A bacteriolytic muramidase from the basidiomycete *Schizophyllum commune*

W. D. GRANT,* B. A. PROSSER and R. A. ASHER

Cawthron Institute, Private Bag, Nelson, New Zealand

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The basidiomycete *Schizophyllum commune* produces an extracellular bacteriolytic enzyme when grown on heat-killed cells of *Bacillus subtilis* as sole C, N and P source. The enzyme catalyses the dissolution of isolated *B. subtilis* cell walls at an optimum pH of 3.2–3.4, releasing muramyl reducing groups, which indicates that it is a muramidase. Although low levels of enzyme activity are present when the fungus is grown in the absence of bacteria, full enzyme production appears to be induced by bacterial cells and repressed by glucose. Whole bacteria are not lysed by the enzyme at pH 3.3, but are rendered osmotically fragile, and lyse when the pH is raised to 7 or higher. The muramidase is effective against several Gram-positive bacteria but did not lyse any of the Gram-negative species tested.

Introduction

Fermor & Wood (1981) found that several basidiomycetes were well suited to growth on bacterial cells as a substrate and that *Schizophyllum commune* was one of the most vigorous colonizers of heat-killed *Bacillus subtilis* cells in solid media. This work was confirmed recently (Grant *et al.*, 1986) when it was shown that *S. commune*, in common with other fungi, was able to grow in liquid culture at the expense of heat-killed cells or isolated cell walls of *B. subtilis* in buffered mineral media. The cells or walls were extensively degraded as fungal growth took place and wall-degrading enzyme activity was detected in the culture fluids. Out of 12 fungi chosen for their ability to synthesize such enzymes, *S. commune* produced the most effective bacteriolysis, judged on the joint criteria of rate of dissolution of buffered wall suspensions and extent of lysis of bacteria in the fungal cultures. The enzyme responsible was shown to be a glycosidase with a pH optimum of 3.2–3.4 (Grant *et al.*, 1986). In this investigation we describe the production and properties of this enzyme: it is an extracellular muramidase capable of lysing the walls of several Gram-positive species, and is produced when bacterial cells are present in the fungal growth medium. The enzyme is of potential importance in comparative studies on the active sites of polysaccharide hydrolases (Yaguchi *et al.*, 1983).

Methods

Organisms. *Schizophyllum commune* strain 59A was obtained from the culture collection of the Forest Research Institute, Rotorua, New Zealand. *Bacillus subtilis* 168, obtained from the Microbiology Unit, University of Oxford, UK, was used as a fungal growth substrate, and walls from this organism were used in the assay of wall-lytic enzymes (Grant *et al.*, 1986). With the exceptions of *Bacillus stearothermophilus* B65 and *B. subtilis* W23, which were from our laboratory stock, all bacteria were numbered strains from recognized culture collections (see Table 1).

Bacterial cultures. All bacteria were grown in Nutrient Broth (Oxoid) with the exception of the lactobacilli and streptococci, which were cultured on Lactobacilli MRS Broth (Difco). Cultures were grown to mid-exponential phase, harvested by centrifugation, washed with distilled water, then used as substrate in the bacteriolytic enzyme assay. Cells of *B. subtilis* 168 for use as fungal growth substrate were heat-killed (100 °C, 15 min), washed once with distilled water, then freeze-dried from distilled water suspension.

Bacterial cell walls. Cell walls were prepared from mid-exponential phase cultures of *B. subtilis* 168 by breakage of the cells in a Braun MSK homogenizer and purification by the procedure of Anderson *et al.* (1978), in which autolytic enzymes are denatured during treatment with SDS at 100 °C.

