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Autolysis of Lactococci: Detection of Lytic Enzymes by Polyacrylamide Gel Electrophoresis and Characterization in Buffer Systems

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Lactococcal strains were screened for bacteriolytic activity against *Micrococcus luteus* cells, lactococcal cells, and cell walls. Thirty strains were screened for bacteriolytic activity against cells and cell walls incorporated into agar medium. Enzymes from all strains hydrolyzed the substrates; however, the activity against *Micrococcus* cells was much higher than against *Lactococcus* cells or cell walls. Electrophoretic profiles of bacteriolytic activities of culture supernatants, sodium dodecyl sulfate-treated cell extracts, cell wall fractions, and cell extracts were analyzed in sodium dodecyl sulfate-polyacrylamide gels containing *M. luteus* cells or lactococcal cell walls as the substrate. The 22 strains tested contained two to five lytic bands in the culture supernatant, ranging in size between 32 and 53 kDa. The cell extracts, the sodium dodecyl sulfate-treated cell extracts, and the cell wall fractions revealed two lytic bands of 47 and 53 kDa. Effects of external factors on autolysis of some strains were determined in buffer systems. Optimal autolysis was observed in the exponential growth phase at pH 6.0 to 7.5 and at a temperature of 30°C. Two of three strains tested seemed to contain a glycosidase, and all three strains contained an *N*-acetylmuramyl-L-alanine amidase or an endopeptidase.

Lactococci are important starter organisms in the production of cheese and cultured milk products. Autolysis of starter bacteria releases intracellular enzymes into the curd. This enzyme leakage influences cheese ripening, and rapidly autolyzing strains may be selected to decrease the ripening time (3, 4).

Bacteria contain one or more autolytic enzymes that can degrade their own cell walls (22, 28). These enzymes are characterized by their hydrolytic bond specificity. The four common types of autolysins are (i) *N*-acetylmuramidases, which liberate free reducing groups of *N*-acetylmuramic acid; (ii) *N*-acetylglucosaminidases, which liberate free reducing groups of *N*-acetylglucosamine; (iii) *N*-acetylmuramyl-L-alanine amidases, which hydrolyze the bond between *N*-acetylmuramic acid and L-alanine; and (iv) peptidases, which hydrolyze the main peptides and the bridge peptides (21). Although the physiological function(s) of autolytic enzymes remains unclear, they are believed to play an important role in cell wall growth and turnover and cell separation (21, 28). The ability of bacteriolytic enzymes to renature after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has made it possible to detect multiple forms of cell wall hydrolases in the renatured-substrate-containing gel (7, 16, 20).

In the present work, lytic enzymes of different lactococci were studied by using agar plates and SDS-polyacrylamide gels containing *Micrococcus luteus* cells or lactococcal cells or cell walls as substrates for the enzymes. Effects of different physical and chemical factors on autolytic properties were studied by using buffer systems. The specificity of the autolytic enzymes involved in cell wall degradation was also analyzed.

MATERIALS AND METHODS

Bacteria and growth conditions. The different strains used were obtained from the culture collection of the Department of Food Science, Agricultural University of Norway, Ås; the Department of Food Science and Nutrition, University of Minnesota, St. Paul; the National Collection of Dairy Organisms, Reading, United Kingdom; and the American Type Culture Collection, Rockville, Md. (Table 1). The strains were grown at 30°C in M17 broth (26) with the addition of 0.5% (wt/vol) glucose (M17G broth). The strains were stored at −80°C in M17G broth or in 10% (wt/vol) reconstituted skim milk (Difco), both supplemented with 15% (vol/vol) glycerol. Exponentially growing cultures were used as the inoculum (1%).

Isolation of bacterial cells. Cells from the exponential growth phase were harvested by centrifugation at 14,000 × *g* for 10 min at 4°C. The pellet was washed in 0.05 M potassium phosphate buffer (pH 7.0) and in 0.05 M Tris-HCl buffer (pH 7.0). The cells were resuspended in 0.05 M Tris-HCl buffer (pH 7.0) before lyophilization.

Preparation of native cell walls. Cells (2 to 3 g [dry weight]) were suspended in 20 to 30 ml of 0.05 M potassium phosphate buffer (pH 7.0) by stirring for 30 min at 4°C. The cells were disrupted by three passages through a refrigerated French pressure cell at 15,000 lb/in^2 (about 103 mPa; Carver Laboratory Press). The cells were cooled on ice between passages. Whole cells were removed by centrifugation at 1,000 × *g* for 15 min at 4°C. The cell walls were obtained from the supernatant by centrifugation at 14,000 × *g* for 15 min at 4°C. The walls were washed in 0.05 M potassium phosphate buffer (pH 7.0) before lyophilization.

