

Release of Autolytic Enzyme from *Streptococcus faecium* Cell Walls by Treatment with Dilute Alkali

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The autolytic enzyme (endo- β -1,4-*N*-acetylmuramoylhydrolase) of *Streptococcus faecium* (*S. faecalis* ATCC 9790) was released in a soluble form from insoluble cell wall-autolytic enzyme complexes by treatment with dilute NaOH at 0°C. Treatment of cell wall-enzyme complexes, obtained from either exponential- or stationary-phase cells, with 0.008 to 0.01 N NaOH gave maximum yields of autolytic enzyme activity. At a fixed concentration of NaOH, the yield of autolysin increased with increasing wall densities and was accompanied by the release of methylpentose and phosphorus in amounts proportional to the autolysin. Since extraction of wall-enzyme complexes with 4.5 M LiCl at 0°C also removed methylpentose and phosphorus, release of enzyme with NaOH did not appear to result from hydrolysis of covalent linkages. The autolytic enzyme activity released from intact cells, or cell walls, was predominantly in the latent (proteinase activable) form which could be activated by trypsin or a proteinase present in commercial bovine plasma albumin.

Several bacterial species are known to possess autolytic cell wall peptidoglycan hydrolase activities (autolysins) which are capable of hydrolyzing bonds in their insoluble, protective cell wall (4). The single peptidoglycan-hydrolyzing activity present in *Streptococcus faecium* (*S. faecalis* ATCC 9790) has been shown to be an endo- β -1,4-*N*-acetylmuramoylhydrolase that is bound to the cell wall by a very firm electrostatic linkage (10). In disrupted cells, autolysin activity was found only in the cell wall fraction from which it could be removed in a soluble form, either by complete dissolution of the wall substrate (14) or by treatment with very high concentrations of salt, such as 4.5 M LiCl (10). Additionally, enzyme activity in walls is present in two forms, an active form and a latent form which can be activated by several different proteinases (13, 14).

Autolytic dissolution of walls, due to the action of the endogenous active form of the enzyme (in the absence of a proteinase), releases soluble enzyme of both the naturally active and the latent forms in a ratio of about 1:4. The long incubation periods required for this self-digestion process usually result in relatively low recoveries of enzyme activity, especially in the absence of stabilizing agents such as glycerol or

albumin. Autolytic dissolution of walls in the presence of a proteinase (e.g., trypsin) results in a faster rate of wall hydrolysis and in higher yields of autolysin, which is then entirely in the (e.g., trypsin-) activated form. Both types of wall autolysates contain all the products of wall dissolution, including low-molecular-weight peptidoglycan fragments and soluble non-peptidoglycan polymers (e.g., teichoic acids) of the wall attached to hydrolyzed peptidoglycan fragments (13, 14).

In contrast to wall autolysates, autolysin activity removed from walls by high salt concentrations should be relatively free of wall polymers. Such preparations contain only latent autolysin activity and, under optimum conditions, have been obtained in yields of about 50% of the activity apparently present in the initial wall-enzyme complexes. Extraction of concentrated wall suspensions was even less efficient. Also, the high salt concentrations in the extracts must be substantially reduced both for assays of enzyme activity and for the initial steps of further purification.

Observations that the *N*-acetylmuramoylhydrolase activity of *Lactobacillus acidophilus* could be released from isolated wall-enzyme complexes by a very brief treatment with 0.01 N NaOH at 0 to 4°C (3) led to the present studies of extracting the *S. faecalis* autolysin. As a source of enzyme, alkaline extracts have major advantages over wall lysates and LiCl extracts,

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since the enzyme can be conveniently and efficiently removed in relatively high yield and specific activity from concentrated wall suspensions. In addition, alkaline extracts do not contain significant amounts of the products of wall hydrolysis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. faecium* was grown in S broth (1% tryptone, 1% yeast extract, 2% glucose, 0.3 M sodium phosphate, pH 7) at 37°C. The culture (20 liters) was harvested in late exponential phase (0.48 mg of cellular dry weight per ml) by continuous-flow centrifugation at 0 to 4°C and washed several times with ice-cold, double-distilled water (LOG cells). A second batch of cells was grown in the supernatant broth collected from the first 20-liter culture. These latter cells were harvested as described above after 17 h at 37°C when they were well into the stationary phase (STAT cells).