Fungal cultures. *S. commune* was grown statically at 25 °C on (i) Sabouraud's liquid medium (SL) (Oxoid) at pH 5 with 0.4 µM-thiamin and 0.04 µM-biotin supplements, (ii) a defined glucose/salts medium (GS) containing KH₂PO₄, 7.4 mM; NH₄NO₃, 12.5 mM; MgSO₄, 2 mM; CaCl₂, 2 mM; H₃BO₃, 46 µM; MnCl₂, 9 µM; ZnCl₂, 0.73 µM; CuSO₄, 0.32 µM; (NH₄)₆Mo₇O₂₄, 0.032 µM; D-glucose, 0.1 M; thiamin, 0.4 µM; biotin, 0.04 µM; buffered with 0.05 M-MES to pH 5, and (iii) a bacteria/salts medium (BS) which was GS medium with glucose, KH₂PO₄ and NH₄NO₃ omitted and replaced by heat-killed freeze-dried *B. subtilis* cells (2.5 mg ml⁻¹) as C, P and N source. Starter cultures (10 ml, GS medium) were inoculated from potato dextrose agar

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Abbreviation: HEWL, hen egg-white lysozyme.

plates, incubated for 4 d, harvested, and washed with sterile GS salts (GS medium with glucose, KH_2PO_4 and NH_4NO_3 omitted). Mycelium was fragmented with glass beads on a wrist-action shaker and used to inoculate experimental growth flasks.

Harvesting and biomass estimation. In quantitative growth and enzyme production experiments, duplicate 25 ml cultures were harvested by centrifugation (5000 g, 15 min) at 24 h or 48 h intervals. Pellets were washed once with distilled water. The wash was combined with the original supernatant and made up to a known volume before filtering (0.45 μm) and freezing. Pellets were dried (105 °C) to constant weight, or freeze-dried if fungal biomass was to be measured by ergosterol extraction and estimation. For the latter method, a modification of the procedure of Seitz *et al.* (1979) was used. Mycelium was homogenized in an Ultraturrax homogenizer (3 $\times$ 30 s bursts) in cold methanol (25 ml), filtered (Whatman GFC paper), then washed with methanol (2 $\times$ 5 ml). The filtrate was made up to 40 ml with methanol then saponified by refluxing for 30 min with KOH (4 g) and ethanol (10 ml). After addition of water (10 ml) to the cooled solution, ergosterol was extracted with 3 $\times$ 25 ml redistilled hexane. The combined extracts were rotary evaporated (30–35 °C) to dryness, dissolved in methanol (5.0 ml) then passed through a 0.2 μm Teflon filter (Gelman Acrodisc CR). Ergosterol was measured by HPLC on a Waters μ Bondapak C-18 column, eluted with methanol/water (95:5, v/v) at a flow rate of 2 ml min⁻¹, measuring absorbance at 282 nm. Mycelial ergosterol contents were calibrated against dry weight measurements of *S. commune* after different periods of growth in GS medium. The linear relationship obtained was used to estimate fungal biomass in BS cultures, where bacterial cells and cell debris precluded measurement by direct weighing.

Enzyme assays and analytical methods. Wall-degrading enzyme activity was assayed by measuring the rate of decrease of optical density ($\Delta\text{OD}_{600} \text{ h}^{-1}$), over several hours, of a mixture containing *B. subtilis* walls (600 μg) suspended in buffer to a final volume of 310 μl , and culture supernatant (100 μl) incubated at 25 °C (Grant *et al.*, 1986). One unit is defined as the quantity of enzyme in 100 μl of supernatant catalysing a ΔOD_{600} of 1.0 in 1 h under the assay conditions. Culture filtrates were concentrated where necessary by ultrafiltration (Amicon YM-10 membrane). The pH optimum of wall-degrading activity in culture supernatants was determined by assaying over the pH range 2.5–9.8 using sodium formate/formic acid (2.5–4.8), sodium acetate/acetic acid (4.8–5.8), MES (5.8–7.1), TES (7.1–8.0), glycine/NaOH (8.5–9.4), and $\text{NaHCO}_3/\text{NaOH}$ (9.4–9.8) buffers, all at ionic strength 0.05.