Preparation of SDS-treated cell walls. Lyophilized cells (1 g) were resuspended in 80 ml of 4% (wt/vol) SDS by stirring for 90 min at room temperature. Cell walls were isolated by the method of Potvin et al. (20), with some modifications. The cell suspension was sonicated at 0°C in four cycles of 5 min each at the maximum power setting (MSE 100 W 7100 ultrasonic disintegrator). Cells that were not disrupted were removed by centrifugation at 1,000 × *g* for 15 min. The other centrifugations were done at 14,000 × *g* for 15 min at 20°C.

Screening of lytic activity. Bacteria were grown on the surface of M17G agar containing either 0.2% (wt/vol) autoclaved, lyophilized *M. luteus* cells, lactococcal cells, or SDS-treated cell walls. Agar plates containing *M. luteus* cells were incubated for 48 h at 30°C, and agar plates containing lactococcal cells or cell walls were incubated for 2 to 6 days at 30°C. Lytic activity was detected as clear zones in the agar around the colonies. The hydrolysis zone is defined as the $\text{diameter}_{\text{colony + hydrolysis zone}} - \text{diameter}_{\text{colony}}$. Freeze-dried cells of *M. luteus* ATCC 4698 were obtained from Sigma Chemical Co.

Preparation of lytic-enzyme fractions. The supernatants obtained after heating and centrifugation in the preparation of SDS-treated cell walls (20) were used as the SDS cell extracts. Culture supernatants were obtained after centrifugation of overnight cultures (10 ml) at 14,000 × *g* for 10 min, unless stated otherwise. The cells were washed twice in 0.05 M Tris-HCl buffer (pH 7.0) before resuspension in 2.5 ml of 0.05 M Tris-HCl buffer (pH 7.0). The cells were sonicated as described in the preparation of SDS-treated cell walls (see above). Cell extracts were obtained after centrifugation of sonicated cells at 19,000 × *g* for 10 min at 4°C. The cell wall fractions were obtained after resuspension of the pellet in 2.5 ml of 0.05 M potassium phosphate buffer (pH 7.0). The protein concentration was measured in samples by using a protein assay kit (Bio-Rad Laboratories,

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TABLE 1. Lactococcal strains used in this investigation

Species	Strain	Source[a]
L. lactis subsp. *lactis*	INF-L2	A
L. lactis subsp. *lactis*	INF-L4	A
L. lactis subsp. *lactis*	INF-L-2_1 a1[b]	A
L. lactis subsp. *lactis*	INF-L-2_1 a2[b]	A
L. lactis subsp. *lactis*	INF-L-2_1 b[b]	A
L. lactis subsp. *lactis*	INF-L-2_1 c[b]	A
L. lactis subsp. *lactis*	INF-L-4_3	A
L. lactis subsp. *lactis*	INF-L-4_4 a[b]	A
L. lactis subsp. *lactis*	INF-L-4_4 b19[b]	A
L. lactis subsp. *lactis*	INF-L-4_4 b20[b]	A
L. lactis subsp. *lactis*	INF-L-5_4 a[b]	A
L. lactis subsp. *lactis*	INF-L-5_4 b1[b]	A
L. lactis subsp. *lactis*	INF-L-5_4 b2[b]	A
L. lactis subsp. *lactis*	INF-L-5_4 c[b]	A
L. lactis subsp. *lactis*	INF-L-$6_{4'}$ a[b]	A
L. lactis subsp. *lactis*	LM 0230	B
L. lactis subsp. *lactis*	LM 2306	B
L. lactis subsp. *lactis*	LM 2336	B
L. lactis subsp. *cremoris*	Cornell	B
L. lactis subsp. *cremoris*	INF-C12	A
L. lactis subsp. *cremoris*	INF-C15	A
L. lactis subsp. *cremoris*	INF-C1200	A
L. lactis subsp. *cremoris*	BC101	B
L. lactis subsp. *cremoris*	INF-C-41_2 a[b]	A
L. lactis subsp. *cremoris*	INF-C-41_3 a[b]	A
L. lactis subsp. *lactis* biovar diacetylactis	INF-E2-6	A
L. lactis subsp. *lactis* biovar diacetylactis	INF-E2-6 59[c]	A
L. lactis subsp. *lactis* biovar diacetylactis	NCDO 176	C
L. lactis subsp. *lactis* biovar diacetylactis	ATCC 15346	D
L. lactis subsp. *lactis* biovar diacetylactis	INF-FD Dia	A
L. lactis subsp. *lactis* biovar diacetylactis	INF-D1	A
L. lactis subsp. *lactis* biovar diacetylactis	INF-D3	A
L. lactis subsp. *lactis* biovar diacetylactis	INF-D5	A
L. lactis subsp. *lactis* biovar diacetylactis	INF-D6	A

