Partial characterization of a major autolysin from *Mycobacterium phlei*

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Autolytic enzyme profiles of fast- and slow-growing mycobacteria were examined using SDS-PAGE zymography with incorporated mycobacterial peptidoglycan sacculi as substrate. Each species tested (*Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium aurum*, *Mycobacterium fortuitum* and *Mycobacterium kansasii*) appeared to produce a different set of enzymes on the basis of differing number and molecular masses. A major autolysin from *M. phlei* was purified to apparent homogeneity by DEAE-cellulose chromatography, preparative gel electrophoresis and Mono Q FPLC. This enzyme had an estimated molecular mass of 38 kDa, an isoelectric point of 5.5 and a pH optimum of pH 7.5. Digestion of purified peptidoglycan by the enzyme resulted in the appearance of reducing sugars, suggesting that the 38 kDa autolysin is a β -glycosidase. Partial internal amino acid sequence of the autolysin was determined and should facilitate identification, cloning and overexpression of the encoding gene.

Keywords: *Mycobacterium phlei*, autolysin, β -glycosidase

INTRODUCTION

Mycobacteria have a unique cell-wall structure that is characterized by a large number of lipids with unusual structures, such as mycolic acids with associated short-chain fatty acids and peptides. These wall components are anchored through arabinogalactan to the peptidoglycan layer. For pathogenic species, the cell wall is believed to: be partly responsible for the ability to colonize host cells; stimulate a myriad of immune responses; evade attack by host immune strategies; and promote chronic debilitating diseases (Barclay & Wheeler, 1989). Diverse immune reactions have been attributed to several components of the wall including proteins (Hunter *et al.*, 1989), muramyl dipeptide (Puzo, 1990), sulfolipids (Barclay & Wheeler, 1989; Puzo, 1990), glycolipids, lipooligosaccharides and lipoarabinomannan (Ellner & Daniel, 1979; Moreno *et al.*, 1988). As the backbone of the cell wall, to which all other substances are bound, the peptidoglycan is of

utmost importance. Little is known about cell-wall turnover in mycobacteria, which is a pre-requisite for bacterial growth and division; such turnover must ultimately be controlled by peptidoglycan metabolism (Höltje, 1996; Koch, 1990; Rogers *et al.*, 1980; Shockman *et al.*, 1996; Tomasz, 1984).

During growth and division, modification of the bacterial cell wall requires coordinated functions of peptidoglycan hydrolases and peptidoglycan synthases, including the penicillin-binding proteins (PBPs). The PBPs and peptidoglycan hydrolases were recently proposed to form a multi-enzyme complex in *Escherichia coli* to expand the existent structure of its murein sacculus (Höltje, 1996, 1998). Lytic peptidoglycan hydrolases can be described as autolysins since their uncontrolled action can result in the breakdown of the structural integrity and protective properties of the wall. These autolysins, like all enzymes, have discrete specificity and comprise three major classes: β -glycosidases (β -N-acetylmuramidase and β -N-acetylglucosaminidase), N-acetylmuramoyl-L-alanine amidase and D,D-endopeptidases, which hydrolyse peptide cross-bridges (reviewed by Shockman & Höltje, 1994; Höltje, 1998). In view of their potentially lethal action, the tightly controlled function of the autolysins seems to be an essential role in the metabolism of the murein sacculus (Koch, 1990;

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Rogers *et al.*, 1980; Shockman *et al.*, 1996; Tomasz, 1984).

Our long-term goal is to understand how autolysins are involved in cell-wall turnover in mycobacteria, and how the turnover products (which are most likely a cascade of immunodominant substances) interact with hosts to induce, for example, immunogenic and pathogenic responses. Although autolysis in mycobacteria was observed over two decades ago (Kilburn & Best, 1977), to date, the individual autolysins have not been isolated and studied. This is probably due to the low expression level of autolysins and consequent difficulties in detection, especially in slow-growing bacteria. Moreover, the difficulties associated with the *in vitro* growth of mycobacterial cultures has further hindered progress.

The recent development of a zymogram-based method for the detection of autolysins from Gram-negative bacteria (Bernadsky *et al.*, 1994; Watt & Clarke, 1994) has provided a sensitive means to study the mycobacterial enzymes; here, we identify for the first time autolysins from both fast- and slow-growing mycobacterial species, and partially characterize a major autolysin from *Mycobacterium phlei*.

