeared as the result of the overexpression of a gene which is normally expressed at a low level in all strains or as the result of a gene transfer from another species, as occurs for the proteinases of lactococci and some species of lactobacilli.

The uniqueness of the *S. thermophilus* proteinase activity should motivate additional biochemical and genetic studies. The liberation of this proteinase from the cell wall would facilitate its purification, which is a prerequisite for studying its specificity with the caseins and how it participates in cell metabolism. The cloning of the proteinase gene(s) and the study of its sequence would contribute to an understanding of proteinase gene expression and proteinase secretion; eventually, such studies could be used to determine the origin of the proteinase gene(s) and to transfer the proteinase gene(s) to other strains.

ACKNOWLEDGMENTS

We are grateful to Harry Laan for providing the polyclonal antibodies, Jan Kok for providing plasmid pGKV552, Allan Hillier for reviewing the language, and all of our colleagues at the Station de Recherches Laitières for their help.

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