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Characterization of Autolysins from *Mycobacterium smegmatis*

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This study demonstrates, for the first time, the autolytic enzymes associated with mycobacterial cell walls. Based on the release of radioactivity and ninhydrin-reactive material from isolated cell walls, it was shown that maximum activity occurs during the late log phase of growth and at a buffer pH of about 8.0. Chemical analyses of autolytic digests of isolated cell walls indicated that at least three autolysins are active under the conditions used. These are *N*-glycolylmuramic acid-L-alanine amidase, an aminopeptidase that releases L-alanine, and an endopeptidase that solubilizes and L-alanyl-D-glutamic acid dipeptide. No other endopeptidase, carboxypeptidase, or glycosidase activity was detected.

Many bacteria have been shown to contain one or more cell wall-lytic enzymes. These enzymes, called autolysins, have been placed into three classes with respect to their activity. Glycosidases act on the strands of glycan in murein. The amide bond between L-alanine and *N*-acetylmuramic acid is cleaved by an amidase, and endopeptidases hydrolyze at various points in the peptides of cell walls (4).

Despite considerable descriptive information on autolysins from numerous bacteria (4), their physiological function remains conjectural. Most postulations concerning potential roles for various autolysins are related to cell wall growth, cell division, and cell separation (6). An additional consequence of autolytic enzyme activity may occur in species of the genus *Mycobacterium*, however.

Wax D preparations from *M. smegmatis* consist of mycolic acid linked to an arabinogalactan, whereas those from *M. tuberculosis* and *M. kansasii* are mycolates of an arabinogalactan linked to fragments of peptidoglycan (7). It was suggested by Lederer (7) that autolysin activity could control the nature of these wall-associated oligomers. The absence of peptidoglycan from the wax D of *M. smegmatis*, for example, could reflect autolysin cleavage of this material after synthesis. In view of the interesting and potentially important immunological adjuvant activity associated with wax D, this study was undertaken to characterize the autolysins of *M. smegmatis* so that their potential relationship to wax D composition can be determined.

MATERIALS AND METHODS

Organism. The organism used in this study, *M. smegmatis* derived from ATCC 607, was obtained from the culture collection of the Mycobacteriology Branch, Center for Disease Control, Atlanta, Ga.

Culture conditions. *M. smegmatis* was grown in 7H-9 broth (Difco Laboratories, Detroit, Mich.) supplemented only with 0.2% glycerol and 0.05% Tween 80 (Atlas Powder Co., Wilmington, Del.). The 7H-9 albumin-dextrose enrichment was not used.

A sufficient quantity of cells to prepare cell walls was obtained by inoculating the organisms from a seed flask (absorbancy at 620 nm [A_{620}] of 0.8) into 15 1-liter flasks containing 250 ml of medium and grown in a New Brunswick shaker at 37°C. The cells were harvested by centrifugation when the growth reached an A_{620} of 0.6 (unless otherwise stated) and washed three times in cold 0.01 M potassium phosphate buffer (pH 7.0).

Cell wall isolation. Washed cells were suspended in enough cold 0.01 M potassium phosphate buffer (pH 7.0) to make a thick slurry (about 3 g of cells per 10 ml of buffer). An equal volume of glass beads (0.17-mm diameter) was added, and breakage was accomplished with a Braun cell homogenizer. The cell suspensions were subjected to six 1.5-min burst cycles with intermittent cooling in a dry ice-acetone bath. Unbroken cells and the beads were removed by centrifugation at 3,000 × *g* for 20 min, and the cell walls were isolated by centrifugation of the supernatant solution at 30,000 × *g* for 20 min. Microscope examination at this stage confirmed that the pellet contained fewer than 5% intact cells. The walls were then washed four times in cold 0.01 M potassium phosphate buffer (pH 7.0).

Tritium-labeled cell walls were prepared by this same procedure by adding 0.125 mCi of [^{3}H]diaminopimelic acid (300 mCi/mmol) and 4.4 M L-

lysine to the 7H-9 growth medium. This procedure was shown by others (3) to preferentially direct diaminopimelic acid into cell walls rather than protein. This was confirmed with *M. smegmatis* in this study.