[a] Sources: A, Department of Food Science Culture Collection, Agricultural University of Norway, Ås; B, Department of Food Science and Nutrition, University of Minnesota, St. Paul; C, National Collection of Dairy Organisms, Reading, United Kingdom; D, American Type Culture Collection, Rockville, Md.
[b] Different morphological variants: a, a1, a2, b, b1, b2, c, b19, b20.
[c] Plasmid-free strain of *L. lactis* subsp. *lactis* var diacetylactis INF-E2-6.

Richmond, Calif.). Preparations of samples were repeated at least three times, and representative results are shown.

Lytic activity from the clear zones surrounding the colonies on agar plates containing *M. luteus* was eluted as described by Potvin et al. (20).

SDS-PAGE. SDS–12% (wt/vol) polyacrylamide gels containing 0.2% (wt/vol) autoclaved bacterial cells or SDS-treated cell walls were used to detect lytic enzymes. SDS-PAGE was carried out as described by Laemmli (14). Electrophoresis was performed by using a Bio-Rad Mini-Protean II cell unit (gel size, 73 by 102 by 0.75 mm) at room temperature in the anodal direction at 20 mA until the blue dye reached the bottom of the gel. Protein standards contained phosphorylase *b* (molecular weight, 97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400) (Bio-Rad).

Detection of lytic activity. After electrophoresis, the gels were washed with 250 ml of distilled water under gentle shaking for 30 min. The gels were then incubated for 12 to 16 h at 37°C with gentle shaking in 0.025 M Tris-HCl buffer (pH 7.0) containing 1% (vol/vol) Triton X-100 (16, 20), unless otherwise stated. Lytic activity appeared as clear bands in the opaque gel and as dark bands after the gels were photographed against a dark background. Molecular masses were determined by comparison with standards on the same gel stained with Coomassie blue (after the photograph mentioned above had been obtained).

The effect of pH on the bacteriolytic enzyme profiles was tested by incubating the gels after electrophoresis in 0.05 M potassium phosphate buffers (pHs 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) and in 0.05 M glycine-NaOH buffer (pH 9.0), all containing Triton X-100 as mentioned above.

Autolysis in buffer systems. Bacteria were harvested by centrifugation at 14,000 × *g* for 10 min at 4°C after different culture times. The cells were washed in Ringer's solution and then in 0.05 M potassium phosphate buffer (pH 7.0) or 0.05 M Tris-HCl buffer (pH 7.0). The cells were resuspended in different buffer systems and at different temperatures. Optical density at 600 nm (OD_{600}) was measured in a Corning colorimeter (model 252) with special glass cuvettes. The initial OD_{600} was adjusted to about 0.7. The samples were incubated in a water bath at 30°C, unless stated otherwise. Autolysis was monitored by measuring the decrease in OD_{600}. Autolysis was characterized by the following two parameters: the rate of autolysis, expressed as the decrease in OD_{600} per minute during the first 60 min; and the extent of autolysis, expressed as the percent decrease of OD_{600} after a certain time (15).

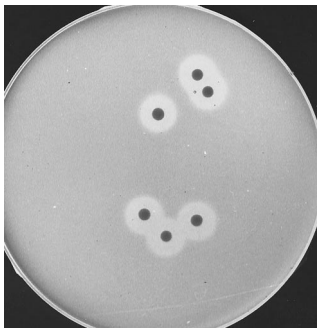


FIG. 1. Lytic zones surrounding *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 grown on M17G agar containing lyophilized *M. luteus* cells (0.2%).

The influence of pH on autolysis was measured in 0.05 M potassium phosphate buffers ranging from pH 5.0 to pH 8.0 and in 0.05 M glycine-NaOH buffer (pH 9.0). The effect of temperature on autolysis was determined in 0.05 M potassium phosphate buffer (pH 7.0) at 4, 10, 20, 30, 40, and 50°C. Effects of monovalent and divalent cations, as well as the chelating agent EDTA, on autolysis were measured in 0.05 M potassium phosphate buffer (pH 7.0).

Liberation of reducing sugars and free amino groups. Native cell walls (4 to 8 mg/ml) were resuspended in 0.05 M potassium phosphate buffer (pH 7.0) and used as a substrate for determination of reducing sugars and free amino groups liberated from the cell walls. The wall suspensions were incubated at 30°C. Samples were centrifuged at 20,000 × *g* for 10 min at 4°C to remove unhydrolyzed cell walls. Reducing sugars released during hydrolysis of the cell walls were determined by the method of Park and Johnson (19) as modified by Thompson and Shockman (27). Free amino groups liberated during hydrolysis of the cell walls were determined by the method of Ghuysen et al. (9).

