Effects of High Pressure on the Viability, Morphology, Lysis, and Cell Wall Hydrolase Activity of Lactococcus lactis subsp. cremoris

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Viability, morphology, lysis, and cell wall hydrolase activity of Lactococcus lactis subsp. cremoris MG1363 and SK11 were determined after exposure to pressure. Both strains were completely inactivated at pressures of 400 to 800 MPa but unaffected at 100 and 200 MPa. At 300 MPa, the MG1363 and SK11 populations decreased by 7.3 and 2.5 log cycles, respectively. Transmission electron microscopy indicated that pressure caused intracellular and cell envelope damage. Pressure-treated MG1363 cell suspensions lysed more rapidly over time than did non-pressure-treated controls. Twenty-four hours after pressure treatment, the percent lysis ranged from 13.0 (0.1 MPa) to 43.3 (300 MPa). Analysis of the MG1363 supernatants by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed pressure-induced lysis. Pressure did not induce lysis or membrane permeability of SK11. Renaturing SDS-PAGE (zymogram analysis) revealed two hydrolytic bands from MG1363 cell extracts treated at all pressures (0.1 to 800 MPa). Measuring the reducing sugars released during enzymatic cell wall breakdown provided a quantitative, non-denaturing assay of cell wall hydrolase activity. Cells treated at 100 MPa released significantly more reducing sugar than other samples, including the non-pressure-treated control, indicating that pressure can activate cell wall hydrolase activity or increase cell wall accessibility to the enzyme. The cell suspensions treated at 200 and 300 MPa did not differ significantly from the control, whereas cells treated at pressures greater than 400 MPa displayed reduced cell wall hydrolase activity. These data suggest that high pressure can cause inactivation, physical damage, and lysis in L. lactis. Pressure-induced lysis is strain dependent and not solely dependent upon cell wall hydrolase activity.

Cheddar cheese maturation is a complex process involving many biochemical changes. Such changes include carbohydrate, protein, and lipid breakdown. Secondary catabolic changes also occur, such as deamination, decarboxylation, beta-oxidation, and ester formation. Proteolysis is considered by most researchers to be the principal ripening reaction in Cheddar cheese (14, 15). The starter culture is a major source of proteolytic enzymes in cheese. The proteolytic system of Lactococcus lactis, the most widely used Cheddar cheese starter, has been studied thoroughly and includes a cell wall-associated proteinase, peptide and amino acid transporters, and intracellular peptidases. The end products of proteolysis, peptides and amino acids, contribute to the quality attributes of Cheddar cheese during maturation. However, peptidolysis does not readily occur in the cheese until the starter bacteria lyse, releasing intracellular peptidases in the cheese matrix, where they have direct access to their peptide substrates (4, 19). Therefore, the degree and rate of peptidase release after cell lysis are of great importance for cheese maturation and flavor development (3, 5, 9, 11, 19, 37, 38).

Surveys have shown that considerable variation exists in the degree of autolysis of industrial lactococcal starter strains grown in complex media or when cells are resuspended in inorganic buffers (27). Many researchers believe that cell autolysis is a major factor in cheese maturation (5, 11, 12). Fast-er-lysing cells result in accelerated proteolysis (38). Lysis may be caused by bacterial enzymes or by induction of a prophage (5, 13). Bacteria can contain one or more autolysins, or cell wall hydrolases, that degrade their own cell walls (6, 23). The major enzyme responsible for the autolysis of Lactococcus lactis subsp. cremoris MG1363 cells is N-acetylmuramidase (AcmA) (7). AcmA is a lysozyme-like enzyme that hydrolizes the N-acetylmuramyl-1,4-β-N-acetylglycosamine bonds of the peptidoglycan (5, 23, 28). Therefore, the N-acetylmuramidase liberates free reducing groups of N-acetylmuramic acid (23).