Solubilization of cell wall autolysins. *M. smegmatis* cell walls (1.0 g [wet weight]) were added to 10.0 ml of cold 5 M LiCl and stirred for 15 min. The suspension was centrifuged at 30,000 × *g* for 20 min, and the supernatant solution was dialzyed overnight at 4°C against two 5-liter changes of 0.01 M potassium phosphate buffer (pH 7.8). The extracted protein was assayed by the method of Lowry et al. (9).

Autolysis measurements. Several different procedures were employed to detect and quantitatively measure the lysis of *M. smegmatis* cell walls. (i) Cell walls were suspended in 0.01 M potassium phosphate buffer (pH 7.8) to an A_{540} of 1.0 and incubated at 37°C with or without agitation, and the absorbance (after resuspension) was read periodically with a Gilford model 240 spectrophotometer. (ii) Cell wall suspensions were incubated at 37°C in the buffer described above, and samples were withdrawn periodically and rapidly frozen in an acetone-dry ice bath. These samples were later thawed at room temperature and centrifuged at 30,000 × *g* for 20 min. The supernatant solutions containing soluble wall fragments were then analyzed for ninhydrin-reactive material (14) or reducing groups (11). (iii) Radioactively labeled cell walls were treated as in method ii, and the solubilized radioactivity was assayed, using 0.5-ml samples and 10 ml of Aquafluor (New England Nuclear Corp., Boston, Mass.). Each sample was counted in a Nuclear-Chicago Isocap/300 liquid scintillation counter.

Isolation of soluble wall fragments. Isolated cell walls were suspended in 0.01 M potassium phosphate buffer (pH 7.8), the optical density was adjusted to approximately 1.0 (A_{540}), and the mixture was incubated at 37°C for 8 h. After centrifugation at 30,000 × *g* for 20 min, the supernatant solution was extracted for 90 min (with stirring) at room temperature with chloroform-methanol (1:1, vol/vol). After low-speed centrifugation (30 × *g* for 5 min) the upper, aqueous-methanol phase was removed and concentrated to about 1.0 ml by flash evaporation at 40°C.

Chemical characterization of solubilized cell wall material. Samples of soluble wall fragments, prepared as described above, were applied as a single band to prewashed Whatman 3MM filter paper and subjected to high-voltage electrophoresis with a Savant flat-plate electrophoresis apparatus. The buffers used were prepared by the method of Best and Mattingly (2) and consisted of the following: pyridine-acetic acid-water (11:8:981, vol/vol/vol) at pH 5.0 and acetic acid-formic acid-water (94:42.5:863.5, vol/vol/vol) at pH 2.0. Both buffer systems were run at 45 V/cm (2.2 mA/cm). After electrophoresis, 1-inch (2.54-cm) guide strips were cut and analyzed for areas of radioactivity with a Packard radiochromatogram scanner, model 7201. Subsequently, the guide strips were developed with ninhydrin. Material to be analyzed further was eluted with water and concentrated by lyophilization.

Analytical methods. Amino acids were assayed by a program described previously (2), using a Beckman model 120 B automatic amino acid analyzer. Hexosamines were assayed by the procedure of Morgan and Elson (10). Amino acids were assayed directly or after hyrolysis with redistilled 6 N HCl at 110°C for 18 h, and amino sugars were measured after hydrolysis with 2 N HCl for 4 h. The HCl was always removed by evaporation and desiccation.

The determination of D-alanine was based on the procedure of Ghuysen et al. (5), whereby D-alanine is oxidatively deaminated to pyruvate by D-amino acid oxidase, and the pyruvate is reduced by lactate dehydrogenase in the presence of excess reduced nicotinamide adenine dinucleotide (NADH). The absorbance was read on an Aminco Bowman spectrophotofluorometer with the sensitivity adjusted to give maximum deflection of the galvanometer. The decrease in fluorescence due to the oxidation of NADH to NAD is proportional to the amount of D-alanine originally present.

Chemicals. Tritiated diaminopimelic acid was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Lactate dehydrogenase, NADH, D-amino acid oxidase, and amino acids were purchased from Sigma Chemical Co., St. Louis, Mo. The dehydrated media were purchased from Difco Laboratories, Detroit, Mich.