RESULTS

Screening of lactococci for lytic activity. Thirty lactococcal strains showed lytic activity against *M. luteus* cells on agar plates. One strain is shown in Fig. 1. The strains showed differences in lytic activity after the incubation period. The hydrolysis zone varied between 1 and 6 mm in diameter. Twenty-six of the strains tested had hydrolysis zones of 3 mm or larger. Four of the 26 strains, *Lactococcus lactis* subsp. *lactis* INF-L2, *L. lactis* subsp. *lactis* INF-L4, *L. lactis* subsp. *cremoris* INF-C12, and *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6, with different-sized hydrolysis zones, were also tested for autolytic activity against lactococcal cells and cell walls. Only weak hydrolysis zones were seen in these strains.

Detection of lytic enzymes by SDS-PAGE. Culture supernatants of 22 strains of lactococci were screened for bacteriolytic activity with *M. luteus* cells as the substrate in SDS-PAGE. The number of lytic bands varied between two and five, and the molecular masses of the lytic enzymes varied between 32 and 53 kDa (Table 2; Fig. 2). The intensity of the lytic bands also showed large variations.

After different culture times (0, 4, 6, 12, and 24 h), the culture supernatants of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 showed different lytic activities. One main enzyme band, 47 kDa, and two weaker bands, 32 and 39 kDa, were observed in the culture supernatants of exponential-phase cells (4 and 6 h). As the culture reached stationary phase, the intensity of these bands increased, especially the lytic bands at 32 and 47 kDa; however, a new band of 53 kDa also appeared. The optimum pH for bacteriolytic activity in the gel was in the range of 6.0 to 7.0. The intensity of the enzyme bands of 32, 39,

TABLE 2. Molecular size of bacteriolytic enzymes from the supernatants of 22 lactococcal strains in SDS-polyacrylamide gels containing *M. luteus*

Strain	Intensity of bacteriolytic band[a]:				
	B1 (32 kDa)	B2 (39 kDa)	B3 (43 kDa)	B4 (47 kDa)	B5 (53 kDa)
L2	+	++	+	++	−
L4	++	+	−	+++	+
L-2_1 a1	+	−	−	+++	+
L-2_1 a2	+	−	−	+++	+
L-2_1 c	+	++	++	+++	+
L-4_3	+	++	+	++	−
L-4_4 a	+	++	+	++	−
L-5_4 b1	−	−	−	+++	+
L-5_4 b2	−	++	++	+++	+
L-5_4 c	−	−	−	+++	+
L-$6_{4'}$ a	+	++	++	+++	−
LM 0230	++	+	−	+++	−
LM 2306	++	+	−	+++	−
LM 2336	++	+	−	+++	−
C12	−	−	−	++	+
C1200	−	+	+	++	−
BC101	+	+	−	+++	−
C-41_3 a	+	+	++	+++	−
E2-6	++	+	−	+++	+
NCDO 176	+	++	++	+++	+
FD Dia	+	+++	++	+++	−
D1	+	+++	++	+++	−

[a] +++, very high intensity; ++, high intensity; +, low intensity; −, no band detected.

and 53 kDa decreased considerably at higher pH values, while the enzyme band of 47 kDa was less influenced by the pH.

Autolytic activity in the lactococcal supernatants was also tested with lactococcal cell walls as the substrate. With its own cell walls as a substrate, *L. lactis* subsp. *cremoris* INF-C12 showed bands identical to those detected with *M. luteus* as the substrate. Another strain, *L. lactis* subsp. *lactis* INF-L2, showed the same number of bands; however, a new band of 53 kDa was observed and the 32-kDa band was missing. Cell walls from *L. lactis* subsp. *cremoris* INF-C12 and *L. lactis* subsp. *lactis* INF-L2 were also used as substrates for lytic enzymes from the supernatants of 12 other lactococcal strains. Ten of the strains

