Proteolytic Activity by Strains of Lactobacillus plantarum and Lactobacillus casei

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ABSTRACT

Growth, acid production, and hydrolysis of milk proteins in sterile skim milk at 37°C were determined for seven strains of Lactobacillus plantarum, two of Lactobacillus casei, and one of L. casei ssp. pseudoplantarum. Hydrolysis of milk protein was monitored by SDS-PAGE. Lactobacillus plantarum NRRL B-4004 was the most proteolytic and began to hydrolyze β-casein after 125 h; hydrolysis was complete after 215 h. All strains of L. plantarum preferentially degraded β-casein of milk, whereas αs1-casein was partially hydrolyzed by some strains. Lactobacillus casei NRRL B-1922 and L. casei NRRL-B 441 did not hydrolyze β-casein even after 265 h. Lactobacillus casei ssp. pseudoplantarum ATCC 25598 partially hydrolyzed β-casein after 215 h. The pH of milk at the end of proteolysis was above 4.0 with L. plantarum, 3.7 to 3.8 with L. casei, and 4.1 with L. casei ssp. pseudoplantarum ATCC 25598. Four strains of L. plantarum had maximum viable cell populations ranging from 1.2 \times 10^6 to 2.0 \times 10^6 cfu/ml. Two strains of L. plantarum exhibited minimal growth, whereas the viable cell population of the two strains of L. casei varied markedly. The viable cell population of L. casei ssp. pseudoplantarum at the end of proteolysis was similar to that of L. plantarum at 2.0 \times 10^6 cfu/ml.

(Key words: lactobacilli, proteolysis, cheese ripening)

INTRODUCTION

Proteolysis is generally recognized as the principal reaction in the ripening of many varieties of cheese (11). In addition to proteolysis, cheese ripening involves degradation of milk fat and lactose and production of volatile constituents, which contribute to the complex association of chemical compounds in the product. Enzymes of starter bacteria, rennet, or other milk coagulant (7), milk (if cheese is made from raw or heat-treated milk), and nonstarter lactic acid bacteria are responsible for proteolysis, which is important during maturation of cheese (21, 28) and contributes directly to development of the desired texture and flavor intensity in cheeses (5, 11). The flavor is attributed, in part, to production of small peptides and free amino acids through protein degradation, and degradation of amino acids by decarboxylation, transamination, or other metabolic processes during cheese ripening (27).

Lactobacillus casei and Lactobacillus plantarum occur in most hard and semi-hard cheeses as adventitious bacteria, and they may contribute to flavor development during the ripening process. Evaluating strains of lactobacilli by monitoring electrophoretic patterns generated by bacterial proteolysis may be of practical value in selection of strains for potential use in cheese making. A qualitative screening procedure to detect casein hydrolysis by Streptococcus lactis (Lactococcus lactis ssp. lactis) using SDS-PAGE has been reported by Hill and Gasson (13). We applied this approach to lactobacilli; thus, the objective of this study was to screen seven strains of L. plantarum, two of L. casei isolated from cheese, and one of L. casei ssp. pseudoplantarum for their growth characteristics and proteolytic activity.

MATERIALS AND METHODS

Cultures and Cultural Conditions

Lactobacillus casei NRRL B-441 and NRRL B-1922 as well as Lactobacillus plantarum...
Colonies of lactobacilli were counted. The chemically sterilized skim milk was then plated on MRS agar (20) and treated skim milk was confirmed with the aero­hydrogen peroxide completely. Sterility of bic plate count method using plate count agar. Microbial Growth

Measurement of pH

The pH was determined with an Orion pH Meter (Model 601 A, Digital Ionalyzer, Orion Research Inc., Cambridge, MA).