METHODS

Bacteria and growth conditions. *M. phlei*, *M. smegmatis*, *M. aurum*, *M. fortuitum* and *M. kansasii* used in the present study were from our own collection (Paul & Beveridge, 1992, 1994). *M. kansasii* was grown in 7H9 broth (Difco) supplemented (per litre) with 6 egg yolks, 100 ml oleic acid/albumin/dextrose/catalase (OADC; Difco), 100 000 units penicillin G and 0.05 g chloramphenicol (antibiotics are routinely added to prevent the growth of any contaminants during the slow growth of mycobacteria). All other mycobacterial species were grown in brain-heart infusion broth (BBL, Becton Dickinson), supplemented with 0.5% (v/v) glycerol and 0.05% (v/v) Tween 80. Routinely, bacteria were grown to OD₆₀₀ 0.8–1.0 (late-exponential phase) at 37 °C with occasional shaking.

Preparation of peptidoglycan. Peptidoglycan sacculi were isolated from the various species of mycobacteria by the method of Hoyle & Beveridge (1984).

Zymogram analysis. Zymogram analysis of peptidoglycan hydrolases was performed as previously described (Bernadsky *et al.*, 1994; Li *et al.*, 1996; Watt & Clarke, 1994) with isolated peptidoglycan sacculi (1%, w/v) from *M. fortuitum*, *M. kansasii*, *M. phlei* or *M. smegmatis* as substrate in the gels. In some cases, the period of destaining was shortened to facilitate the visualization of minor bands, resulting in different background intensities between gels. For presentation, gels were digitized using a Gel Doc 1000 UV fluorescent gel documentation system (Bio-Rad) as previously described (Watt & Clarke, 1994).

To test the influence of protease inhibitors on the activity of autolysins during zymogram analysis, a cocktail of 10 protease inhibitors (Complete; Boehringer Mannheim) was added to samples prior to electrophoresis. BSA was used as a positive-control substrate.

Purification of the major *M. phlei* autolysin. All steps during

purification were carried out at 4 °C or on ice unless otherwise noted.

Crude cellular extract. Freeze-dried cells (1.5 g) were washed once in Milli-Q water and resuspended in 160 ml 20 mM sodium phosphate buffer, pH 8.0 (extraction buffer). The bacterial suspension was sonicated (Braun, Sonic 2000) for 20 min at 150 W. Most (99%) of the cells were broken after sonication, as visualized by microscopy. To this cell extract, DNase and RNase were added to a final concentration of 50 µg ml⁻¹ each. The extract was kept on ice for 1 h and then subjected to centrifugation at 9000 g for 10 min to separate supernatant (crude cellular extract) from the remaining particulate matter.

DEAE-cellulose chromatography. The extraction buffer was used to pack and equilibrate a 50 × 2.2 cm column of DEAE-cellulose (Sigma). The clarified cell extract was applied at a flow rate of 1 ml min⁻¹ and non-adsorbing materials were washed from the column using the extraction buffer. The autolysin was eluted by applying a linear NaCl gradient (0–0.75 M) in 20 mM sodium phosphate buffer, pH 6.5, and 5 ml fractions were collected. The protein concentration in each fraction was monitored by measuring its absorbance at 280 nm and the NaCl concentration in each fraction was determined as described below. For quick detection of fractions containing active enzyme, 100 µl from each of 10 fractions were combined and freeze-dried for zymogram analysis. Positive combined fractions were analysed further by zymography to precisely locate the enzyme-containing fractions. Individual fractions containing autolytic activity were pooled, freeze-dried, resuspended in a small amount of deionized water and dialysed against 6 l deionized water for 2 d with at least two changes of water during this period.

Preparative gel electrophoresis. This was performed in a 12% polyacrylamide separating gel under non-denaturing (native) conditions using a model 491 Prep Cell (Bio-Rad). The flow rate of the buffer through the elution chamber was set to 1 ml min⁻¹. Fractions of 2.5 ml per tube were collected after the tracking dye had migrated to the elution chamber. The protein concentration in each fraction was monitored by measuring its absorbance at 280 nm while enzymic activity was assessed by the zymogram method described above. Active fractions were pooled, freeze-dried and dialysed against 20 mM sodium phosphate buffer, pH 8.0.