Foods can be exposed to high pressures with minimal effect on texture and consistency (22). High pressures can activate or inactivate enzymes (10). For example, pressurizing Lactobacillus helveticus cells to 400 MPa at 30°C for 10 min increased the activities of aminopeptidase and X-prolyl dipeptidyl amino-peptidase (21). Recent work has also demonstrated that high pressure promotes membrane damage and permeability in Escherichia coli O157 (2). The effects of high hydrostatic pressure on bacteria and enzymes suggest its potential to accelerate or arrest cheese ripening by affecting cell permeability and enzyme activity.

The present study investigated the effects of high pressure on the viability, structure, lysis, and cell wall hydrolase activity of two L. lactis subsp. cremoris strains (MG1363 and SK11) in buffer suspensions. This will provide a better understanding of the biochemical and microbiological basis for changes that occur in pressure-treated cheeses.

MATERIALS AND METHODS

Sample preparation and pressure treatment. L. lactis subsp. cremoris MG1363 and SK11 were selected for study because they are well characterized and their lysis properties have been previously studied (5, 13). MG1363 is derived from a...
cheese-making strain (16), and SK11 is used in pilot-scale cheese production (20). MG1363 and SK11 cells were grown to late exponential phase in G-M17 and L-M17 broth (Difco, Detroit, Mich.) (32), respectively, at 30°C. The cell pellet was collected from 15 ml of culture, washed once in cold, sterile phosphate-buffered saline (PBS) at pH 7, and then resuspended in 15 ml of PBS. The cell suspension was aseptically transferred into a sterile plastic bag (Nasco, Fort Atkinson, Wis.) that was then heat sealed. Finally, the sample was pressure treated to a pressure in the range of 100 to 800 MPa for 5 min at 25°C in a Quintus high-pressure food processor (Flow Pressure Systems, Kent, Wash.).

The initial temperature of the pressure-transmitting fluid (1 part distilled water:1 part Houghton-Safe 620-TY) was controlled to account for adiabatic heating (Houghton International, Valley Forge, Pa.). An untreated control was left at 25°C at atmospheric pressure (0.1 MPa) for 5 min while each pressure-treated sample was being pressurized.

Cell viability evaluation. After the pressure treatment, serial dilutions of the control and pressure-treated samples were aseptically prepared using 0.1% peptone water. The samples were transferred to G-M17 (MG1363) or L-M17 (SK11) agar plates to enumerate viable cells.

Transmission electron microscopy (TEM). After the pressure treatment, both the treated and untreated samples (1 ml) were centrifuged at room temperature and the supernatants were discarded. The cell pellets were resuspended and fixed overnight (24 h) in 4% glutaraldehyde in PBS (pH 7.4) at 4°C. Then, the samples were washed three times in PBS, pH 7.0, and resuspended in 1% osmium tetroxide for 1.5 h at room temperature. After two washes in PBS (pH 7.0) and removal of most of the buffer, the cells were resuspended in 2% warm agarose, and the mixture was allowed to solidify on ice. The specimens were cut into pieces not larger than 1 mm³ and left in a vial of PBS, pH 7.0, overnight at 4°C. After two washes in distilled water, the specimens were en bloc stained in 1% uranyl acetate for 1.5 h. After two washes in distilled water, the samples were dehydrated in graded ethanol concentrations according to the following schedule: 50% for 45 min, 70% for 45 min, 80% for 50 min, 95% for 1 h, and 100% for 1.5 h. Each ethanol concentration was changed three times during the specified time period. After dehydration, the samples were placed in propylene oxide (±95%) for 20 min with two solution changes and infiltrated in 1:1 propylene oxide Spurr resin overnight under a vacuum (20 in. of Hg). Then, the samples were infiltrated with 100% Spurr resin over 24 h with two solution changes, placed into a Beem capsule with fresh Spurr resin, and put under a vacuum for 4 h. The Spurr-embedded sample was polymerized in a vacuum oven for 8 h at 60°C. The sections were cut at 70 nm on a Reichert Ultracut E ultramicrotome and placed on Formvar-coated 200-mesh copper grids. Grids were stained in 2% aqueous uranyl acetate for 4 h, followed by Reynolds lead citrate for 5 min. Grids were examined in a Phillips CM 12 transmission electron microscope at 60 kV.