Bacteriolytic enzyme activity, i.e. lysis of whole bacterial cells, was assayed by incubating suspensions of freshly-grown bacteria in formate buffer (ionic strength 0.025) with culture supernatant or supernatant concentrates at 25 °C. Samples (2 $\times$ 100 μl) were transferred at intervals into 200 μl 0.5 M-TES buffer pH 8.6 and 200 μl of the formate buffer (control) and the OD_{600} values of both were read 5 min after transfer. Control experiments in which the bacteria were incubated with GS salts instead of culture supernatant were performed for each bacterial species, with aliquots being transferred to the two buffers at appropriate intervals in each case. Optical density changes caused by autolysis or dilution effects were corrected using the results from the controls.

Glucose was measured with a commercial glucose oxidase analytical kit (Tekit 952 DM, Searle Diagnostics).

Reducing groups released by the action of enzyme on cell walls were measured by the method of Park & Johnson (1949). Reducing terminal residues were identified and estimated by two-dimensional paper chromatography as previously described (Grant *et al.*, 1984) after NaBH_4 reduction and acid hydrolysis (Ghuysen *et al.*, 1966).

Results

Growth of S. commune and production of wall-degrading enzyme

Extracellular bacterial wall-degrading activity was produced by *S. commune* when it was grown at either pH 7 or pH 5 on heat-killed *B. subtilis* cells as sole C, N and P source, although higher activity was consistently obtained at the lower pH. Quantitative growth studies and enzyme activity measurements were carried out on cultures of this fungus at pH 5 and 25 °C in the three different media: BS, a suspension of heat-killed *B. subtilis* cells in mineral salts; GS, a glucose/salts medium, and SL, a glucose/peptone broth (Fig. 1). The specific activities per mg fungal dry weight are shown in Fig. 2.

Properties of wall-degrading enzyme

The pH optimum of 3.2–3.4 reported for the wall-lytic activity in *S. commune* culture fluids after growth on *B. subtilis* (Grant *et al.*, 1986) was confirmed. The pH profiles of the enzymes present in supernatants from SL and GS cultures were similar to that in the BS culture fluids, with optima at pH 3.2–3.3.

Hydrolysis of *B. subtilis* walls by *S. commune* culture supernatants was accompanied by the release of reducing groups but not amino groups (Grant *et al.*, 1986). The time course of appearance of reducing groups is shown in Fig. 3. Duplicate determinations of the muramic acid residues susceptible to NaBH_4 reduction after wall digestion with *S. commune* supernatant showed that a mean of 70% (individual determinations 63% and 77%) possessed free reducing groups, whereas <10% (individual determinations 0% and 16%) of the wall glucosamine residues were available for NaBH_4 reduction. This indicated that the enzyme activity was that of a muramidase, and this was confirmed by the detection of muramicitol but not glucosaminitol on paper chromatograms of acid hydrolysates of the reduced wall digests. In addition, the concentrated culture fluids did not catalyse the hydrolysis of the β -N-acetylglucosaminidase substrate 4-nitrophenyl-N-acetyl- β -D-glucosaminide (Maruhn *et al.*, 1976).

Control of synthesis of the wall-degrading enzyme

The results in Figs 1 and 2 indicated that the enzyme was probably induced by the presence of bacterial cells. When a culture of *S. commune* in GS medium was supplemented with heat-killed *B. subtilis* (final concentration 2.5 mg ml⁻¹) after several days' growth, wall-

Fig. 1. Fungal dry weight (filled symbols) and extracellular wall-degrading enzyme activity (open symbols) of *S. commune* grown in (a) *Bacillus subtilis* (BS), (b) glucose/salts (GS), and (c) Sabouraud's liquid (SL) media. Duplicate cultures in each medium were harvested daily in the early part of the growth period, then less frequently. Fungal dry weights in (a) were obtained from a calibration curve relating dry weight to mycelial ergosterol content. Note the interrupted dry weight scale in (c).