Mono Q anion-exchange FPLC. A Mono Q HR 5/5 anion-exchange column was equilibrated with 10 mM NaCl in 20 mM sodium phosphate buffer, pH 8.0, for at least 2 h at room temperature. Fractions from the preparative gel electrophoresis (above) were applied to the column after the flow rate was set to 1.0 ml min⁻¹. The column was first eluted with the same buffer as above for 10 min and then a linear NaCl gradient (0.01–1.0 M over 70 min) in the same phosphate buffer was applied. Fractions of each peak were collected, freeze-dried and analysed for activity by zymography. The purity of the active fraction was analysed by SDS-PAGE and 2-D gel electrophoresis.

Turbidometric assay of enzymic activity. Autolytic activity of various samples was quantitatively measured by the turbidometric assay as described by Dolinger *et al.* (1989). A standard assay consisted of a sample of autolysin added to 0.5 mg (wet wt) *M. smegmatis* cell walls in 20 mM Tris/HCl buffer, pH 7.0, containing 10 mM MgCl₂, 0.1% (w/v) Triton X-100 and 0.05% (w/v) NaN₃ in a final volume of 1.0 ml. Digestion of cell-wall fragments was monitored by measuring the decrease in OD₅₄₀ of the suspension over time. One enzymic unit was

defined as the amount of activity causing a decrease in OD₅₄₀ of 0.001 after 24 h incubation at 37 °C.

Determination of molecular mass. A Superdex 75 HR 10/30 gel filtration column was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, for 2 h at room temperature. The autolysin sample was applied to the column at a flow rate of 0.3 ml min⁻¹. The column was eluted with the same buffer and the elution volume of the autolysin was recorded. In parallel experiments, blue dextran and protein molecular mass standards (Sigma) were applied to the column to obtain a calibration curve.

pH optimum of the autolysin. The pH optimum of the major autolysin was determined by using the recently developed spot assay (Watt & Clarke, 1997) with modifications. Briefly, molten 4% Noble agar, held at 60 °C, was mixed with peptidoglycan sacculi suspended in buffers of various pHs to give plates containing 0.1% (wet wt; w/v) peptidoglycan. The buffers were: 20 mM sodium citrate/phosphate, pH 4.0–pH 7.0; 20 mM sodium phosphate, pH 6.0–8.0; 20 mM Tris/HCl, pH 7.0–9.0. MgCl₂, Triton X-100 and NaN₃ were added to all buffers as above. Samples (5 µl) of the enzyme fractions were spotted as single drops onto plates and the diameter of clear zones was quantitatively measured after incubation of the plate for 24 h at 37 °C.

Isoelectric point determination. Isoelectric focusing was done in a vertical polyacrylamide mini-gel system (Bio-Rad) according to the method of Robertson *et al.* (1987). The pH gradient of the gels was determined by measuring the pH of each slice cut from one side of the gel after it had been soaked in deionized water, or by comparing the enzyme with protein standards of known pI (IEF standards in the range of 4.8–10.6; Pharmacia).

Amino acid composition analysis. Protein bands were electroblotted from an SDS-PAGE gel onto an Immobilon-P transfer membrane (Millipore) and amino acid composition analysis was performed as previously described (Li *et al.*, 1993).

Internal amino acid sequencing. The protein of interest was excised from a Coomassie blue-stained polyacrylamide gel and digested *in situ* with trypsin. Amino acid sequencing of digested peptides by nano-electrospray mass spectrometry was performed as previously described (Wilm *et al.*, 1996).

Specificity of the autolysin. After digestion of peptidoglycan samples by the autolysin at 37 °C for 24 h in the renaturing buffer, reducing sugar content was measured colorimetrically by the method of Lever (1972), while the appearance of free amino groups was assayed by the method of Ghuyssen *et al.* (1966).

Analytical techniques. The NaCl concentrations in the eluate from DEAE-cellulose chromatography were determined by measuring the conductivity of a sample with a series of solutions of known NaCl concentrations as standards. Protein concentrations were estimated by either UV-absorbance spectroscopy at 280 nm or the Bio-Rad Protein Assay Kit with BSA as the standard.