Cell lysis evaluation. After pressure exposure, the treated and untreated samples were aseptically transferred to a sterile cuvette and held at 25°C. The absorbance at 600 nm (Abs600) was monitored over time up to 24 h at 25°C. The percentage of cell lysis was calculated as follows: (Abs600 - Abs150)/Abs150 × 100, where the Abs150 is the initial absorbance and Abs600 is the absorbance measured after t hours of incubation (4). Using this calculation method, L. lactis cultures that were completely lysed (by bacteriolysis) yielded values of 94 to 95% lysis.

Supernatant evaluation. After the pressure treatment, both the treated and untreated samples were incubated for 24 h at 25°C. The supernatant from 1 ml of cell suspension (10⁷ CFU/ml) was combined 1:1 with Laemmli sample buffer (17), and this combination was heated for 5 min at 100°C. Ten microliters of each sample was electrophoresed through a sodium dodecyl sulfate (SDS)–10% (wt/vol) polyacrylamide gel. Electrophoresis was performed by using a Mini-Protean II cell unit (Bio-Rad, Hercules, Calif.) as described by Thompson and Shockman (34). The gels were incubated with gentle shaking for 5 h. After the electrophoresis, the detection of proteins was accomplished with the Silver Stain Plus kit (Bio-Rad). Relative band intensities were determined using the software described above.

(ii) Analysis of free reducing sugars. The free reducing sugar content of the pressure- and non-pressure-treated MG1363 cell suspensions was measured. Cells were pressure treated and held at 25°C for 24 h. At times 0 and 24 h, 15 ml of the cell suspension (10⁷ CFU/ml) was centrifuged at 20,000 × g for 10 min at 4°C to remove cells and unhydrolyzed cell walls. The supernatant was analyzed for free reducing sugars released during hydrolysis of the cell walls by the method of Park and Johnson (26) as modified by Thompson and Shockman (34). The release of reducing sugars was reported as the change in reducing sugar content: (μmoles − μmoles₀), where μmoles₀ is the initial content and μmoles is the content after t hours of incubation.

Statistical analysis. All data shown are the results of at least three independent replications of each experiment. Mean comparisons (Tukey's honestly significant difference test) and standard deviations were calculated using JMPIn (SAS Institute Inc., Cary, N.C.) and SigmaPlot (version 5.0; SPSS Inc., Chicago, Ill.).

RESULTS

Cell viability after pressure treatment. The cell viability was measured directly after pressure treatment and followed similar patterns for both strains (Fig. 1). MG1363 and SK11 were inactivated (<10 CFU/ml) at pressures of 400 to 800 MPa. Cell viability was not significantly affected at pressures of 100 and 200 MPa. At 300 MPa, the MG1363 population decreased by 7.3 log cycles, whereas the SK11 population decreased by 2.5 log cycles; thus, SK11 is more tolerant than MG1363 to pressure treatment at 300 MPa.

Pressure-induced cellular damage detected by TEM. Cell pellets collected directly after high-pressure treatments (0.1,
300 and 800 MPa) were analyzed using TEM. Similar results were seen for both MG1363 and SK11 (Fig. 2). Cell envelope and intracellular damage was apparent after exposure to 300 and 800 MPa compared to the non-pressure-treated control (0.1 MPa). Deformation (Fig. 2c and f), shedding (Fig. 2e), and partial loss (Fig. 2f) of the cell envelope were observed in pressure-treated samples. Intracellular regions of lower density occurred at both 300 and 800 MPa. All effects were more pronounced at 800 MPa.

Cell lysis after pressure treatment. The lysis of pressure- and non-pressure-treated cell suspensions was monitored over 24 h. Measurements of $A_{600}$ before and immediately after pressure treatment indicated that no immediate change in $A_{600}$ occurs due to pressure (data not shown). Pressure-induced cell lysis after 24 h differed between the two strains. Pressure-treated MG1363 cell suspensions lysed more rapidly than non-pressure-treated controls over time (Fig. 3). Twenty-four hours after pressure treatment, the extent of lysis ranged from 13.0 to 43.3%, with the control at 0.1 MPa lysing 13.0%. The sample treated at 300 MPa lysed the most extensively (43.3%). The sample treated at 300 MPa lysed significantly more than those treated at 100, 200, and 600 to 800 MPa ($P < 0.05$). There was no statistical difference between the samples treated at 300, 400, and 500 MPa. Lysis increased with increasing pressure up to 300 MPa, and then, as pressure rose above 300 MPa, lysis gradually decreased. Therefore, the intermediate pressure levels promoted lysis to a greater extent than the lower or higher pressures. The lysis of pressure-treated SK11 suspensions did not differ significantly from that of the non-pressure-treated