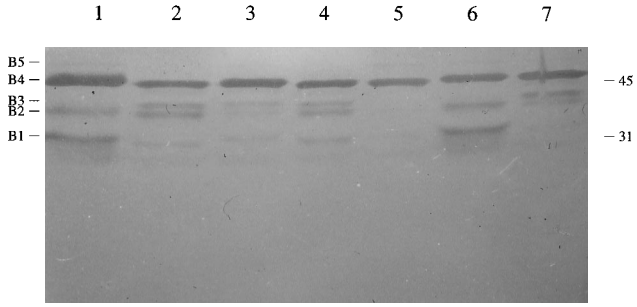


FIG. 2. Separation of lytic enzymes in the supernatants of lactococcal strains by SDS-PAGE. The gel contained 12% acrylamide and 0.2% *M. luteus* cells. After electrophoresis, proteins were renatured in 0.025 M Tris-HCl (pH 7.0) containing 1% Triton X-100. Lanes: 1, *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6; 2, *L. lactis* subsp. *lactis* INF-L2; 3, *L. lactis* subsp. *lactis* INF-L-2_1 c; 4, *L. lactis* subsp. *lactis* INF-L-4_4 a; 5, *L. lactis* subsp. *cremoris* INF-C12; 6, *L. lactis* subsp. *lactis* INF-L4; 7, *L. lactis* subsp. *lactis* INF-L-5_4 b2. Lytic enzyme bands (B1 to B5) are identified on the left. Molecular masses of standards (in kilodaltons) are indicated on the right.

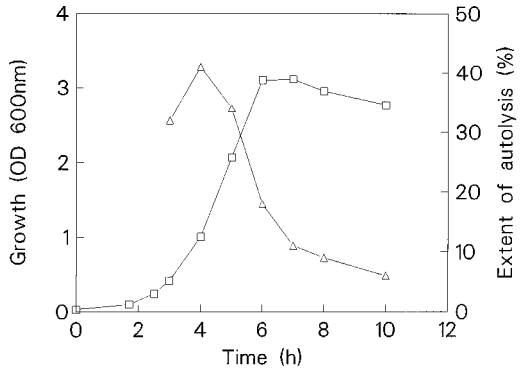


FIG. 3. Autolysis (△) of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 as a function of growth (□). Cells were harvested in different growth phases, washed, and resuspended in 0.05 M potassium phosphate buffer (pH 7.0). Autolysis was monitored by measuring the decrease in OD_{600}. The extent of autolysis after 8 h of incubation at 30°C is indicated.

showed a reduced number of lytic bands compared with the results obtained with *M. luteus* as the substrate.

Different cell fractions from *L. lactis* subsp. *lactis* INF-L2, *L. lactis* subsp. *lactis* INF-L4, *L. lactis* subsp. *cremoris* INF-C12, and *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 were further studied for bacteriolytic activity. All strains contained two lytic bands (47 and 53 kDa) in the cell extract, in the SDS-treated cell extract, and in the cell wall fraction. The band of 47 kDa showed very high/high intensity, and the band of 53 kDa showed high/low intensity.

Elution of proteins from the clear lytic zone surrounding the lactococcal colonies grown on *M. luteus*-containing agar plates followed by SDS-PAGE showed only one lytic band, of 47 kDa. Lytic activities were detected for 29 of 30 tested strains by SDS-PAGE of gels containing *M. luteus* cells. One strain, *L. lactis* subsp. *cremoris* Cornell, showed a very small hydrolysis zone on *M. luteus*-containing agar plates and no lytic band after elution and SDS-PAGE.

Effect of growth phase, pH, and temperature. The extent of autolysis of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 varied depending on the age of the cells (Fig. 3). Maximum autolysis in phosphate buffer was observed for cells from the exponential growth phase. At stationary phase, the bacteria showed a marked decrease in autolysis. Identical results were obtained with Tris-HCl buffer (pH 7.0).

The effect of pH on autolysis of exponential-phase cells in phosphate buffer and glycine-NaOH buffer was studied (Fig. 4). The pH optimum for autolysis was in the range of 6.0 to 7.5.

The influence of temperature on the extent of autolysis of exponential-phase cells was tested for three strains. Maximum autolysis was observed at 30°C. Autolysis of two of the strains as a function of temperature is shown in Fig. 5. *L. lactis* subsp. *lactis* INF-L4 showed about 70% autolysis after incubation at 30°C, while *L. lactis* subsp. *lactis* INF-L2 showed about 30% autolysis. The highest rate of autolysis was observed at 40 and 50°C during the first hour of incubation (data not shown), after which the activity was rapidly reduced.