RESULTS AND DISCUSSION

Autolytic enzyme profiles

The autolytic enzyme profiles of fast- and slow-growing mycobacteria were examined by zymogram analysis with peptidoglycan purified from the various myco-

Fig. 1. Autolysin profiles of mycobacteria by zymography using 12.5% polyacrylamide gels containing 1% (wet wt) peptidoglycan sacculi from *M. smegmatis* as substrate. (a) Analysis of extracts from *M. phlei*. Lane 1, SDS-PAGE with Coomassie blue staining of crude cellular extract; lane 2, zymogram analysis of crude cellular extract. (b) Zymogram profiles of crude extracts from *M. kansasii* (lane 1), *M. smegmatis* (lane 2), *M. aurum* (lane 3) and *M. fortuitum* (lane 4). Arrows denote the poorly detected bands. Positions of prestained low-molecular-mass markers (in kDa) are indicated on the left of each panel.

bacterial species as substrate. The typical lytic profile of autolysins from *M. phlei* in a zymogram gel with peptidoglycan sacculi from *M. smegmatis* as substrate is shown in Fig. 1 (a), lane 2, and those of *M. kansasii*, *M. smegmatis*, *M. aurum* and *M. fortuitum* are shown in Fig. 1 (b). The approximate molecular masses of the autolysins in these mycobacteria are: *M. phlei*, 38 kDa (major) and 33 kDa (Fig. 1a, lane 2); *M. kansasii*, 64 kDa (major) and 61 kDa (minor, arrowhead, only clearly seen on the zymogram gel) (Fig. 1b, lane 1); *M. smegmatis*, 34 kDa (minor, arrowed) and 31 kDa (major) (Fig. 1b, lane 2); *M. aurum*, 16 kDa (major), 95 kDa and 104 kDa (minor) (Fig. 1b, lane 3); *M. fortuitum*, 38 (arrowed), 42, 45, 73 (arrowed) and 76 kDa (Fig. 1b, lane 4). Virtually identical enzyme profiles of these mycobacteria were observed when peptidoglycan sacculi from either *M. phlei*, *M. fortuitum*, or *M. kansasii* were used as substrates in the zymogram analysis. Of the autolysins observed in the zymograms, the 38 kDa enzyme of *M. phlei* appeared to have the highest activity based on the size of the clearing zone and, because of this, it was the one that we chose to purify and characterize. Despite its apparent strong activity in the zymogram gel, staining with either Coomassie blue (Fig. 1a, lane 1; arrowed) or silver nitrate suggested that the 38 kDa autolysin was a minor cellular protein.

Autolytic activity has previously been detected in *M. smegmatis* by chemical analysis of autolytic digests of isolated cell walls (Kilburn & Best, 1977). The detected activities included *N*-glycoylmuramoyl-L-alanine amidase, aminopeptidase and endopeptidase activities. However, no particular protein(s) associated with these enzyme activities was thereafter identified or purified.

Table 1. Purification of the 38 kDa autolysin from *M. phlei* from 1 l of cell culture grown to late exponential phase

Purification step	Total vol. (ml)	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Recovery (%)	Purification factor
Crude cell extract	160	1230	7800	6.4	100	1
DEAE-cellulose	20	40	3300	82	42	13
Preparative gel electrophoresis	5	3	875	292	11	46
Mono Q anion exchange FPLC	2	0.32	408	1275	5.2	199

To our knowledge, no other investigations have been reported on mycobacterial autolysins. Our results indicated that all tested strains of mycobacteria possess two or more autolytic enzymes.