![FIG. 2. Transmission electron micrographs of non-pressure- and pressure-treated L. lactis subsp. cremoris MG1363 (a, b, and c) and SK11 (d, e, and f). Pressures used were 0.1 MPa (a and d), 300 MPa (b and e), and 800 MPa (c and f). Bar = 0.50 μm.](http://aem.asm.org/)

![FIG. 3. Percent lysis of pressure-treated (0.1 to 800 MPa) L. lactis subsp. cremoris MG1363 log-phase cell suspensions held at 25°C for 24 h posttreatment. Error bars represent ±1 standard deviation ($n = 3$). Values with the same letter are not statistically different ($α = 0.05$).](http://aem.asm.org/)
control across all pressure levels (P < 0.05). All SK11 suspensions displayed less than 8.7% lysis. These data indicate that high pressure can induce subsequent cell lysis and that the extent of lysis is pressure and strain dependent.

Detection of released intracellular proteins by SDS-PAGE. Though no lysis of SK11 cell suspensions was observed, this did not preclude the possibility that membrane permeability was increased by the pressure treatment. Therefore, the supernatants of pressure- and non-pressure-treated samples were evaluated for protein content by SDS-PAGE 24 h posttreatment. Pressure-treated cell suspensions of MG1363 and SK11 differed in their release of intracellular proteins. For MG1363, protein bands were present at all pressures (0.1 to 800 MPa), indicating release of intracellular proteins (Fig. 4). However, higher relative intensities arose after exposure to pressures of 200 MPa and more. The band intensity of the sample treated at 200 MPa was 1.70 times greater than that of the control, while the intensities of the bands resulting from treatment at 300 and 400 MPa were 2.55 and 2.18 times greater than those of the control, respectively. The relative intensities of samples treated at 500 MPa and higher pressures were approximately 1.9 times greater than those of the control. Pressure-treated cell suspensions of SK11 did not release protein at any pressure (data not shown), indicating that cell permeability to large molecules was not induced by pressure treatment of this strain. These data correlate with the pressure-induced cell lysis observations described above.

Cell wall hydrolase activity assayed by renaturing SDS-PAGE. Cell extracts of both MG1363 and SK11 were analyzed for cell wall hydrolase activity directly after exposure to high pressure. M. hyodeikiticus cells were used as the substrate for zymogram analysis. The cell extracts of MG1363 displayed two lytic bands at all pressure ranges (0.1 to 800 MPa) (Fig. 5). The intensity of the bands did not vary significantly at different pressures (data not shown). There were no lytic bands detected with the cell extracts of SK11 (data not shown). This experiment led to three possible conclusions: MG1363 cell wall hydrolizing enzymes are resistant to high pressures, zymogram analysis is not sensitive enough to detect the differences in activity, or pressure-damaged enzymes are renatured by this assay.

Cell wall hydrolase activity assayed by evolution of free reducing sugars. To analyze cell wall hydrolase activity quantitatively without denaturing and renaturing proteins, the supernatant of pressure-treated MG1363 was monitored for evolution of free reducing sugars. During cell wall hydrolysis, peptidoglycan is broken down, releasing free reducing sugars into the surrounding medium. Therefore, measuring the reducing sugar concentration in the supernatant quantifies cell wall hydrolase activity. After 24 h, the sample treated at 100 MPa had released a significantly larger amount of free reducing sugars (45.4 μmol) than had all other samples (Fig. 6). There was no significant difference between the samples treated at 0.1, 200, and 300 MPa. Liberation of reducing sugars was lower in the samples treated at 400 to 800 MPa. Pressure treatment at 100 MPa activated cell wall hydrolase activity or increased cell wall accessibility to the enzyme, while higher pressures (400 to 800 MPa) inactivated its activity or decreased accessibility.