RESULTS AND DISCUSSION

Even though autolytic enzymes have not been characterized in the mycobacteria, preliminary experiments suggested their presence. For example, aging cultures slowly lose turbidity, and the addition of cell wall antibiotics such as D-cycloserine to growing cultures produces a fairly rapid lysis. In contrast to the behavior of isolated cell walls of other organisms, however, those from *M. smegmatis* exhibited little or no absorbance change during an 8-h incubation at 37°C in 0.01 M potassium phosphate buffer (pH 7.8). There was a release or solubilization of radioactivity from isolated cell walls labeled with [^{3}H]diaminopimelic acid (Fig. 1). In addition, it was observed that the rate of release of this radioactivity closely parallels the release of soluble, ninhydrin-reactive material from *M. smegmatis* cell walls (Fig. 2). As indicated in this graph, no detectable reducing groups were solubilized during the 8-h incubation.

Additional experiments with isolated cell walls of this organism indicated that autolysin activity was most prevalent when the walls were obtained from late-exponential-phase cells (Fig. 3a), and the pH optimum for this activity was about 8.0 (Fig. 3b).

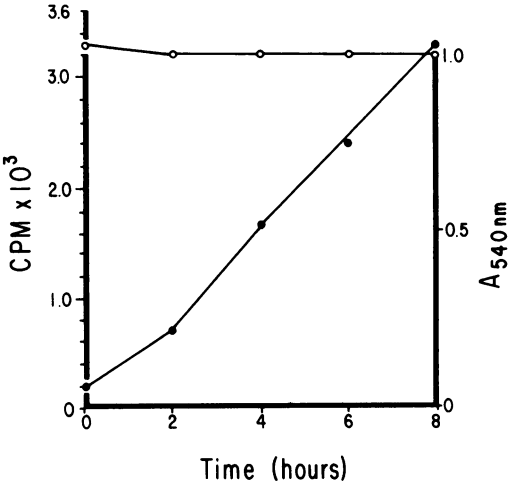

FIG. 1. *Cell wall absorbance and release of soluble cell wall fragments as a function of time. Cell walls were prepared from a culture grown in the presence of [³H]diaminopimelic acid. The walls were suspended in 0.01 M potassium phosphate buffer (pH 7.8) and incubated at 37°C. A_{540} (○) was determined, and 1.0-ml samples were removed, centrifuged, and assayed for soluble radioactivity (●).*

Solubilization of *M. smegmatis* cell wall autolysins with lithium chloride. Pooley et al. (13) showed with *Streptococcus faecalis* that high salt concentrations would extract wall-associated autolysin. *M. smegmatis* cell walls were prepared and extracted as described above. The autolytic enzyme activity of the concentrated LiCl-extracted protein was determined by adding the extract back to *M. smegmatis* walls that had been extracted with LiCl and boiled for 10 min in distilled water to inactivate autolysin remaining after salt extraction. Quantitatively more ninhydrin-reactive material was released by the native LiCl extract than by the boiled control (Fig. 4). Samples taken at time zero were subtracted from all subsequent readings to eliminate the contribution of the added extract. The specific activity of the nonboiled, LiCl-extracted enzyme preparation was 0.139 μmol/h per mg of protein, based on a standard ninhydrin curve obtained using L-leucine.

Characterization of soluble fragments released from *M. smegmatis* cell walls. Isolation and chemical characterization of solubilized wall fragments were undertaken to determine the nature of autolysin responsible for the indigenous autolysin activity associated with the *M. smegmatis* cell walls. In initial experiments, the cell wall lysates were subjected to electrophoresis at pH 2.0 and 5.0, but in both instances the radioactivity remained at the origin. Since mycobacterial cell walls contain approximately 60% lipid (by weight), an alternative procedure was devised in which the autolysate was extracted at room temperature with chloroform-methanol (1:1) prior to electrophoresis. After extraction of lipids, the suspension was centrifuged at 30 × *g* for 5 min. Samples from both the water-methanol and chloroform phases indicated that essentially all radioactivity was in the water-methanol layer.