The observation that autolysins in one species of mycobacteria could act on peptidoglycan of different species suggests common structural features among the cell walls of these species. These mycobacteria are generally thought to possess A1 γ peptidoglycan containing the characteristic D-alanyl-*meso*-diaminopimelic acid interpeptide linkage (Schleifer & Kandler, 1972), but an unusual *meso*-diaminopimelyl-*meso*-diaminopimelic acid cross-linkage has also been detected in both *M. smegmatis* and *M. tuberculosis* (Wietzerbin *et al.*, 1974). There have been no reports suggesting that the glycan moiety is O-acetylated (Clarke & Dupont, 1992). Thus, the overall structure of peptidoglycan in mycobacteria, even though complexed with a variety of additional unusual secondary constituents, seems comparable to that of several Gram-positive (e.g. *Bacillus subtilis*) and most Gram-negative bacteria. These bacteria also have A1 γ peptidoglycan and they express their individual complement of autolysins (Bernadsky *et al.*, 1994; Foster, 1992; Goodell & Schwartz, 1977; Hölftje, 1996; Schwartz *et al.*, 1969; Watt & Clarke, 1994). Clearly, mycobacterial autolysins from one species are capable of degrading the cell walls from other mycobacteria and it is not unusual for an autolysin to have rather broad activity. For example, those isolated from *Pseudomonas aeruginosa* have been shown to hydrolyse the A1 γ peptidoglycan from different genera and species of Gram-negative bacteria (Watt & Clarke, 1997). However, the presence of either O-acetyl substituents on the peptidoglycan of *Proteus mirabilis* or teichoic acids in Gram-positive bacteria prevented hydrolysis by the *P. aeruginosa* 26 kDa autolysin, suggesting that this and other enzymes from different Gram-negative bacteria may not act on highly substituted A1 γ peptidoglycan such as that produced by the mycobacteria.

Purification of the *M. phlei* 38 kDa enzyme

Purification of the 38 kDa autolysin to apparent homogeneity was achieved by a four-step procedure summarized in Table 1 and Fig. 2. Most of the enzyme

Fig. 2. SDS-PAGE analysis of samples obtained at various steps of purification of *M. phlei* 38 kDa autolysin. Lane 1, crude cellular extract; lane 2, DEAE-cellulose eluate containing autolysin; lane 3, preparative gel electrophoresis eluate containing autolysin; lane 4, fraction 10 from the Mono Q column. Material loaded into lanes 1, 2 and 3 is from the same number of cells, but 5-fold more material was loaded into lane 4. The arrow shows the position of the 38 kDa autolysin. Positions of prestained low-molecular-mass markers (in kDa) are indicated on the left.

activity present in the cells was liberated by ultrasonication in dilute buffer (Fig. 1a, lane 2) suggesting a surface (i.e. cell wall) location for the enzyme; only trace activity was detected in the remaining cell debris. An extraction of the cell sonicate with 5 M LiCl recovered a similar amount of autolytic activity but given the added need of a dialysis step to remove the salt, this extraction protocol was not used for routine purifications. After binding the autolysin to DEAE-cellulose, lipids and unadsorbed proteins in the supernatant were readily removed. Among many proteins bound to the DEAE-cellulose only a small proportion (fractions 24–59), including the 38 kDa autolysin, eluted with 0.03–0.1 M NaCl (see Fig. 3b for zymographic analysis of fractions 11–70). The majority of other bound proteins were

Fig. 3. Chromatography of *M. phlei* extract on DEAE-cellulose. (a) Elution profile of proteins as detected by absorbance at 280 nm (solid line) using a linear NaCl gradient (dotted line) in 20 mM sodium phosphate buffer, pH 6.5. (b) Zymogram analysis of fractions 11–70 of eluate (20 µg total protein of each fraction). The four combined fractions from 21 to 60 contained the enzyme activity. Position of a prestained low-molecular-mass marker (in kDa) is indicated on the left.

eluted with NaCl concentrations higher than 0.1 M (Fig. 3a).

Native preparative gel electrophoresis was then applied to the partially purified autolysin. Although many contaminating proteins were removed, resulting in a 46-fold purification of the enzyme, considerable enzyme activity was lost during this step (Table 1). The protein concentration in each fraction was too low to be monitored, but active fractions were easily detected by zymogram analysis. Eventual purification of the enzyme was achieved by Mono Q anion-exchange chromatography of these active fractions from preparative gel electrophoresis. A typical protein elution profile is shown in Fig. 4(a). The enzyme eluted as a single peak in 0.23 M NaCl (Fig. 4a, fraction 10) and its autolytic activity was clearly seen after zymogram analysis (Fig. 4b).

To our knowledge, this represents the first report of the isolation and purification of a mycobacterial autolysin. Although we were successful in obtaining the purified protein, the purification protocol has limited applications because of the apparent minor quantity of total autolysin expressed in the cells and the difficulty of its recovery (Fig. 1a, lane 1; Table 1). For this reason, in future experiments we will attempt to isolate the encoding gene and overexpress it in a suitable host system.