DISCUSSION

Five-minute pressure treatments at ≥400 MPa inactivated cell suspensions of late-exponential-phase L. lactis cultures. Pressure treatments of 100 and 200 MPa had no significant effect on cell viability, while treatment at 300 MPa inactivated part of the population. SK11 was more tolerant to pressure treatment at 300 MPa than was MG1363. Studies on the pressure resistance of Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, and Salmonella species have shown appreciable differences between strains within the same species (1, 2, 22, 25). Intraspecies strain variation in pressure tolerance has also been reported for two species of lactic acid bacteria. Casal and Gómez (8) reported differences in viability of three L. lactis subsp. lactis strains after pressure treatment in milk sus-

FIG. 4. SDS-PAGE analysis of intracellular proteins in the supernatant of pressure-treated (0.1 to 800 MPa) L. lactis subsp. cremoris MG1363 cell suspensions held at 25°C for 24 h posttreatment. Lanes 1 to 9 represent treatments at 0.1, 100, 200, 300, 400, 500, 600, 700, and 800 MPa, respectively. The relative band intensities with respect to the control are shown below the gel.

FIG. 5. Renaturing SDS-PAGE of L. lactis subsp. cremoris MG1363 cell extracts from pressure-treated (0.1 to 800 MPa) cell suspensions. M. hyodeikiticus cells (0.2% lyophilized) were used as the substrate for zymogram analysis. Lanes 1 to 9 represent treatments at 0.1, 100, 200, 300, 400, 500, 600, 700, and 800 MPa, respectively.
treatment caused bud scars on the surfaces of the cells and that membrane integrity was lost in most of the cell population. These observations suggest that the cellular wall or membrane could be a target of high-pressure treatment, but intracellular damage should not be overlooked. The intracellular regions of low density observed in L. lactis (Fig. 2) are similar to those observed in pressure-treated L. monocytogenes by Mackey et al. (18). These authors suggest that the low-density regions are caused by transient membrane invaginations under pressure that are subsequently reversed upon pressure release, leaving the low-density regions adjacent to the cell membrane. Interestingly, these low-density regions were not observed in high-pressure-treated S. enterica serovar Thompson (18), suggesting that the gram-positive cell wall may be associated with this phenomenon.

High-pressure treatment induced lysis over time posttreatment in L. lactis MG1363, but not in SK11. Pressure-treated MG1363 cell suspensions lysed to a greater extent over time than did non-pressure-treated controls. No pressure-induced lysis of L. lactis SK11 was observed. MG1363 and SK11 have inherently different lysis characteristics. MG1363 possesses a well-characterized cell wall hydrolase, AcmA, that is involved in stationary-phase cell lysis (5). Cells grown in G-M17 begin to lyse after approximately 10 h of growth. SK11 does not lyse after 24 h of growth in L-M17 medium but displays thermoresistible lysis when shifted to 40°C, likely due to induction of a prophage (13). The fact that pressure-induced lysis of SK11 was not observed suggests that the prophage was not induced or was inactivated by pressure treatment. The difference in susceptibility to pressure-induced lysis between the Lactococcus strains may be due to differences in modes of lysis, membrane properties, or other factors. In the only other report of pressure-induced bacterial cell lysis, Tholozan et al. (33) reported, but did not quantify, lysis of L. monocytogenes treated at 500 and 600 MPa. Further study of a larger group of strains differing in their lysis characteristics is required to better understand the factors involved and allow predictions of pressure-induced lysis in other strains.

Though no lysis of SK11 cell suspensions occurred, this did not preclude the possibility that membrane permeability increased as a result of the pressure treatment. In recent studies, high pressure was shown to induce membrane permeability or leakiness (25, 39). Therefore, the supernatants of pressure- and non-pressure-treated samples were assessed for an increase in intracellular protein concentration. SK11 did not release detectable amounts of protein into its supernatant at all pressures, including the non-pressure-treated control. The supernatants for MG1363 contained intracellular proteins at all pressures, including the non-pressure-treated control, concuring with the observation that some cell lysis was observed with all treatments.