Preparation of native cell walls. Aqueous suspensions of LOG or STAT cells were disrupted in a Ribicell fractionator (Ivan Sorvall Inc., Norwalk, Conn.) operated at 50,000 lb/in² at 0 to 15°C. After disruption, the unbroken cells and wall fragments were sedimented by centrifugation (15,000 × *g*, 20 min). The sediment was washed once with ice-cold 0.15 M sodium phosphate, pH 7.5, and then washed three times with ice-cold, double-distilled water before being stored at -70°C. After each sedimentation, only the upper portion of the sediments, containing the wall fragments, was retained, removing intact cells from the final wall preparations.

Extraction of autolysin. Cold, aqueous NaOH was added directly to ice-cold aqueous suspension of walls and after rapid mixing was immediately centrifuged (50,000 × *g*, 10 min). The supernatant solution containing the soluble autolysin was immediately removed and neutralized with HCl, and Tris-hydrochloride was added to yield a final concentration of 0.01 M at pH 7. The entire process was carried out at 0 to 4°C.

Soluble autolysin was also prepared (i) by extraction of wall-enzyme complexes with 4.5 M LiCl at 0°C (10) and (ii) from autolysates of cell wall-autolytic enzyme complexes incubated in 0.01 M sodium phosphate, pH 7, in the absence or presence of trypsin as described previously (14). After self-hydrolysis was complete (no further decrease in turbidity of the wall suspension), the insoluble material was removed by centrifugation (13,000 × *g*, 30 min), and the supernatant was retained as crude soluble autolysin.

Assay for autolytic enzyme activity. Enzyme activity was measured by adding 25- to 100- μ l samples to 1.5-ml suspensions of *S. faecium* sodium dodecyl sulfate-treated walls (0.2 mg/ml) in 0.01 M Tris-hydrochloride, pH 7. The rate of dissolution of sodium dodecyl sulfate-inactivated walls by the enzyme at 37°C was monitored by the loss of turbidity at 450 nm measured with a Gilford model 300 spectrophotometer (2). Total (active plus latent) activity was determined in the presence of trypsin (0.5 μ g/ml), and naturally active autolysin was measured in the absence of trypsin. One unit of enzyme activity is defined as that

producing a loss of 0.001 optical density unit of wall substrate per h determined from the linear decrease in optical density with time (2).

Analytical methods. Protein was measured by the method of Lowry et al. (9) with bovine serum albumin as the standard; methylpentose was measured by the method of Dische and Shettles (15) with rhamnose as the standard; and phosphorus was measured by the method of Lowry et al. (8) with KH₂PO₄ as the standard. Carbohydrate (neutral sugars) was determined by the phenol-sulfuric acid method (5) with glucose as the standard.

Other materials. Trypsin (2× crystallized) was purchased from GIBCO Laboratories, Grand Island, N.Y.; soybean trypsin inhibitor (154 U/mg) was purchased from Worthington Biochemicals Corp., Freehold, N.J.; and bovine plasma albumin (BPA; fraction V powder) was purchased from Armour Pharmaceutical Co., Kankakee, Ill.

RESULTS

Effect of NaOH concentration on extraction of autolysin activity. Significant amounts of autolysin activity were removed from STAT walls when a concentration of 0.003 M NaOH was used (Fig. 1A). However, optimal removal of autolysin from the walls and recovery in the supernatant fluid were obtained at 0.008 to 0.01 N NaOH (pH, as measured with a glass electrode, 11.1 to 11.5). Higher NaOH concentra-

