

Fluorimetric assay of mycobacterial group-specific hydrolase enzymes

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SUMMARY Fluorogenic substrates prepared from 4-methylumbelliferone provide a simple, convenient method for detecting and assaying group-specific hydrolase activity in small quantities of whole mycobacteria. Physico-chemical properties of the enzymes such as pH dependence and heat stability may also be studied. A technique is described for studying glycosidase, hexosaminidase, acid phosphatase, and acyl esterase activity in seven mycobacterial species. Such techniques will be a useful aid to the taxonomy, identification, and quantification of mycobacteria, especially slowly growing strains in which biochemical properties are very difficult to detect by other techniques.

Enzymic activities in many bacteria can be rapidly and accurately estimated. Unfortunately this is not the case in the genus *Mycobacterium*, in which even the so-called rapid growers have a relatively low metabolic rate. Enzymes may be studied in detail by extracting the required enzyme from a large mass of disrupted organisms (Diaz and Wayne, 1974), but tests requiring small quantities of whole bacteria are preferable for routine identification and taxonomic studies. For this, very sensitive systems for detecting enzyme activity are needed.

A range of substrates linked to 4-methylumbelliferone (4-methyl-7-hydroxy-coumarin) are commercially available for detecting and assaying group-specific hydrolase enzymes. After enzymic hydrolysis the free 4-methylumbelliferone fluoresces intensely in alkaline solution with a corresponding absorption of ultraviolet light at 362 nm (Leaback, 1975).

Grange and Clark (1977) found 4-methylumbelliferone-linked substrates suitable for spectrophotometric estimations of mycobacterial glucosidase and arylsulphatase. I investigated the possibility of using 4-methylumbelliferone-linked substrates to study the presence, quantity, and properties of a range of mycobacterial acid group-specific hydrolases, including glycosidases, hexosaminidases, acid phosphatases and acyl esterases.

Material and methods

Mycobacterium chelonae ATCC 19237, *M. duvalii* NCTC 514, *M. fortuitum* ATCC 6841, *M. smegmatis*

ATCC 607, *M. kansasii* (clinical isolate), *M. marinum* (clinical isolate), and *M. tuberculosis var. bovis* BCG (Pasteur) were maintained on Löwenstein Jensen medium.

The following 4-methylumbelliferyl (4-MU)-linked substrates were purchased from Koch Light Laboratories Ltd: 4-MU- β -D-glucopyranoside, 4-MU- β -D-galactopyranoside, 4-MU- α -L-arabinopyranoside, 4-MU- α -D-mannopyranoside, 4-MU- β -D-glucuronide, 4-MU-phosphate, 4-MU-2-acetamido-2-deoxy- β -D-glucopyranoside, and 4-MU-2-acetamido-2-deoxy- β -D-galactopyranoside. The last two substrates were for estimating N-acetyl glucosaminidase and N-acetyl galactosaminidase respectively. The acyl esters of fatty acids were 4-MU acetate, 4-MU propionate, 4-MU butyrate, 4-MU heptanoate, 4-MU nonanoate, 4-MU oleate, 4-MU elaidate, and 4-MU palmitate.

The bacteria were harvested from Löwenstein Jensen medium and suspended in distilled water at a concentration of 20 mg wet weight/ml. Clumps of bacteria were dispersed by agitation on a vortex mixer. In preliminary studies the bacteria were washed, but this was found to be unnecessary and was subsequently omitted.

The substrates were dissolved in dimethyl sulfoxide (grade 1, Sigma Biochemicals Ltd) to give 0.04-M stock solutions except for the oleate, elaidate, and palmitate esters, which, owing to solubility, were prepared as 0.02-M solutions. The stock solutions were stored at -20°C . Immediately before use 0.2 ml phosphatase, glycosidase, and hexosaminidase substrates were added to 0.8 ml of buffer at the required pH; 0.2 ml of acyl esterase substrates were added to 9.8 ml of buffer.

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All enzyme activities, with the exception of acid phosphatase, were estimated in phosphate buffer prepared by mixing 0.2-M solutions of KH_2PO_4 and Na_2HPO_4 to obtain pH values of 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. Phosphatase activity was estimated in citrate buffer prepared by mixing 0.2 M solutions of citric acid and trisodium citrate to obtain pH values from 3.5 to 7.0.

Sodium glycinate buffer was prepared by adding 1.0 N NaOH to a 0.2-M solution of glycine to give a pH of 10.5.