After the lipid was removed, five ninhydrin-positive bands and two radioactive areas, each migrating toward the cathode, were detected upon electrophoresis (Fig. 5). As indicated in this chromatogram, only one area contained both an intense ninhyrin reaction and significant radioactivity. For this reason, only the

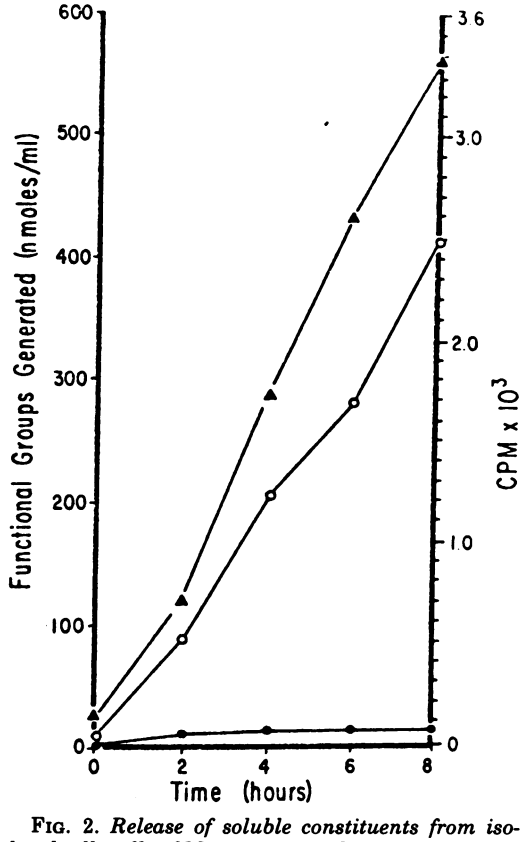

FIG. 2. *Release of soluble constituents from isolated cell walls of M. smegmatis. Cell walls that had been labeled with [³H]diaminopimelic acid were suspended to an A_{540} of 1.0 in 0.01 M potassium phosphate buffer (pH 7.8) and incubated at 37°C. Samples were removed at the times indicated and assayed for radioactivity released (▲), solubilized ninhydrin-reactive material (○), and soluble reducing groups (●).*

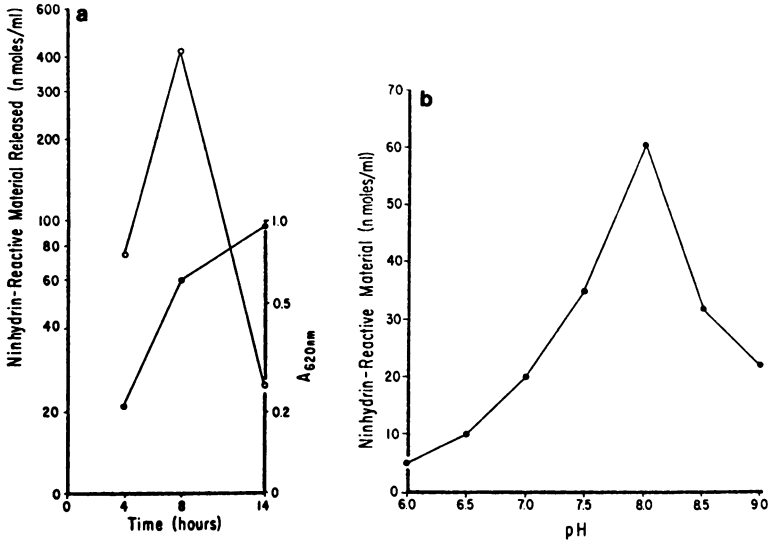

FIG. 3. *(a) Autolysin activity in cell walls from M. smegmatis harvested at different phases of growth. Isolated cell walls were prepared from cells of M. smegmatis at the stages of growth indicated (●). The relative release of ninhydrin-reactive material was compared (○). (b) Effect of pH on cell wall lysis. The buffers used were morpholinopropane sulfonic acid (pH 6.0 to 6.5), potassium phosphate (pH 7.0 to 8.0), and borate-potassium phosphate (pH 8.5 to 9.0). All buffers were 0.01 M. Cell walls were suspended to an A_{540} of 1.0 and incubated for 6 h in the respective buffer prior to the assay.*