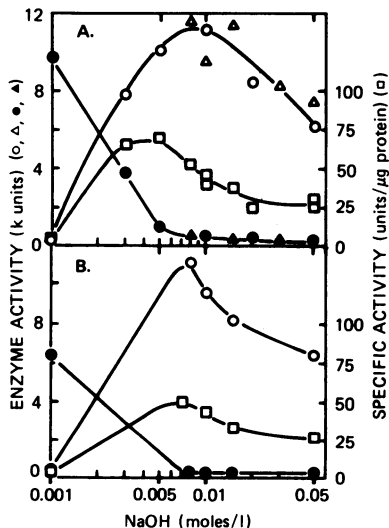


FIG. 1. Removal of autolytic enzyme activity from *S. faecium* cell walls with increasing concentrations of alkali. (A) STAT cell walls (28 mg/ml); (B) LOG cell walls (12 mg/ml). Symbols: ○ and Δ, autolytic activity released to the supernatant fraction; ● and ▲, activity which remained with the wall sediment; □, specific activity of total autolytic enzyme activity (assayed in the presence of proteinase) in supernatant fractions. Different symbols represent experiments performed on different dates.

tions resulted in lower recoveries of autolysin activity. Increased amounts of protein extracted from the walls at NaOH concentrations higher than 0.01 N (data not shown) and decreased amounts of enzyme activity were reflected by a decrease in specific activity of extracted autolysin (Fig. 1A). A similar relationship between NaOH concentration and release and recovery of autolysin activity was noted for LOG walls (Fig. 1B). Similarly, extraction of autolysin from LOG walls was optimum at 0.008 to 0.01 N KOH (data not shown). In earlier experiments, 90 to 95% of the autolytic enzyme activity recovered was in the latent form and required proteinase activation for expression. Apparently, the more labile active form (13) was more easily inactivated by the alkali and was less easily recovered. Reduced recovery of activity at NaOH concentrations higher than 0.01 N is consistent with the reported lability of this activity (13), which was also noted when NaOH extractions were, for various reasons, prolonged or performed at temperatures above 0°C. In contrast, enzyme activity was reasonably stable at acidic pH (data not shown).

Effect of wall concentration on extraction of autolysin activity. Increased amounts of autolysin activity were removed and recovered from wall-enzyme complexes when the initial concentration of the walls was increased (Fig. 2A). The major limiting factor was the technical problem of cleanly separating wall sediments from the supernatant fluid within the 10-min centrifugation period, especially at wall concentrations higher than 40 mg/ml. The specific activity of the extracted enzyme also increased with increasing wall concentration (Fig. 2B). NaOH extracts of LOG walls yielded approximately twice the level of enzyme activity compared with STAT walls (Fig. 2A), but the specific activity of the STAT wall extracts was about 1.5 times that of LOG walls (Fig. 2B). Apparently, NaOH extracted more nonenzyme protein from LOG walls than from STAT walls. Because of the technical limitation mentioned above, concentrations of about 20 mg of walls per ml were deemed the most appropriate for effective autolysin removal and recovery.

Along with autolysin activity and protein, 0.01 N NaOH also removed relatively small amounts of methylpentose (presumably rhamnose) and phosphorus from LOG walls (Fig. 2C). Although the amounts of methylpentose and phosphorus extracted increased with increasing wall concentration in a manner similar to that observed for autolysin activity (Fig. 2C), two sets of observations suggest that the cold-alkali treatment is not hydrolyzing covalent bonds in the wall. First,