Microbial Growth

Samples were appropriately diluted with 0.5% peptone solution, plated on MRS agar (20), and plates were incubated at 37°C for 2 d before colonies of lactobacilli were counted. Polyacrylamide Gel Electrophoresis

The extent of proteolysis in skim milk was determined by PAGE. Electrophoresis was done with a Vertical Slab Gel Electrophoresis Unit (Model 600-SE, Hoefer Scientific Instruments, San Francisco, CA) at room temperature according to the methods of Laemmli (15) and Laemmli and Favre (16) using a 12% running gel and 4% stacking gel. Samples for electrophoresis were prepared according to the method described by Basch et al. (3). Two hundred and fifty microliters of sample were mixed with 2.5 ml of Tris-EDTA (.166 M Tris and 1 mM EDTA, pH 8.0) and 2.5 ml of 7% SDS. A 1-ml portion was then mixed with 200 µl of β-mercaptoethanol, and the mixture was heated at 100°C for 5 min. Then 200 µl of bromophenol blue (.05%) and 200 µl of glycerol were added. Twelve microliters of this mixture were then applied to each sample slot in a slab gel. Purified αs1-, β- and κ-caseins as well as low molecular size markers: α-lactalbumin (14.2 kDa), trypsin inhibitor (20.1 kDa), trypsinogen (24.0 kDa), carbonic anhydrase (29.0 kDa), glyceraldehyde-3-phosphate dehydrogenase (36.0 kDa), ovalbumin (45.0 kDa), and bovine serum albumin (66.0 kDa) were used to compare with the different protein bands on the gel. Gels were exposed to 30 mA/slab. All chemicals were from Sigma Chemical Co., St. Louis, MO.

After electrophoresis, gels were stained and destained using the methods of Laemmli (15) and Laemmli and Favre (16). They were scanned with a densitometer (DC0-1B, Gelman, Ann Arbor, MI) equipped with an integrator. Proteolysis of milk was noted by disappearance of the original protein band from densitometric scans.

RESULTS AND DISCUSSION

Polyacrylamide gel electrophoresis was performed to monitor hydrolysis of casein fractions in skim milk by the different strains of lactobacilli so differences in peptides generated by bacterial proteolysis could be compared. Two strains of L. casei, seven of L. plantarum, and one of L. casei ssp. pseudoplantarum were incubated in chemically sterilized skim milk, and pH of the medium, viable cell population, and extent of proteolysis of skim milk were monitored.

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This study was done using skim milk sterilized with hydrogen peroxide. We could not sterilize skim milk with the conventional heat treatment of 121°C for 15 min because this would result in degradation of milk proteins by heat rather than by proteolytic activity of the lactobacilli.

Growth and acid production by different strains of lactobacilli in skim milk are shown in Figures 1, 2, and 3. Initially, about 1 x 10^7 cfu/ml were in the skim milk, but after incubation for about 265 h, populations of four strains of L. plantarum had decreased to 1.2 x 10^6 to 2.0 x 10^6 cfu/ml, whereas populations of two strains of L. plantarum had increased. The population of L. plantarum ATCC 14917 increased 1.4-fold to 1.4 x 10^7 cfu/ml whereas that of L. plantarum NRRL B-813 increased ca. 8-fold to ca. 8 x 10^7 cfu/ml. However, about 98% of cells of L. plantarum ATCC 8014 were inactivated at the end of proteolysis (determined as the time required for complete degradation of α_s1- and β-caseins).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Viable count (cfu/ml)</th>
<th>Time for initial hydrolysis (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL B-1931</td>
<td>7.80</td>
<td>4.83</td>
</tr>
<tr>
<td>NRRL B-1927</td>
<td>7.25</td>
<td>5.00</td>
</tr>
<tr>
<td>ATCC 8014</td>
<td>6.23</td>
<td>4.24</td>
</tr>
<tr>
<td>NRRL B-813</td>
<td>5.02</td>
<td>7.97</td>
</tr>
<tr>
<td>A:2K20</td>
<td>5.95</td>
<td>4.08</td>
</tr>
<tr>
<td>ATCC 14917</td>
<td>7.33</td>
<td>4.88</td>
</tr>
<tr>
<td>ATCC 25598</td>
<td>7.14</td>
<td>4.20</td>
</tr>
<tr>
<td>NRRL B-1922</td>
<td>6.86</td>
<td>3.74</td>
</tr>
<tr>
<td>NRRL B-441</td>
<td>3.40</td>
<td>3.80</td>
</tr>
<tr>
<td>L. casei ssp. pseudoplanarum</td>
<td>7.25</td>
<td>4.43</td>
</tr>
<tr>
<td>ATCC 25598</td>
<td>7.25</td>
<td>4.43</td>
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<tr>
<td>ATCC 25598</td>
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Figure 1. Growth and acid production by different strains of Lactobacillus plantarum in chemically sterilized skim milk at 37°C. NRRL B-1931: log colony-forming units per milliliter (●), pH (●); NRRL B-1927: log colony-forming units per milliliter (●), pH (●); NRRL B-4004: log colony-forming units per milliliter (●), pH (●); NRRL B-813: log colony-forming units per milliliter (●), pH (●).