Effects of different cations and chelating agent. All monovalent cations used reduced the extent of cell autolysis of *L. lactis* subsp. *lactis* INF-L4 at concentrations higher than 0.1 M, but the rate of autolysis was reduced only by NH_4^+, at concentrations higher than 0.01 M (Table 3). However, different divalent cations influenced the autolysis to various extents (Table 3). The rate and extent of autolysis were reduced by Cu^{2+} and Co^{2+} at 10^{-4} M or higher concentrations. In addition, a

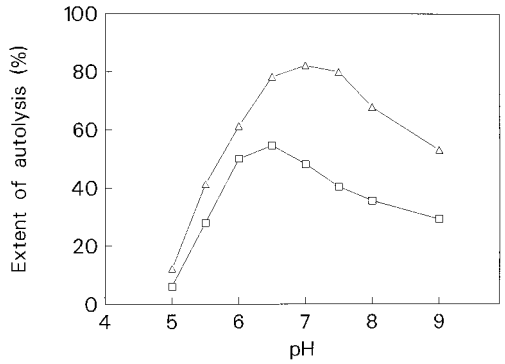


FIG. 4. Influence of pH on autolysis of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 in 0.05 M potassium phosphate buffer (pHs 5.0 to 8.0) and in 0.05 M glycine–NaOH buffer (pH 9.0). The extent of autolysis was measured after 20 h (□) and 6 days (△) of incubation at 30°C.

slight reduction of the extent of autolysis was observed for Mn^{2+} at 10^{-3} M. EDTA did not influence the rate or the extent of autolysis.

Specificity of the autolysin(s). The specificities of the autolysins of three strains were studied by detection of newly exposed groups of the peptidoglycan after hydrolysis of the cell wall. An increase in the number of reducing groups will be detected if the enzyme is a muramidase or a glucosaminidase, and an increase in the number of free amino groups will be detected if the enzyme is an amidase or an endopeptidase. Autolysis of native cell walls of *L. lactis* subsp. *cremoris* INF-C12 and *L. lactis* subsp. *lactis* INF-L4 was accompanied by an increase in the number of both reducing groups and free amino groups, while *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 showed only an increase in free amino groups (Fig. 6). Different cell wall-associated proteolytic activities are found in different *Lactococcus* strains, and the enzymes are inhibited by phenylmethylsulfonyl fluoride or diisopropylfluorophosphate (12, 25). Autolysis of native cell walls and liberation of free amino groups were therefore tested in the presence of phenylmethylsulfonyl fluoride. All strains tested showed an increase in free amino groups when the inhibitor was present. One of the strains is shown in Fig. 6C. Autolysis of some lactococcal strains appeared to be the result of a glycosidase and an amidase or an endopeptidase.

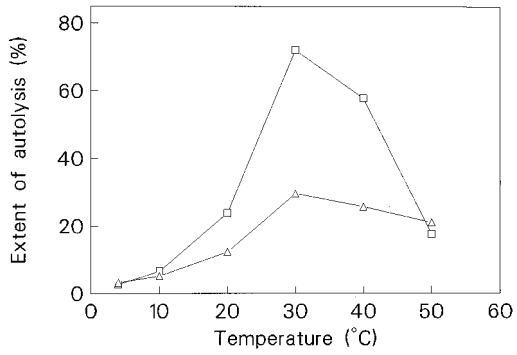


FIG. 5. Effect of temperature on autolysis of cells in 0.05 M potassium phosphate buffer (pH 7.0). Symbols: □, *L. lactis* subsp. *lactis* INF-L4; △, *L. lactis* subsp. *lactis* INF-L2. The extent of autolysis after 8 h of incubation is indicated.

TABLE 3. Effect of monovalent cations on the autolysis of *L. lactis* subsp. *lactis* INF-L4 in buffer[a]