Fig. 2. Specific activities of wall-degrading enzyme in cultures of *S. commune* grown in BS (○), GS (□) and SL (△) media (see Fig. 1). Data are means of specific activities of the duplicate cultures.

degrading activity was higher than that of an unsupplemented control culture. Although the extent of increased enzyme production varied considerably in such cultures, high levels of activity were consistently produced in response to addition of bacterial cells when the glucose in the medium was replaced with glycerol. Fig. 4 shows one of three similar experiments in which wall-lytic enzyme activity appeared in response to the addition of heat-killed bacteria. Increased activity was never detected in the unsupplemented control cultures. The final enzyme

activity in the supplemented cultures varied from 0.10 to 0.17 units.

In other experiments, glucose was added (final concentration 20 mg ml^{-1}) to cultures of the fungus growing on heat-killed bacteria (BS medium) (Fig. 5). Glucose addition at day 3 delayed the appearance of wall-degrading activity in the culture fluid until the glucose had been utilized. Essentially the same results were obtained when glucose was added at day 0 (not shown). However, addition at day 6, when appreciable levels of activity were already present, did not prevent further increase in activity with time.

Enzyme action on whole bacteria

S. commune culture fluids which would rapidly degrade isolated *B. subtilis* walls were unable to lyse suspensions of the live bacteria at pH 3.3. Prolonged incubation of cells with 20-fold concentrates of such culture fluids had only very slight effects on the optical density of the suspension. Microscopic examination of the treated bacteria, however, showed that although individual cells were intact and indistinguishable in appearance from normal *B. subtilis*, virtually all of the typical exponential-phase filaments of the bacteria had been converted to single cells. It was subsequently discovered that these cells lysed instantly on addition of dilute alkali, and it seems likely that at least some of the cell wall had been removed by the action of the enzyme. Thus, to measure the action of *S. commune* culture fluids against whole bacteria, it was necessary to incubate at low pH then to

Fig. 3. Time course of lysis of *B. subtilis* cell walls by *S. commune* culture fluid. (a) Optical density of cell wall suspension; (b) increase in reducing groups during cell wall dissolution. Data points represent means of duplicate determinations. Variation between duplicates was less than $\pm 5\%$. ●, Walls plus culture fluid; ○, walls plus BS medium base; △, culture fluid alone.

Fig. 4. Wall-degrading activity in cultures of *S. commune* on addition of heat-killed *B. subtilis* cells (2.5 mg ml^{-1} final concentration) after 3 d (○) and 6 d (●) growth on GS medium in which glucose was replaced by glycerol as carbon source. □, Unsupplemented control culture. Data points represent single determinations of enzyme activity in culture fluid samples. Arrows, points of addition of bacterial cells.

transfer samples at intervals to an alkaline buffer before measuring optical density. Controls were samples diluted identically in the incubation buffer. Identical experiments in which GS salts were used in place of the fungal culture fluid produced no loss of optical density at either pH over several hours. The most rapid lysis of *B. subtilis* occurred when the pH of the incubation buffer was 3.8.

Twenty-seven bacterial strains were tested in this way for their susceptibility to the action of *S. commune* culture fluid (Table 1). Where activity was low or absent, the experiment was repeated using a 20-fold concentrate of the culture fluid.

Table 1. Lysis of bacteria by *S. commune* culture fluid

Organism	Relative lytic activity*
<i>Arthrobacter citreus</i> NCIB 8915	3
<i>A. globiformis</i> NCIB 8602	76
<i>A. globiformis</i> NCIB 7811	0
<i>Bacillus cereus</i> NCTC 8035	28
<i>B. licheniformis</i> NCTC 6346	72
<i>B. megaterium</i> NCTC 10342	57
<i>B. pumilus</i> NCTC 8241	152
<i>B. stearothermophilus</i> B65	57
<i>B. subtilis</i> 168	100
<i>B. subtilis</i> ATCC 6633	170
<i>B. subtilis</i> W23	45
<i>Brevibacterium albidum</i> ATCC 15831	0
<i>Corynebacterium aquaticum</i> NCIB 9460	0
<i>Enterobacter aerogenes</i> NCTC 10006	0
<i>Escherichia coli</i> NCTC 8196	0
<i>Lactobacillus bulgaricus</i> NCDO 1489	28
<i>L. casei</i> ATCC 7469	0
<i>L. plantarum</i> ATCC 8014	79
<i>Micrococcus luteus</i> NCTC 2665	13
<i>Proteus vulgaris</i> NCTC 4635	0
<i>Pseudomonas aeruginosa</i> NCTC 6749	0
<i>Serratia marcescens</i> NCTC 10211	0
<i>Staphylococcus aureus</i> NCTC 4163	0
<i>Staph. epidermidis</i> ATCC 14990	45
<i>Streptococcus faecium</i> NCTC 7171	4
<i>Strep. lactis</i> NCTC 6681	2
<i>Strep. salivarius</i> ATCC 13419	0