Figure 2. Growth and acid production by different strains of Lactobacillus plantarum in chemically sterilized skim milk at 37°C. A:2K20: log colony-forming units per milliliter (●), pH (●); ATCC 8014: log colony-forming units per milliliter (●), pH (●); ATCC 14917: log colony-forming units per milliliter (●), pH (●).

Figure 3. Growth and acid production by different strains of Lactobacillus casei in chemically sterilized skim milk at 37°C. NRRL B-1922: log colony-forming units per milliliter (●), pH (●); NRRL B-441: log colony-forming units per milliliter (●), pH (●); ssp. pseudoplanarum ATCC 25598: log colony-forming units per milliliter (●), pH (●).
The pH of milk after 265 h was above 4.0, and it did not vary markedly among the seven strains of *L. plantarum*. Even upon extended incubation for up to 580 h, the pH of milk containing *L. plantarum* did not fall below 4.0. In contrast, incubation of *L. casei* NRRL B-1922 and *L. casei* NRRL B-441 in milk for 310 h caused the pH to decrease from 6.5 to 3.7 or 3.8, whereas milk with *L. casei* ssp. *pseudoplantarum* had a slightly higher final pH of 4.1 (data on pH not shown). Behavior of *L. casei* ssp. *pseudoplantarum* was quite similar to that of the strains of *L. plantarum*.

The population of *L. casei* ssp. *pseudoplantarum* at the end of proteolysis was $2.0 \times 10^6$ cfu/ml, which was similar to values obtained for *L. plantarum* strains. There was, however, marked variation in the populations of the two strains of *L. casei*. The viable cell count of *L. casei* NRRL B-1922 after 310 h of incubation in skim milk was $7.2 \times 10^6$ cfu/ml compared with an initial population of $1.0 \times 10^7$ cfu/ml. The population of *L. casei* NRRL B-441 at the end of proteolysis had decreased to $2.5 \times 10^3$ cfu/ml (Table 1).

Both strains of *L. casei* used in our study were isolated from cheese. Observations similar to ours were reported (1) for lactobacilli, which also were isolated from cheese. These lactic acid bacteria grew poorly in milk, probably because available nitrogen was lacking. Nath and Ledford (23) observed that some fractions from Cheddar cheese inhibited growth of *L. casei* var. *casei*, but with increased ripening time, the inhibitory effect disappeared. They showed that the cheese fractions were derived through hydrolysis of $\alpha$-casein during the ripening process since $\beta$- and $\kappa$-caseins in...
Cheddar cheese were not altered during this process. Their result would explain, in part, the gradual increase in numbers of lactobacilli during cheese ripening even though these bacteria grow poorly in milk.