To determine the potential contribution of cell wall hydrolase activity to pressure-induced lysis, we examined this activity after pressure treatment. High pressures can activate or inactivate enzymes (10, 30, 31). Cell wall hydrolase activity was determined by two methods. Renaturing SDS-PAGE with or was inactivated by pressure treatment. The difference in inherent lysis characteristics. MG1363 possesses a well-characterized cell wall hydrolase, AcmA, that is involved in stationary-phase cell lysis (5). Cells grown in G-M17 begin to lyse after approximately 10 h of growth. SK11 does not lyse after 24 h of growth in L-M17 medium but displays thermoresistible lysis when shifted to 40°C, likely due to induction of a prophage (13). The fact that pressure-induced lysis of SK11 was not observed suggests that the prophage was not induced or was inactivated by pressure treatment. The difference in susceptibility to pressure-induced lysis between the Lactococcus strains may be due to differences in modes of lysis, membrane properties, or other factors. In the only other report of pressure-induced bacterial cell lysis, Tholozan et al. (33) reported, but did not quantify, lysis of L. monocytogenes treated at 500 and 600 MPa. Further study of a larger group of strains differing in their lysis characteristics is required to better understand the factors involved and allow predictions of pressure-induced lysis in other strains.

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To determine the potential contribution of cell wall hydrolase activity to pressure-induced lysis, we examined this activity after pressure treatment. High pressures can activate or inactivate enzymes (10, 30, 31). Cell wall hydrolase activity was determined by two methods. Renaturing SDS-PAGE with M. hyoaelekites as the substrate suggested that pressure had no effect on MG1363 cell wall hydrolase activity. SK11 displayed no lytic activity in this assay. These results prompted our in-
vestigation of cell wall hydrolase activity using a quantitative assay that does not require denaturation and renaturation of proteins. The end products (free reducing sugars) of cell wall hydrolysis were analyzed to measure enzyme activity with and without pressure treatment (26, 34). The data acquired by this assay contradict those observed with renaturing SDS-PAGE. Either renaturing SDS-PAGE was not sensitive enough to detect these differences in activity or the renaturation step returned the enzyme to its native state.

Cell wall hydrolase activity may not be the only contributor to pressure-induced lysis of L. lactis subsp. cremoris MG1363. Though treatment at 100 MPa yielded optimum hydrolase activity, 300 MPa was the optimum pressure to induce lysis. Likewise, though treatment at 400 to 800 MPa reduced cell wall hydrolase activity compared to the control, cells treated at these pressures lysed more extensively than the control. Purtle et al. (27) reported that the high rate of autolysis in L. lactis subsp. cremoris 2250 is not due to AcmA activity only. Changes in membrane lipid or protein structure, lipid-protein interactions, diffusion rate, and exposure of active sites all could also be contributing to pressure-induced lysis.

Pressure has the potential to accelerate or arrest cheese ripening by promoting or inhibiting autolysis of some starter culture strains. We demonstrated autolysis enhancement of one L. lactis strain post-pressure treatment in a buffer suspension. Cells in a cheese matrix are in a vastly different environment than the buffer used in this study. Salt, lactic acid, peptides, amino acids, and other solutes may impact autolysis in a pressure-treated cheese. Previous studies on bacterial lysis in cheese measured the activity of an intracellular marker enzyme to assess release of intracellular contents into the cheese matrix (24, 36). A different approach may be necessary to study cell lysis in pressure-treated cheeses, because pressure can inactivate or activate many enzymes (10). A pressure-insensitive intracellular marker or an alternative method to assess lysis in cheese must be established before cell lysis in pressure-treated cheeses can be evaluated.

This is the first report to investigate pressure effects on Lactococcus structure, quantify pressure-induced bacterial cell lysis, and measure pressure effects on cell wall hydrolase activity. Though the mechanism behind pressure-induced cell lysis is not understood, it is evident from this study that it is strain and pressure dependent and may involve changes to the cell envelope structure and cell wall hydrolase activity.

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