* Activity against *B. subtilis* 168 set at 100.

Fig. 5. Effect of addition of glucose (20 mg ml⁻¹, final concentration) on production of wall-degrading activity by cultures of *S. commune* growing on BS medium. (a) Control culture; (b) glucose added at day 3 (arrow); (c) glucose added at day 6 (arrow). ●, Wall-degrading activity (data points are single determinations of enzyme activity in culture fluid samples); △, glucose concentration in culture fluid (means of duplicate determinations: variation between duplicates less than $\pm 5\%$ of the mean).

Discussion

When *S. commune* was grown on a medium containing heat-killed *B. subtilis* cells as sole C, N and P source, an extracellular enzyme activity was produced which degraded the bacteria and which could also catalyse the dissolution of isolated *B. subtilis* cell walls (Fermor & Wood, 1981; Grant *et al.*, 1986). Cell wall hydrolysis was accompanied by the release of free reducing groups but no amino groups (Grant *et al.*, 1986). Since we have now shown that 90% of the released reducing groups were muramic acid residues, and that the culture filtrates of the fungus contained no β -N-acetylglucosaminidase activity, we conclude that the enzyme is a muramidase or 'lysozyme', viz. a hydrolase specific for the muramyl glycosidic linkage in the cell wall peptidoglycan. As this linkage occurs only in bacterial or endospore wall structures, it seems likely that the enzyme is produced by the fungus as part of a specific mechanism for degradation of bacterial cells. We have recently confirmed that the enzyme is also produced when the fungus grows at the expense of viable bacteria (W. D. Grant, unpublished results), although the interfering effects of bacterial autolysis make this unsuitable as an experimental system.

Many microbial muramidases are optimally active in acid conditions (e.g. Hash, 1963; Morita *et al.*, 1978; Hayashi *et al.*, 1984), but the pH optimum of 3.3 for the *S. commune* enzyme is surprisingly low. This characteristic could provide a test of the accepted muramidase mechanism, as exemplified by hen egg-white lysozyme (HEWL). The HEWL active site contains two residues, Glu-35 and Asp-52, which are essential for activity, with Glu-35 in the protonated and Asp-52 in the ionized form

(Blake *et al.*, 1967), and although the muramidase from the fungus *Chalaropsis* appears to have analogous catalytic residues (Fouche & Hash, 1978) its pH optimum (4.6) is higher than that of the *S. commune* enzyme. An acidic residue with an unusually low pK would be required to allow maximal activity at pH 3.3. Although Rupley *et al.* (1974) showed that the pK of Asp-52 in HEWL could be as low as 3.8, it is clear that a study of the active site of the *S. commune* enzyme could provide further insights into muramidase mechanism(s). Such a study would also be of interest because of the known homology between the active sites of endoglucanases from this fungus and that of HEWL (Yaguchi *et al.*, 1983; Paice *et al.*, 1984). It is interesting in this regard that one of these *S. commune* endoglucanases possesses a glutamic acid residue at position 33: Glu-33 is thought to be part of the active site of the muramidase from *Chalaropsis* (Fouche & Hash, 1978).