Our study was, in part, aimed at obtaining information on proteolysis of milk proteins by different strains of *L. plantarum* that might have a possible role in cheese ripening and to compare the results with those from two strains of *L. casei*, NRRL B-1922 and NRRL B-441, which were isolated from cheese. The protein fraction of skim milk most extensively hydrolyzed was β-casein. Evidence for this is the less intense protein band or partial loss of the protein band, which corresponded to standard β-casein, on the electrophoretogram of skim milk. All strains of *L. plantarum* preferentially degraded β-casein of milk, whereas α₄₁-casein was partially hydrolyzed by some strains. *Lactobacillus plantarum* NRRL B-1931 completely hydrolyzed β-casein after 265 h (Figure 4, lane 11). However, β-casein was not completely hydrolyzed by *L. plantarum* NRRL B-1927 or ATCC 8014 after the 265-h incubation period (Figure 5, lane 10; Figure 6, lane 1) (data for *L. plantarum* NRRL B-813, A:2K20, B-4004, and ATCC 14917 are not shown). *Lactobacillus casei* sp. *pseudoplantarum* ATCC 25598 also partially hydrolyzed β-casein (Figure 7). The electrophoretic patterns for hydrolysis of α₄₁- and β-casein by *L. casei* NRRL B-1922 and NRRL B-441 were compared with that of *L. plantarum* NRRL B-4004 (data not shown). β-Casein was hydrolyzed completely by *L. plantarum* NRRL B-4004 after 215 h of incubation at 37°C, whereas it was not hydrolyzed by the two strains of *L. casei*. However, α₄₁-casein

*Figure 5. Polyacrylamide gel electrophoretic pattern of skim milk subjected to proteolysis by *Lactobacillus plantarum*: NRRL B-1927 at 37°C. Lane 1, control at 265 h; lane 2, 0 h; lane 3, 74 h; lane 4, 102 h; lane 5, 102 h; lane 6, 150 h; lane 7, 166 h; lane 8, 215 h; lane 9, 240 h; lane 10, 265 h; lane 11, α₄₁-casein; lane 12, β-casein; lane 13, γ-casein; lane 14, molecular size markers (top to bottom): 66.0 kDa; 45.0 kDa; 36.0 kDa; 29.0 kDa; 24.0 kDa; 20.1 kDa; 14.2 kDa.*
was partially degraded by \textit{L. plantarum} NRRL B-4004 but was not hydrolyzed by \textit{L. casei}.

The time for initial hydrolysis of the $\alpha_\text{s}1$- or $\beta$-casein fractions of skim milk, pH of the medium during initial proteolysis, and viable cell count are given in Table 1. The incubation time required for complete proteolysis determined by disappearance of protein band from gel as measured densitometrically differed among the strains even though the initial inoculum level was the same at $1.0 \times 10^7$ cfu/ml because different strains had different rates for initial proteolysis. From information in Figures 4, 5, 6, and 7, it is evident that $\alpha_\text{s}1$-casein was not hydrolyzed by \textit{L. plantarum} NRRL B-1927 and \textit{L. casei} ssp. \textit{pseudoplantarum} ATCC 25598, whereas it was partially hydrolyzed by \textit{L. plantarum} NRRL B-1931 and ATCC 8014. Ohmiya and Sato (24) reported that \textit{Lactobacillus bulgaricus} and \textit{Lactobacillus helveticus} hydrolyzed $\alpha_\text{s}1$-casein while $\beta$-casein was more resistant to hydrolysis by these lactic acid bac-


teria. They also showed that the intracellular proteases of \textit{L. bulgaricus} and \textit{L. helveticus} degraded casein fractions in the order of $\alpha_\text{s}1 > \kappa > \beta$-casein (25). $\beta$-Casein was completely hydrolyzed by \textit{Lactobacillus lactis} and partially hydrolyzed by \textit{Lactobacillus acidophilus} and \textit{L. bulgaricus}, whereas $\alpha_\text{s}1$-casein was only partially hydrolyzed by \textit{L. helveticus}, \textit{L. lactis}, and \textit{L. bulgaricus} (8).