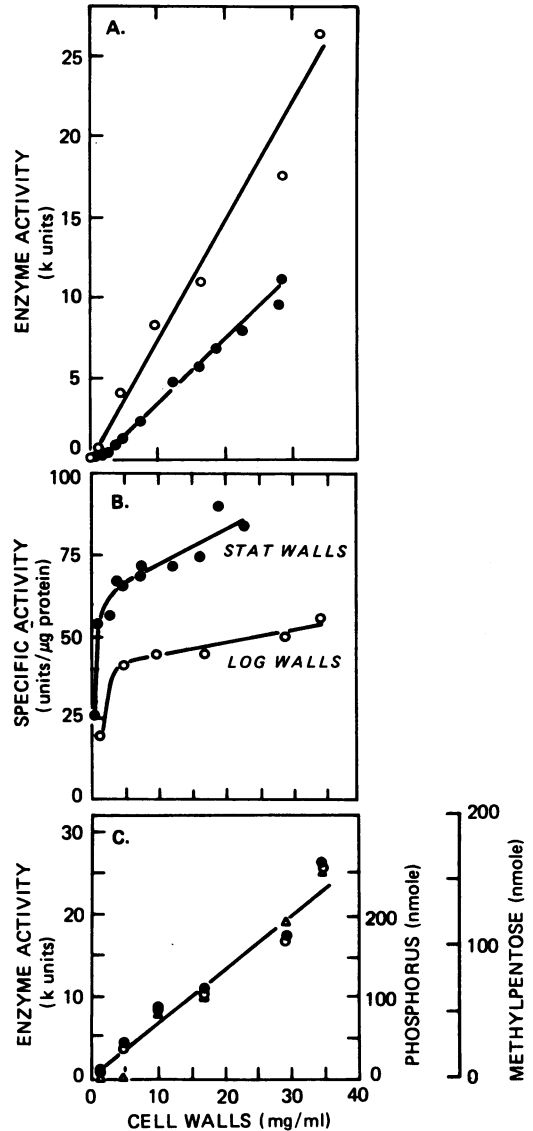


FIG. 2. Release of autolytic enzyme activity from isolated walls of LOG (open symbols) and STAT (closed symbols) *S. faecium* with 0.01 N NaOH (0°C, 10 min) at increasing wall densities. (A) Total autolytic activity released to the supernatant fraction; (B) specific activity of the autolytic enzyme released to supernatant fractions shown in (A); (C) release of methylpentose (Δ), phosphorus (\circ), and autolytic enzyme (\bullet) to the supernatant fraction from LOG walls.

the amounts of methylpentose, phosphorus, and carbohydrate released are about 0.8, 1.8, and 1.3%, respectively, of the total amounts of these two substances present in LOG walls (11). Second, the parallel extractions of the same wall preparations with 4.5 M LiCl removed compa-

able amounts of methylpentose, phosphorus, and carbohydrate (0.9, 2.3, and 2.1%, respectively).

Extraction of autolysin activity from intact cells. NaOH (0.01 N) was also able to remove autolysin activity from intact, exponential-phase cells (Fig. 3). At relatively low cell concentrations, extraction of enzyme activity was approximately as efficient as it was from comparable amounts of walls. At higher cell concentrations, extraction was less efficient, perhaps because of the consumption of hydroxyl ions by the excess of cellular substance. Again, nearly all autolysin activity recovered in the supernatant fluid was in the latent form and required proteinase activation for expression. Specific activities of the NaOH extracts of intact cells were about 10 to 15% of those of similar extracts of comparable amounts of cell walls, presumably due to the concomitant extraction of cytoplasmic proteins from cells.

Comparison of autolysin activity extracted from walls with 0.01 N NaOH with autolysin activity released by autolytic wall dissolution. In the initial series of experiments, 5 to 10% of the total enzyme activity of 0.008 to 0.01 N NaOH extracts was in the active form compared with 20 to 25% usually found in wall autolysates (14). However, in later experiments, more rapid manipulations resulted in relatively good recoveries of the active form in NaOH and LiCl extracts (Table 1). The levels of total autolytic enzyme solubilized by several procedures were compared by assaying the rate with which the soluble enzyme preparations hydrolyzed a standard sodium dodecyl sulfate-inactivated wall substrate. By this criterion, the amounts of total autolytic enzyme solubilized by self-digestion, 0.008 N NaOH, and two successive extractions with 4.5 M LiCl were 348, 311, and 98 U/mg of wall, respectively. If one assumes that the rate with which the native wall-enzyme complexes undergo self-hydrolysis in the presence of trypsin represents total endogenous au-

tolytic enzyme (383 U), then these preparative procedures yielded efficiencies of 91, 81, and 25% of total enzyme solubilized for self-hydrolysis or extraction with 0.008 N NaOH and 4.5 M LiCl, respectively. These values are at best approximate since the initial autolytic enzyme level was determined as the observed rate of dissolution of native cell wall substrate by intrinsically bound, trypsin-activated enzyme, whereas activity of the soluble enzyme preparations was measured by their ability to hydrolyze sodium dodecyl sulfate-inactivated cell wall substrate. Small differences in the kinetics of wall dissolution between these two sets of conditions have been noted in the past (unpublished data).