Fig. 4. Purification of the 38 kDa autolysin by Mono Q anion-exchange chromatography. (a) The enzyme was applied to the column in 20 mM sodium phosphate buffer, pH 8, at 1.0 ml min⁻¹ and eluted with a linear NaCl gradient (0.01–1.0 M) over 70 min. The arrow indicates the fraction containing autolytic activity. (b) Zymogram analysis of fractions 7–13 obtained from Mono Q chromatography (3 µg total protein of each fraction). The position of a prestained low molecular mass marker (in kDa) is indicated on the left.

Properties of the 38 kDa autolysin

Purity of the enzyme was demonstrated by SDS-PAGE, which showed a single band after staining with either Coomassie blue (Fig. 2, lane 4) or silver nitrate (data not shown). Furthermore, the enzyme preparation migrated as a single spot during 2-D gel electrophoresis (data not shown). The enzyme had an apparent molecular mass of 38 kDa by comparison with calibrated SDS-PAGE standards (Fig. 2, lane 4). This compared closely with a molecular mass of 34 kDa derived using Superdex 75 HR 10/30 gel filtration. In addition, gel filtration indicated that the native 38 kDa enzyme was a monomer.

Both prior to and after purification of the protein, several control experiments were performed to exclude the possibility that the clear zones observed on our zymograms were due to either protease activity or non-specific displacement of dye during the staining process, as noted previously (Watt & Clarke, 1994). After treatment with protease inhibitors, the activity of the 38 kDa autolysin was not affected, whereas the activities of trypsin and chymotrypsin were totally inhibited. In addition, trypsin and chymotrypsin did not produce any clear zone in peptidoglycan-containing gels when run without inhibitors (data not shown). The second approach involved renaturing the gels over time. The clear zone in gels increased in intensity as time of renaturation increased from 5 h to maximum renaturation after 18 h.

Table 2. Amino acid composition of the *M. phlei* 38 kDa autolysin

Amino acid*	Amount detected (nmol)	Residues mol ⁻¹ †
Asp/Asn	946	41
Glu/Gln	1080	47
Ser	500	22
His	152	7
Arg	513	22
Thr	392	17
Ala	975	42
Pro	496	22
Tyr	298	13
Val	1010	44
Ile	350	15
Leu	686	30
Phe	302	13
Lys	239	10

* Cys, Met and Trp are not reported as they are oxidized during hydrolysis.

† Based on His = 7 and a molecular mass of 38 kDa for the autolysin.

This time dependence indicated that clearing in the gels was a characteristic of enzymic digestion of the mycobacterial cell walls. Boiling of samples in the absence of SDS-PAGE sample buffer for 4 h permanently denatured the 38 kDa enzyme activity as monitored by the zymogram assay (which also indicated that the clear zone in gels was not due to other heat-stable non-specific interference).

The purified enzyme was tested using a spot assay in which buffers of various pH were incorporated into the agar to determine the activity pH optimum of the autolysin. After incubation for 24 h, the activity of the enzyme on each plate was scored according to the size and transparency of the clearing zone formed. The pH optimum of the 38 kDa autolysin was found to be pH 7.5 in both 20 mM Tris/HCl and 20 mM sodium phosphate buffer. The activity of the enzyme was generally higher in Tris/HCl buffer systems than in other buffers. Isoelectric focusing established the pI of this enzyme to be 5.5 by comparing it with the pH gradient formed in a gel or protein standards with known pI. This estimation was derived from pI values of 5.5–5.7 using the pH-gradient method and pI values of 5.3–5.5 using protein standards.

The amino acid composition of the 38 kDa autolysin is shown in Table 2. Of the total 345 residues calculated, 48% were hydrophobic. The protein contained only 12% basic residues (Arg, His and Lys), which is consistent with an enzyme of moderately acidic pI. Several attempts were made to sequence its N-terminal residues by standard techniques but repeated failure suggested that the N-terminus was blocked. However,

we were able to obtain an internal amino acid sequence from an isolated tryptic peptide by nano-electrospray mass spectrometry (Wilm *et al.*, 1996). The 1816 Da peptide comprised the following sequence: (V,A) or (I/L,G)-V-K-A-T-T-T-E-E-E-T-E-(I/L)-P-A-K. The N-terminal residues in parentheses might be either V-A or I/L-G with each pair in any order. Discrimination between I and L is not possible by this technique because both have the same mass.