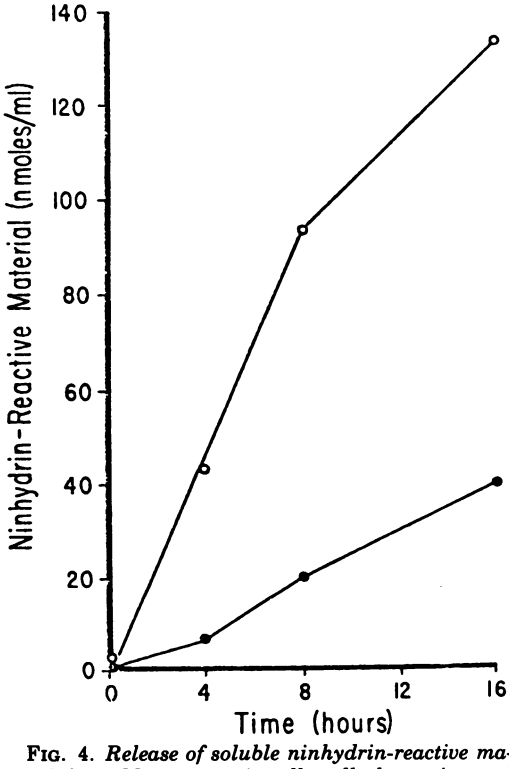

FIG. 4. *Release of soluble ninhydrin-reactive material from M. smegmatis cell walls by native and boiled lithium chloride wall extracts. The reaction mixture contained 0.5 ml of lithium chloride extract (300 μg of protein per ml), boiled extract (●), and nonboiled extract (○). It was added to 4.0 ml of cell walls suspended in 0.01 M potassium phosphate buffer (pH 8.0) and adjusted to an A_{540} of 0.55. The wall substrate had been treated previously with trypsin and boiled for 5 min in distilled water.*

material designated as band 3 was studied further. The water eluate from this band was lyophilized and acid hydrolyzed, and the amino acid content was determined. The analyses showed the presence of alanine, glutamic acid, and diaminopimelic acid in a molar ratio of 5:1:1, respectively. The amount of alanine present, in relation to the other amino acids, was considerably greater than the amount known to be present in wall peptides of this organism. Petit et al. (12) established a molar ratio of 1.6:1:1 in their analyses, for example. Thus, free alanine in band 3 was suspected.

To obtain better resolution of band 3 material, a sample was streaked (adjacent to an L-alanine standard) on prewashed Whatman no. 1 paper and subjected to descending chromatography in butanol-acetic acid-water (120:30:50, vol/vol/vol) for 16 h. The locations of the three ninhydrin-reactive areas are shown in Fig. 6. Each reactive area was eluted with water and concentrated by lyophilization. A portion of each sample was acid hydrolyzed and analyzed