ESTIMATION OF ENZYME ACTIVITY

For estimating glycosidase, hexosaminidase, and phosphatase activity the reaction mixtures contained 0.1 ml bacterial suspension, 0.1 ml buffered substrate, and 0.8 ml buffer. The reaction mixtures, contained in 15×50 mm screwcap bottles, were incubated in a water bath at 37°C . After incubation the reactions were stopped and the fluorescence developed by adding 1.0 ml sodium glycinate buffer. The reaction mixture estimating acyl esterases contained 0.1 ml bacterial suspension, 0.9 ml buffer, and 1.0 ml buffered substrate. Preliminary studies showed that the optimum pH for this group of enzymes was 8.0 to 8.5 but at this pH the blank value was unacceptably high owing to spontaneous hydrolysis of the substrate. Therefore all reactions were conducted at pH 7.5 and the fluorescence was estimated without modifying the pH.

Fluorescence was measured in 10-mm quartz cuvettes in a Baird Atomic SF 100E spectrofluorimeter at an excitation wavelength of 362 nm and an emission wavelength of 450 nm. The reaction mixtures were maintained at 37°C until the fluorescence was measured, as intensity of fluorescence is temperature-dependent.

The blank tubes contained 0.1 ml distilled water in place of bacteria and the constancy of the light source was checked by means of standard solutions of 4-methylumbelliferone in sodium glycinate buffer.

Phosphatase activity was estimated after three hours' incubation. Glycosidase and hexosaminidase activities were estimated after 18 hours' incubation. As the fluorescence produced by acyl esterase was measured without altering the pH of the reaction mixture these enzyme activities were estimated at intervals throughout the reaction.

Results

GLYCOSIDASE ACTIVITY

All strains had β -D-glucosidase activity with optimum pH of 6.5. α -D-Mannosidase was detectable in *M. chelonae*, *M. fortuitum*, and *M. smeg-*

matris. The activity of this enzyme was maximum at pH 7.0 with an additional smaller peak of activity at pH 6.0 (Fig. 1). A high level of β -D-galactosidase activity was detected in *M. chelonae* (about one-third the rate of β -D-glucosidase) with an optimum pH of 6.5. Only trace amounts of β -D-galactosidase activity were detectable in the other strains. No significant α -L-arabinosidase activity was detectable in any strain.

β -D-glucuronidase activity was detectable in all strains. This enzyme was more pH dependent than the others (Fig. 1). All strains except *M. chelonae* showed a peak of β -D-glucuronidase activity at pH 5.5 but very little activity at pH 4.5. *M. chelonae* showed marked activity at pH 4.5. In an additional study using 0.2-M citrate buffer nine strains of *M. chelonae* all showed an optimum pH of 4.5 or 5.0 for this enzyme.

HEXOSAMINIDASE ACTIVITY

The activities of N-acetyl glucosaminidase and N-acetyl galactosaminidase were very similar in distribution to the glucosidase and galactosidase activities respectively. The former enzyme was present in all strains with an optimum pH of 5.5 except for BCG, in which the optimum pH was 6.0. The latter enzyme was detected only in *M. chelonae*, with an optimum pH of 6.5.

ACID PHOSPHATASE ACTIVITY

This enzyme varied considerably in activity and pH dependence from strain to strain. *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. kansasii*, and BCG showed a relatively high level of activity. *M. duvalii* showed a low level of activity and *M. smegmatis* occupied an intermediate position (Fig. 2).

M. chelonae, *M. smegmatis*, and BCG showed a single peak of activity at pH 5.5 and *M. fortuitum*

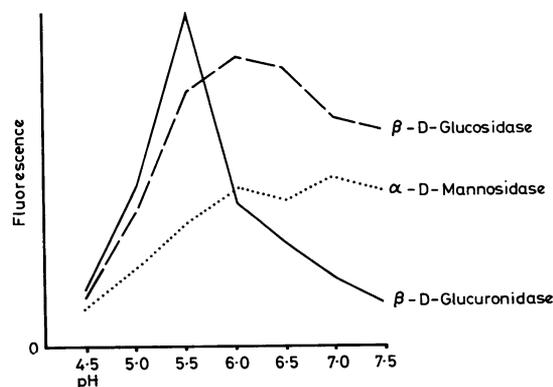


Fig. 1 Effect of pH on β -D-glucosidase, α -D-mannosidase, and β -D-glucuronidase activity of *M. fortuitum*.