The specific activity of the wall-degrading enzyme from *S. commune* grown on peptone-containing medium with high levels of glucose (SL medium) was greater than that obtained from growth on a defined glucose/salts (GS) medium (Fig. 2). This result parallels the findings of Hash (1963) and Hash & Rothlauf (1967) with *Chalaropsis*, and the synthesis of other bacteriolytic enzymes appears to be enhanced by growth of the producing organism in rich media (e.g. Imada *et al.*, 1973; Yokogawa *et al.*, 1973; Hayashi *et al.*, 1983). Although the high activity in *S. commune* broth cultures was rapidly lost as the cultures aged, this could have resulted from the action of proteases which are produced under these growth conditions (W. D. Grant, unpublished results). However, in contrast to other bacteriolytic enzyme systems (Ward & Perkins, 1968; Hash, 1974),

much higher and more stable activities were obtained with a bacterial substrate (Figs 1 and 2), and addition of bacteria to glycerol-grown *S. commune* resulted in increased enzyme activity (Fig. 4). It therefore seems probable that bacteria or bacterial cell components are able to induce synthesis of this enzyme, although the results with SL medium indicate that the induction mechanism is not specific to these components. Glucose repression of enzyme production was indicated by the fact that glucose addition at early stages of growth delayed the appearance of the enzyme activity in BS cultures until after the sugar had been utilized by the fungus (Fig. 5). Although glucose additions after enzyme activity had begun to appear did not prevent further increases in activity (Fig. 5c), it is possible that repression of enzyme synthesis was masked by continued release of enzyme from its binding to the bacterial substrate (Grant *et al.*, 1986). Glucose repression may also be implicated in the inconsistent levels of enzyme activity produced when bacteria were added to glucose-grown cultures.

The failure of *S. commune* culture fluids to lyse viable bacteria at the pH optimum of wall-degrading activity was not unexpected. Hash (1963) found that the pH optimum of the *Chalaropsis* enzyme was 4.6 for cell walls of *Staphylococcus aureus*, but 5.6 for whole cells, although the latter was dramatically affected by the ionic strength of the buffer used. Other workers have reported similar differences in pH optima for bacteriolytic enzyme activity against whole cells or cell walls (Ward & Perkins, 1968; Yokogawa *et al.*, 1975), and difficulties with reproducibility of lysis of whole cells with the *Chalaropsis* enzyme led Hash (1967) to publish a bacteriolysis assay procedure with buffered suspensions of isolated walls as substrate. Preliminary experiments with the *S. commune* culture fluids showed that there was an apparent pH optimum for whole cell lysis of 6.0–6.2, but it was soon discovered that this represented a balance between the action of the enzyme in degrading the walls at a low pH (optimum 3.8) and that of the surrounding medium in effecting osmotic lysis of the resulting spheroplasts (optimum pH > 7). Further investigation of the enzyme-treated cells has shown that they are indeed protoplasts, which are osmotically stable at pH 3–4 (W. D. Grant, unpublished results). Cell damage by lysozyme at acid pH followed by lysis in alkali was first discovered in *Escherichia coli* by Nakamura (1923), and demonstrated in other bacteria by Grula & Hartsell (1954, 1957*a, b*). The relative stability of protoplasts and spheroplasts to osmotic effects at low pH has been described previously (Edebo, 1961; Op den Kamp *et al.*, 1967; van Iterson & Op den Kamp, 1969; Wecke *et al.*, 1982).

The results in Table 1 indicate that, as with other muramidases, the *S. commune* enzyme is active against

several Gram-positive bacteria, the bacilli in particular, but is not effective in lysing Gram-negative species. It is interesting that *Micrococcus luteus*, which is extremely sensitive to lysis by HEWL, is only slowly attacked by the *S. commune* enzyme. The range of bacteria sensitive to the enzyme is similar in some respects to that of the now commonly-used bacteriolytic preparation, mutanolysin, from *Streptomyces globisporus* (Yokogawa *et al.*, 1973, 1974), although the *S. commune* enzyme appears to be less effective on the species of *Lactobacillaceae* tested. This and other fungal muramidases may well be of value in applications which call for low pH conditions, and in this regard could be important as preserving agents in certain foods (Hughey & Johnson, 1987).

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