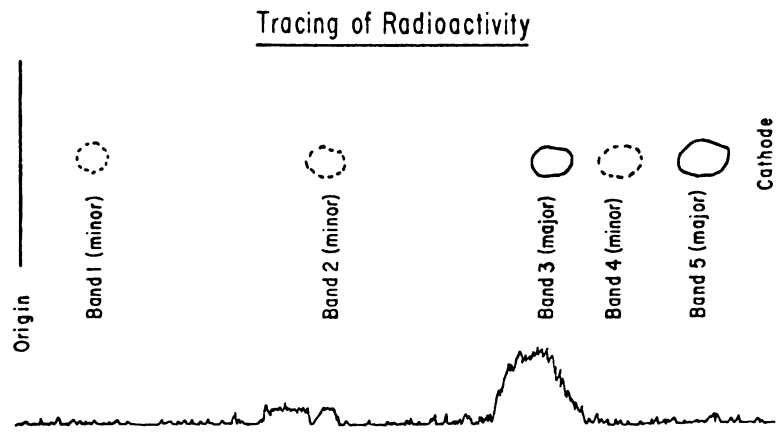

FIG. 5. *Electrophoresis of lipid-extracted cell wall autolysate. M. smegmatis cell walls (2 g) labeled with [^{3}H]diaminopimelic acid were allowed to lyse for 8 h in 0.01 M potassium phosphate buffer (pH 7.8) at 37°C. The suspension was centrifuged at 30,000 × g for 20 min, and the supernatant solution was removed and extracted for 90 min at room temperature in chloroform-methanol (1:1). The water phase was removed and concentrated to 1.0 ml by flash evaporation at 40°C. A 1.0-ml volume was streaked on 3MM paper and subjected to electrophoresis as described in the text. The bands revealed by ninhydrin are indicated by circles, and the staining intensity is characterized as weak (minor) or strong (major).*

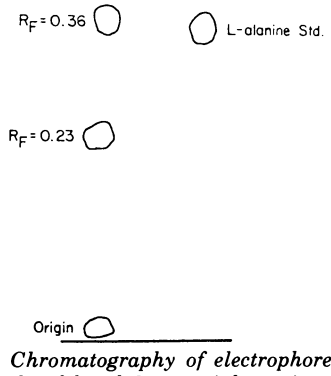

FIG. 6. *Chromatography of electrophoresis band 3. A sample of band 3 material previously eluted from the high-voltage chromatogram (Fig. 5) was streaked on prewashed Whatman no. 1 paper alongside an L-alanine standard. The chromatogram ran for 16 h (descending) in butanol-acetic acid-water (120:30:50, vol/vol/vol) and was developed with ninhydrin.*

TABLE 1. *Chemical characterization of amino acids and peptides released by autolysis of M. smegmatis cell walls*[a]

Band	Location (R_f)	Amino acids	Molar ratio
One	Origin	Ala, Glu, DAP[b]	1.5:1:1
Two	0.23	Ala, Glu	1:1
Three	0.36	Ala	

[a] Data were obtained after resolution of band 3 into three components (bands 1, 2, and 3) by descending chromatography. Each isolated band was eluted and acid hydrolyzed, and the amino acid content and molar ratios were determined by automatic amino acid analysis.

[b] DAP, Diaminopimelic acid.

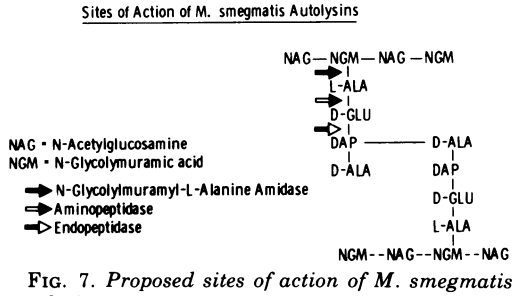

FIG. 7. *Proposed sites of action of M. smegmatis autolysins.*

for amino acid content. Table 1 shows the results of these measurements and confirms the presence of free alanine in band 3 of the autolysis products subjected to electrophoresis.

A configuration analysis of the free alanine established that none of the sample was D-alanine. Therefore, based on the amino acid content, molar ratios, and configuration analysis of the isolated wall fragments, the autolysins responsible for the release of these cell wall constituents are assumed to be an amidase, an amino peptidase, and an endopeptidase. The sites of action of these proposed autolysins on the wall peptide of *M. smegmatis* (7) are indicated by arrows in Fig. 7. Amidase activity (solid arrow) is commonly associated with cell walls (2, 3), but in this organism the enzyme may not be ordinary since the *N*-acyl substituent of muramic acid in mycobacteria is glycolate rather than acetate (1).

Hydrolysis of cell wall peptides at the positions indicated by the other arrows is not usu-

ally observed in autolysin digests. In a similar study with *Bacillus psychrophilus* cell walls, endopeptidase activity (Fig. 7) was detected (except lysine was involved), but no aminopeptidase activity was apparent (2). The absence of D-alanine in the autolytic digests suggests that carboxypeptidase II activity is not present in these cell walls (Table 1).

Additional studies with other strains of mycobacteria will be required to determine whether the autolysins revealed in this study are perhaps responsible for the absence of peptidoglycan in wax D of *M. smegmatis*.

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