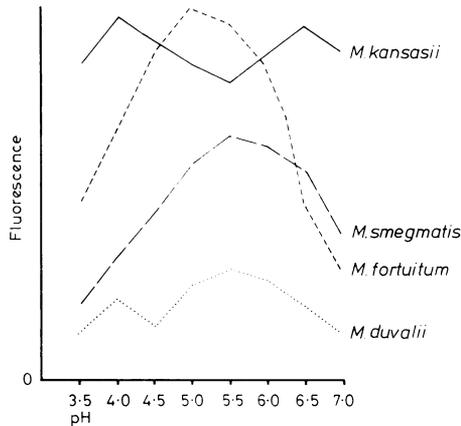


Fig. 2 Acid phosphatase activity of *M. kansasii*, *M. smegmatis*, *M. fortuitum*, and *M. duvalii*.

showed a similar peak at 5.0. *M. marinum* and *M. kansasii* gave two peaks of activity at pH 4 and 6.5. *M. duvalii* gave peaks of activity at pH 4 and 5.5.

Acid phosphatase varied in its heat stability. Preheating the bacteria at 70°C for 30 minutes caused less than 10% inactivation of enzyme (estimated at pH 5.0) in *M. marinum*, *M. kansasii*, and *M. fortuitum* but over 50% in the other four strains.

ACYL ESTERASE ACTIVITY

All the strains hydrolysed the eight acyl esters. In each case the esters of butyric and heptanoic acid were the most rapidly hydrolysed. The other esters were hydrolysed less rapidly in the order propionate > acetate > nonanoate > palmitate > oleate > elaidate. Apart from variation in the absolute reaction rate the seven strains showed no significant difference in acyl esterase activity. As noted in the methods section, it was impracticable to estimate acyl esterase activity at the optimum pH. The reactions were therefore conducted at a pH of 7.5. Phosphate buffered saline pH 7.4 (Dulbecco A, Oxoid) was later found to be equally satisfactory.

Hydrolysis of the butyric acid ester depended on the concentration of bacteria present. Thus reaction mixtures containing a final concentration of *M. fortuitum* of 200, 100, 50, and 25 µg/ml had reaction half times of 5, 11, 21, and 43 minutes respectively. Acyl esterase activity correlated closely with turbidity of bacteria cultured in liquid Sauton medium. During the growth phase very little (< 2%) of the enzyme activity was detectable in culture filtrate but further incubation during the stationary

phase caused an increasing amount of the enzyme to become extracellular.

BLANK VALUES

Excepting acyl esterase substrates at a high pH, all substrates gave a very low constant fluorescence. High blank values were avoided by using freshly prepared buffered substrate and avoiding contamination of the solutions by bacteria and fungi. Such contamination caused high blank values which varied according to the pH. The bacteria, cell-free extracts, and culture filtrates showed no detectable fluorescence at the excitation and emission wavelengths used. Routine use of enzyme blanks in addition to substrate blanks is therefore unnecessary.

Discussion

The aim of this study was to evaluate a technique for detecting, quantifying, and characterising group-specific hydrolases in mycobacteria; not to investigate any particular taxonomic problem. The methods described should present no technical difficulties, but care must be taken throughout to avoid contamination with other bacteria since this can lead to falsely high blanks or results. A spectrofluorimeter was used, but the much less expensive fixed wavelength fluorimeters would be equally suitable. Free 4-methylumbelliferone may also be estimated by measuring ultraviolet absorption at 362 nm in a spectrophotometer (Grange and Clark, 1977). The fluorimeter, however, is more sensitive and the reaction mixtures need not be clarified by centrifugation before measuring the fluorescence. Although fluorescence is expressed in arbitrary units enzyme activity may be quantified by comparing the fluorescence of the test with that of standard solutions of umbelliferone.

Two of the enzymes referred to in this study have been detected by semi-quantitative colorimetric techniques in taxonomic studies of mycobacteria and related genera—namely, β-D-galactosidase (Tsukamura, 1974; Tacquet *et al.*, 1966) and heat-stable acid phosphatase (Saito *et al.*, 1968). Sensitive fluorimetric techniques not only enable enzyme activity to be detected but also the physico-chemical properties such as heat stability and pH dependence to be accurately measured even in small quantities of slowly growing strains. Taxonomically it is much more logical to compare and contrast properties of enzymes which reflect their molecular structure than merely to screen strains for the presence of a given metabolic property. The former approach may also give clues to the evolutionary pathways within a genus, as is the case with immunodiffusion analysis of soluble mycobacterial antigens (Stanford, 1973).

Detection of acyl esterase activity was in itself of no taxonomic value. On the other hand, assay of these enzymes, which appear to be constitutive, may be of value in determining bacterial viability and growth under a range of cultural conditions.

The use of fluorogenic substrates for studying enzyme function is becoming more popular among biochemists and an increasing number of substrates are becoming commercially available. It should therefore be possible to develop a unified and technically easy set of tests for use in taxonomic studies and, subsequently, routine identification of mycobacteria and related genera—especially the very slowly growing strains in which biochemical properties are very difficult to elicit by other techniques.

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