Stabilization of soluble autolysin. In an attempt to stabilize soluble autolysin by the addition of BPA, we noted that BPA converted latent autolysin to the active form (Table 2). This activation of latent autolysin appeared to be due to an undefined proteolytic activity present in commercial preparations of BPA (17).

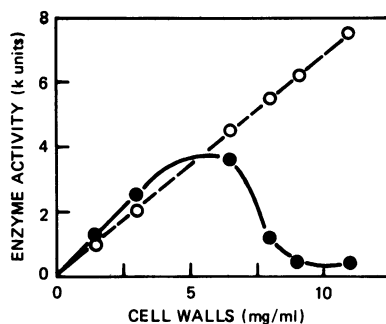


FIG. 3. Release of autolytic enzyme activity with 0.01 N NaOH from various densities of LOG cells (●) or LOG walls (○) of *S. faecium*. The dry weight of cells was determined from the adjusted optical density of the cell suspension (adjusted according to Beer's Law), where the adjusted optical density $\times 0.39 =$ micrograms (dry weight) of cells (16) and, for comparative purposes, is expressed in terms of dry weight of cell walls ($0.25 \times$ cellular dry weight), as determined previously (12).

TABLE 1. Comparison of autolysin recovered from 0.01 N NaOH extraction with that released from autolytic wall dissolution of LOG walls

Method of prepn ^a	Enzyme activity (U/mg of wall)			Sp act (U/ μ g of protein)	Ratio of active to total
	Active	Latent	Total		
Present in walls	102	281	383	5.2	0.27
Self-digestion plus trypsin	348	NA ^b	348	9	NA
NaOH (0.008 N) extraction	88	223	311	11.3	0.28
LiCl (4.5 M) extraction 1 ^c	25	73	98	12.3	0.26
LiCl (4.5 M) extraction 2 ^c	14	23	37	2.6	0.38

^a Soluble autolytic enzyme fractions were prepared, as described in the text, with trypsin present at 0.6 μ g/mg of wall where indicated. The resulting autolysin preparations were assayed as described in the text.

^b NA, Not applicable.

^c Results of two successive extractions of the same walls.

Evidence consistent with the proteinase nature of the activation observed with BPA was the inhibition of its activation (and that of trypsin) by the serine proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) and its rather moderate susceptibility to heat inactivation (Table 3). The low inhibitory activity of PMSF was probably due to its addition directly to the assay system without preincubation with the proteinase. Autolysin activity extracted from walls with 0.01 N NaOH was rapidly inactivated at 50°C (Fig. 4) in a manner similar to that previously reported for autolysin in wall lysates (2). However, heat inactivation was considerably reduced in the presence of high concentrations of glycerol (Fig. 4).

DISCUSSION

Extraction of isolated, native cell walls (or intact cells) with 0.008 to 0.01 N NaOH resulted

TABLE 2. Activation of *S. faecium* autolytic enzyme with trypsin or BPA

Prepn of autolysin ^a	Autolysin activity ^b (U)		
	+ Water	+ Trypsin	+ BPA
Bovine serum albumin (100 µg/ml)	29	112	111
Trypsin (0.2 µg/ml)	110	124	124
BPA (100 µg/ml)	122	118	115

^a Soluble autolytic enzyme was prepared by self-digestion, as described in the text. A cell wall suspension in 0.01 M sodium phosphate, pH 7, was divided into three equal portions (2.0 ml), each of which received 40 µl of the indicated additions before self-digestion.

^b The activity in 0.2 ml of soluble autolysin was assayed, as described in the text, in the presence of trypsin (0.26 µg/ml) or BPA (130 µg/ml), as indicated.