A short sequence of 14 identified residues was not very useful for database searches; a search using the Atlas Retrieval System (ATLAS; National Biomedical Research Foundation, Washington, DC, USA) identified the V-K-A-T-T-T-E, T-T-T-E-E-E-T and E-E-E-T-E-L motifs in a variety of different proteins, none of which were related to peptidoglycan hydrolases. Likewise, and more importantly, a BLAST search of both the *M. tuberculosis* and *M. leprae* genome databases using this amino acid sequence failed to recover a hypothetical protein with predicted autolysin activity, but the P-A-K sequence was found in a hypothetical glycosyl hydrolase from *M. tuberculosis* with homology to α -amylases.

Using *M. smegmatis* peptidoglycan as substrate in 20 mM Tris/HCl buffer, pH 7.0, containing 10 mM MgCl₂ and 0.1% (w/v) Triton X-100, the 38 kDa autolysin (2.06 μ M) released 2.01 ± 0.098 nmol glucose equivalents per hour ($n = 3$) when reaction products were assayed for reducing sugars. This release of reducing sugars was directly proportional to the concentration of enzyme used in the assays (not shown). Free amino groups were not detected in parallel activity assays. These initial results suggest that the 38 kDa autolysin is most probably a β -glycosidase and may be the first identification of an autolysin with glycolytic activity from the mycobacteria. It remains to be determined whether wall-associated polymers, such as mycolic acids and arabinogalactan, are important in the enzyme–substrate interaction. This is especially important since it is known that teichoic acid is involved in the binding of the amidase to the pneumococcal wall (Guidicelli & Tomasz, 1984). As the peptidoglycan-associated substituents of mycobacterial walls account for a substantial proportion of the cell-wall mass, these polymers could also influence the activity of mycobacterial autolysins. Such enzyme–substrate interaction studies with the 38 kDa autolysin will have to await the cloning and overexpression of its gene in view of both the restrictive quantities of enzyme produced by the cells and our failure to obtain sufficient quantities using the current protocol.

Our present studies have demonstrated that each of the five *Mycobacterium* species used in this study produced different sets of autolysins. *M. phlei*, for example, expressed two distinct autolysins, one of which has been purified to homogeneity. The general properties of this enzyme have been revealed and its primary target has been determined to be the glycan strands. Although a temporal study on the expression of the 38 kDa autolysin during cell growth was not conducted, its activity in late-exponential growth would suggest that it plays

an important role in cell-wall turnover, cell growth and division. Interestingly, the maximum autolytic activity was also detected during late-exponential growth in *M. smegmatis* (Kilburn & Best, 1977), suggesting similar functions for the autolysins of this *Mycobacterium* species. Likewise, the major 26 kDa autolysin of *P. aeruginosa* was demonstrated to be important in cell elongation and division, and possibly also in cell-wall turnover (Li *et al.*, 1996). Alternatively, the 38 kDa autolysin could be expressed by dying cells of *M. phlei*.

The lytic action of this 38 kDa glycosidase may also participate in the removal of immunologically important wall materials during cell-wall turnover in *M. phlei*, such as wax D. Intuitively, similar systems could operate in more medically important mycobacteria and contribute to pathogenesis. These complex lipooligosaccharides of peptidoglycan fragments covalently linked to arabinogalactan via a phosphodiester bond and in turn attached to mycolic acids have been obtained *in vitro* by treating cell walls of *M. bovis* BCG with bacteriolytic enzymes (Petit & Lederer, 1984), and thought to occur naturally as an autolytic product (Goren & Brennan, 1979). In animal hosts infected by pathogenic mycobacteria, this and related peptidoglycan turnover products would be highly immunogenic, mitogenic or arthrogenic. The consequences of these interactions with hosts would lead to inflammation or arthritis and may promote chronic debilitating diseases in the hosts (Barclay & Wheeler, 1989; Petit & Lederer, 1984). Clearly, the enzymes responsible for this important aspect of mycobacterial infection require further investigation.

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