The $\alpha$-lactalbumin fraction (molecular size ca. 14.2 kDa) of whey protein was not hydrolyzed by any strain of \textit{L. plantarum}, whereas the $\beta$-lactoglobulin component (molecular size ca. 18.4 kDa) of whey protein was partially hydrolyzed by \textit{L. plantarum} NRRL B-1931 (Figure 4, protein band in lanes 2 or 3 at position A became less intense in lane 11, indicating partial hydrolysis of $\beta$-lactoglobulin). A similar observation was reported on hydrolysis of $\beta$-lactoglobulin by intracellular proteolytic enzymes of \textit{L. plantarum} (9). In contrast, the intracellular enzymes of \textit{L. helveti-
However, it has been observed that whey proteins are completely resistant to the proteolytic enzymes of rennet and starter culture in low fat, semi-hard cheese (6). Although commonly present in small amounts, if at all, the influence of whey proteins on cheese ripening is not well understood. However, it has been observed that whey proteins are completely resistant to the proteolytic enzymes of rennet and starter culture in low fat, semi-hard cheese (6).

In another study (14), we reported that proteolysis of skim milk by cells of *L. helveticus* CNRZ 32, *L. helveticus* ATCC 10797, and *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 12278 required up to 145 h, whereas proteolysis by cell-free extracts of the bacteria was accomplished within 54 h. *Lactobacillus helveticus* ATCC 10797 hydrolyzed α_{s1}- and β-caseins without preference, whereas *L. helveticus* CNRZ 32 preferentially hydrolyzed β-casein but did not hydrolyze α_{s1}-casein. It has been reported that frozen cells of *L. helveticus* CNRZ 32, when incorporated into cheese milk, accelerated ripening of Gouda cheese, which also had good flavor and texture, whereas use of frozen cells of *L. helveticus* ATCC 10797 and *L. delbrueckii* ssp. *bulgaricus* ATCC 12278 resulted in cheese with flavor defects (2). The rate and extent of characteristic flavor development in Cheddar cheese slurries also appeared to be directly related to β-casein hydrolysis (12). Our results indicate that preferential degradation of β-casein but not α_{s1}-casein by *L. helveticus* CNRZ 32 could, in part, account for the superior flavor of cheese made with this

Figure 7. Polyacrylamide gel electrophoretic pattern of skim milk subjected to proteolysis by *Lactobacillus casei* ssp. *pseudoplamarum* ATCC 25598 at 37°C. Lane 1, control at 265 h; lane 2, 0 h; lane 3, 74 h; lane 4, 102 h; lane 5, 120 h; lane 6, 150 h; lane 7, 166 h; lane 8, 215 h; lane 9, 250 h; lane 10, 265 h; lane 11, α_{s1}-casein; lane 12, β-casein; lane 13, κ-casein; lane 14, molecular size markers (top to bottom): 66.0 kDa; 45.0 kDa; 36.0 kDa; 29.0 kDa; 24.0 kDa; 20.1 kDa; 14.2 kDa.
bacterium as compared with cheese made with
L. helveticus ATCC 10797.

Strains of L. plantarum that preferentially
hydrolyzed β-casein may be beneficial in ac­
celerated ripening of Cheddar cheese. Hydroly­
sis of β-casein results in formation of peptides,
which would most likely impart a bitter taste to
cheese. However, as the ripening process con­
tinues, the amino acids liberated from peptides
may be transformed by other enzyme systems,
provided they are present in cheese, to acids,
aldehydes, and amines, which will contribute to
the desired aroma and flavor of cheese (22).
Degradation of β-casein also has been implica­
ted in growth of Streptococcus cremoris
(Lactococcus lactis ssp. cremoris) in milk (10).

In cheese, hydrolysis of β-casein is limited
as opposed to the more extensive degradation
of α-caseins (26). Other workers also reported
similar observations regarding resistance of β-
casein to hydrolysis by several proteolytic en­
zymes, resulting in a higher rate of α_m­
casein hydrolysis when the cheese ripening process
is complete (17, 18, 19). Results from our study
suggest that addition to cheese milk of strains
of L. plantarum, which preferentially degrade
β-casein, might be of practical importance in
accelerating the cheese ripening process.

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