TABLE 3. Inhibition of *S. faecium* autolysin activation

Assay conditions ^a	Enzyme activity (U)	Relative activity
No additions	52	(1.0)
Trypsin (0.5 µg/ml)	636	12
BPA (50 µg/ml)	726	14
BPA (100°C, 5 min) ^b	248	4.8
BPA (100°C, 70 min)	114	2.2
BPA + 1 mM PMSF ^c	426	8.2
BPA + 10 mM PMSF ^d	120	2.3
BPA + isopropanol ^e	705	14
Trypsin + 10 mM PMSF ^d	370	7.1

^a 100-µl portions of alkali-extracted *S. faecium* autolysin were assayed, as described in the text, with the indicated additions.

^b BPA was brought to 100°C for 5 or 70 min before addition to the assay.

^c PMSF dissolved in isopropanol.

^d PMSF at 10 mM was partially insoluble in the aqueous assay and represents saturating conditions.

^e A volume of isopropanol equivalent to the 10 mM PMSF assay was added.

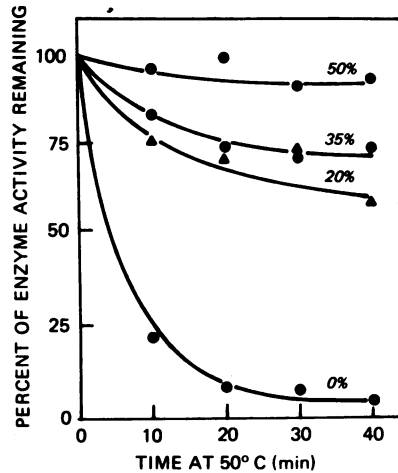


FIG. 4. Thermal inactivation of autolytic enzyme in the presence of glycerol. Alkali-extracted and -neutralized autolysin was mixed with the indicated concentrations of glycerol (vol/vol) and incubated at 50°C. At the times indicated, portions of enzyme were removed to chilled suspensions of sodium dodecyl sulfate-treated walls, and total autolysin activity was subsequently assayed at 37°C, as described in the text.

in the release of wall-bound autolytic enzyme activity. Although the alkaline conditions used were capable of inactivating the autolysin, rapid extraction at 0°C followed by rapid neutralization succeeded in providing extracts of relatively high specific activity. Neither NaOH nor LiCl extractions are selective for the autolysin protein. Other proteins are present in these extracts (unpublished data). The low protein content of these isolated cell walls (e.g., 7.4% in the walls used in the experiment reported in Table 1) results in a reasonable specific activity in wall autolysates (Table 1). However, in view of the relatively large amounts of soluble peptidoglycan fragments and non-peptidoglycan wall polymers present in wall autolysates, either extraction procedure has major advantages over wall autolysis as a method for enzyme solubilization. In contrast to extraction with high salt concentrations (4.5 M LiCl [10]), NaOH extraction was successfully used with dense wall suspensions; the only limitation was the technical problem of completely sedimenting the walls in dense suspensions (greater than 40 mg/ml).

Concurrent extraction of methylpentose- and phosphorus-containing materials raised the possibility that enzyme removal by dilute alkali at low temperatures was due to hydrolysis of covalent linkages in the walls which also released the rhamnose- and phosphorus-containing polymers present in the wall of *S. faecium* (7, 13). Wall teichoic acids have been extracted from

species of *Bacillus* by 0.1 N NaOH for 1 to 10 h at 35°C (6), and a close association of the *N*-acetylmuramyl-L-alanine amidase activity of *Bacillus subtilis* with wall teichoic acid(s) has been postulated (1). Hydrolysis of covalent linkages by the dilute alkali treatment used here seems unlikely since extraction of walls with 4.5 M LiCl at 0°C released similar amounts of methylpentose, phosphorus, and carbohydrate, and the amounts released by either method of extraction were extremely small compared with the total amounts of these materials present in walls. A more likely possibility is that these extraction procedures are removing small amounts of not yet fully assembled precursors which are not covalently linked to the walls. Furthermore, extraction of enzyme activity appeared to depend on the concentration of NaOH used (Fig. 1) rather than on the number of OH⁻ ions present per unit of wall-enzyme complex (Fig. 2A).

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