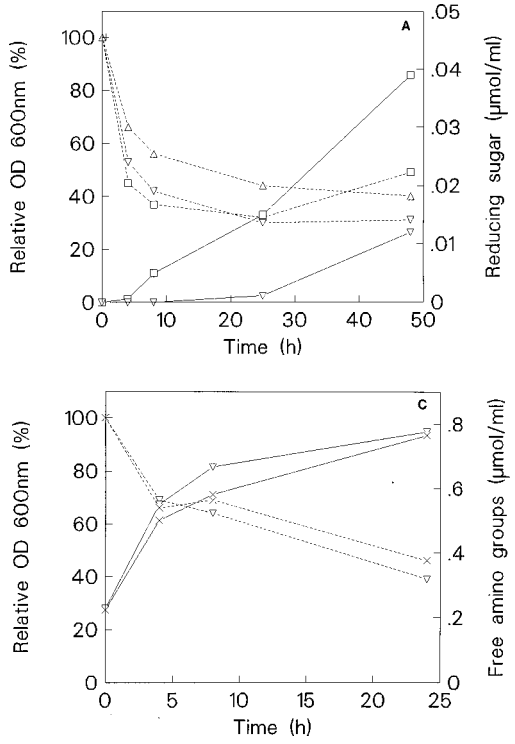
Cation[b]	Concn (M)	Rate of autolysis (10^3)[c]	Extent of autolysis (%)[c]
None		5.5 ± 0.2	95 ± 0.6
Na^+	0.01	5.5 ± 1.5	94 ± 1.2
	0.1	3.2 ± 0.9	85 ± 5.7
	0.2	4.7 ± 2.1	77 ± 6.4
	0.3	4.7 ± 2.0	74 ± 7.2
K^+	0.01	5.2 ± 0.4	93 ± 0.4
	0.1	3.6 ± 0.8	81 ± 5.2
	0.2	4.6 ± 1.6	77 ± 5.5
	0.3	5.1 ± 1.9	76 ± 6.7
NH_4^+	0.01	5.1 ± 1.2	94 ± 1.5
	0.1	2.3 ± 1.3	87 ± 4.6
	0.2	1.1 ± 0.4	66 ± 9.5
	0.3	1.6 ± 0.9	40 ± 9.0
Cu^{2+}	10^{-5}	3.9 ± 0.6	94 ± 1.0
	10^{-4}	1.7 ± 0.5	88 ± 1.2
	10^{-3}	1.1 ± 0.7	70 ± 1.5
Zn^{2+}	10^{-5}	5.9 ± 1.4	95 ± 1.0
	10^{-4}	6.0 ± 1.4	94 ± 0.6
	10^{-3}	6.0 ± 1.4	88 ± 2.0
Mn^{2+}	10^{-5}	6.0 ± 2.1	94 ± 0.6
	10^{-4}	6.3 ± 1.8	94 ± 0.6
	10^{-3}	5.7 ± 1.4	80 ± 1.2
Co^{2+}	10^{-5}	5.3 ± 1.1	93 ± 0.6
	10^{-4}	1.9 ± 0.6	74 ± 10.4
	10^{-3}	0.2 ± 0.2	10 ± 1.5
Mg^{2+}	10^{-5}	6.2 ± 1.5	94 ± 0.6
	10^{-4}	6.4 ± 2.1	94 ± 0.6
	10^{-3}	6.3 ± 2.0	95 ± 0.6
	10^{-2}	4.3 ± 1.1	95 ± 0.6
Ca^{2+}	10^{-5}	6.5 ± 1.8	94 ± 0.6
	10^{-4}	6.0 ± 1.6	95 ± 1.2
	10^{-3}	5.4 ± 1.3	92 ± 1.0

[a] Autolysis was assayed in 0.05 M potassium phosphate buffer (pH 7.0).
[b] Cl^- was the anion used in the experiments.
[c] The rate of autolysis was expressed as the decrease in OD_{600} per minute during the first 60 min, and the extent of autolysis was expressed as the percent decrease in OD_{600} after 24 h of incubation. The experiments were repeated at least three times, and the results presented are the means ± standard deviations.

DISCUSSION

In this report, we present a survey of the lytic activity of 30 lactococcal strains as determined by plating them on M17G agar containing inactivated *M. luteus* cells or lactococcal cells or cell walls. *M. luteus* cells are preferred as the substrate for detection of lytic enzymes from both gram-negative and gram-positive organisms (8, 11, 20). This method has several advantages for the screening of a large number of bacteria for variations in cell hydrolysis activity. The use of *M. luteus* cells or cell walls from the tested strain has also been reported in cloning experiments for the isolation of genes coding for lytic activity (1, 2, 10, 13, 20).

SDS-PAGE of the culture supernatants of 22 strains showed two to five lytic bands; moreover, one of the bands, with a



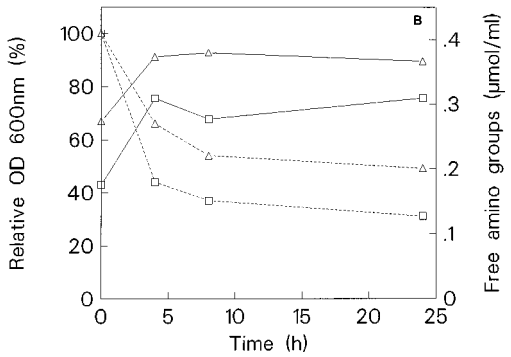


FIG. 6. Autolysis of native cell walls. The degradation of cell walls of *L. lactis* subsp. *cremoris* INF-C12 (□), *L. lactis* subsp. *lactis* INF-L4 (▽), and *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 (△) in 0.05 M potassium phosphate buffer (pH 7.0 at 30°C) was monitored by measuring relative OD_{600} (– –) (A to C), liberated reducing sugar (——) (A), liberated amino groups (——) (B), and liberated amino groups with 1 mM phenylmethylsulfonyl fluoride (×) (C).

molecular mass of 47 kDa and high to very high intensity, was universal for all strains. The intensity and the number of lytic bands were lower when lactococcal cell walls, instead of *M. luteus* cells, were used as the substrate. These observations are in agreement with those of Leclerc and Asselin (16) and Sugai et al. (24). Three lytic bands detected in the supernatant of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 after SDS-PAGE showed considerably reduced intensity when the pH was increased above 7.5, whereas one enzyme band was only very slightly affected by the pH change. This may indicate that *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 contains at least two different types of lytic enzymes.

A study was performed to clarify the location of the lytic activities. Examination of the cell wall fractions, the cell extracts, and the SDS-treated cell extracts from four strains of lactococci showed two lytic bands of 47 and 53 kDa. The 47-kDa band was also always detected in the medium supernatant of all the strains tested. However, the 53-kDa band was observed in the supernatant of only three of the four strains tested. The strains with both enzymes may either release these enzymes during autolysis or transport them through the membrane without cleavage of the proteins. The lytic enzymes with lower molecular masses observed in the supernatants may be degraded forms of the two enzymes of 47 and 53 kDa. The multiplicity of the lytic bands does not necessarily reflect the exact number of cell wall hydrolases. Different modifications such as proteolytic processing and covalent modifications must also be considered (6, 20). The SDS-PAGE method is, moreover, limited to the detection of monomeric enzymes which can renature after exposure to denaturating conditions. However, most cell wall hydrolases known are monomeric enzymes (16). The single enzyme band of 47 kDa detected after elution and SDS-PAGE corresponds to the enzyme of 47 kDa detected in the culture supernatants, the SDS-treated cell extracts, the cell wall fractions, and the cell extracts. This shows the extreme sensitivity of the gel electrophoresis system. Detection of only one lytic band may be due to the presence of small amounts of the enzymes.

Lactococcal cells autolyze spontaneously when they are transferred from M17 broth to a buffer solution. This phenomenon has been observed for many gram-positive organisms (5, 17, 18, 23). The most efficient autolysis was observed for *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 in the middle of the exponential growth phase. Other gram-positive bacteria showed maximum autolysis at the end of the exponential growth phase (5, 17, 18, 23). The optimum pH for autolysis of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 in buffer was confirmed by SDS-PAGE studies of the supernatant. The pH optimum is close to results reported for two *L. lactis* subsp. *cremoris* strains by Mou et al. (18) and three lactococcal strains reported by Vegarud (29). After prolonged incubation, a shift within this pH optimum toward higher pH values was observed. One explanation may be the presence of more than one autolytic enzyme, each with different pH optima and different stabilities. Another possibility is that the cytoplasm contains peptide hydrolases which may degrade the autolytic enzyme(s). The optimum temperature of autolysis was 30°C for three strains tested. The influence of temperature on autolysis of 14 lactococcal strains in broth was studied by Vegarud et al. (30). Five strains showed maximum autolysis at 5°C, six strains showed maximum autolysis at 30°C, and three strains showed maximum autolysis at 10°C.

Monovalent cations, such as Na^+, K^+, and NH_4^+, reduced autolysis when they were present at concentrations higher than 0.1 M. The observations are in agreement with those of Vegarud et al. (30), who observed that autolysis of two of three lactococcal strains was inhibited by NaCl in the medium.

Specificity studies of the autolytic enzymes indicated that two of three strains contained a glycosidase and all three strains contained an amidase or endopeptidase. Specificity studies were also performed with *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 cells as the substrate, and an increase in reducing-sugar groups and free amino groups was observed. The negative results obtained from the reducing-sugar analysis when cell walls of *L. lactis* subsp. *lactis* biovar diacetylactis INF-E2-6 were used as the substrate may be due to other locations or different binding of the autolytic enzyme(s). The glycosidase may be loosely bound to the cell wall or may be intracellular or bound to the cell membrane. The amidase or endopeptidase may be strongly attached to the cell wall, since

the enzyme activity persisted. Mou et al. (18) reported that the autolysin of *L. lactis* subsp. *cremoris* AM1 was an *N*-acetylmuramidase and that the bacteria contained no amidase or endopeptidase activity. This result is different from our findings, especially in relation to *L. lactis* subsp. *cremoris* INF-C12, and may indicate the presence of strain variations.

The results described above, especially the SDS-PAGE studies, the pH studies, and the specificity analysis, suggest that at least two different autolytic enzymes are involved in autolysis of the lactococci. Further studies including purification and characterization of the autolysins must be done to clarify the number of enzymes involved and also what kind of enzyme(s) is involved in autolysis of lactococci.

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