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Identification of extracellular proteins from actinomycetes responsible for the solubilisation of lignocellulose

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Summary we have utilized strains of three actinomycete species, *Actinomadura* sp, *Streptomyces cyaneus* and *Thermomonospora mesophila*, to study the solubilisation of lignocellulose. The production of extracellular proteins was measured for each of the organisms during 17 days growth using medium containing either glucose or ball-milled straw. Some of the extracellular proteins (as identified by SDS gel electrophoresis) were present under both growth conditions, but others were specific to the type of medium or the period of incubation. The levels of proteins were compared with the abilities of the extracellular protein preparations to solubilise a substrate of ^{14}C-labelled lignocellulose. About 6% of the radioactive material were solubilised when the extracellular proteins from the cultures grown on glucose were incubated with the substrate, compared to 20—30% that were solubilised by the extracellular proteins from the cultures grown on ball-milled straw. Partial characterisation of an enzyme from *S. cyaneus* responsible for the solubilisation of lignocellulose was achieved by gel filtration of the extracellular proteins, using Superose 12. Material that eluted from the column with an apparent molecular weight of about 20000 accounted for all of the solubilisation of ^{14}C-labelled (i.e. lignin-derived) moieties. In contrast, when the eluate was tested for the presence of cellulases and xylanases most of the activities were found in fractions containing material with an apparent molecular weight of about 45000. We conclude that in cultures of *S. cyaneus* grown on ball-milled straw, a single extracellular enzyme is responsible for the solubilisation of lignin in lignocellulose, and that this enzyme is unlikely to be a cellulase or a xylanase.

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Introduction

The degradation of lignin in lignocellulose by white rot fungi (e.g. *Phanerochaete chrysosporium*) has received much attention from several research groups. Studies utilising synthetic (Kirk et al. 1983; Nakatsubo et al. 1981; Kawai et al. 1985) and natural substrates (McCarthy et al. 1984) have defined a system whereby lignin biodegradation to CO_2 is a secondary metabolic activity, occurring during severe depletion of nitrogen or carbon sources (Fenn and Kirk 1981). The extracellular enzymes have been characterised (Tien and Kirk 1983; Tien and Tu 1987).

Actinomycetes have the same filamentous habit as fungi and are common in decaying lignocellulose systems such as compost. Indeed evidence has been presented (Phelan et al. 1979; Antai and Crawford 1981; Crawford et al. 1982; McCarthy and Broda 1984) that actinomycetes may also have a role in lignin degradation. Both laboratories have identified a soluble product of the actinomycete activity referred to as "acid precipitable polymeric lignin" (Pometto and Crawford 1986). This material has been partially characterised using HPLC (Borgmayer and Crawford 1985) and NMR spectroscopy (McCarthy et al. 1986). Furthermore, it has been reported (McCarthy and Broda, 1984) that the solubilisation of [^{14}C]-labelled lignocellulose occurs in medium supplemented with yeast extract. There is, therefore, evidence that actinomycetes provide an alternative system for lignin degradation that may have biotechnological advantages; in particular, a soluble

product is formed and the solubilisation occurs during active growth rather than during secondary metabolism.

In this study we demonstrate lignocellulose solubilisation by extracellular enzymes by using cell-free systems derived from three species of actinomycetes, *Streptomyces cyaneus, Thermomonspora mesophila* and *Actinomadura* sp. We have characterised the production of the proteins, using SDS gel electrophoresis, from each of the organisms grown on media containing either ball-milled straw or glucose and correlated this with the ability of crude preparations of the proteins to solubilise a ^{14}C-labelled lignocellulose substrate. Finally, partial characterisation of the enzyme responsible for the solubilisation of the lignocellulose was achieved for *S. cyaneus* using gel filtration.

Material and methods

Bacterial Strains and Culture Conditions.
Thermomonospora mesophila DSM43048, *Streptomyces cyaneus* MT 813, and *Actinomadura* sp. MT809 (McCarthy and Broda, 1984) were maintained as suspensions of spores and hyphae in 20% (v/v) glycerol at −80° C. *T. mesophila* was cultured on CYC agar (Cross and Attwell 1974) adjusted to pH 8.0 and *S. cyaneus* and *Actinomadura* sp. were cultured on nutrient broth (0.65% w/v, Oxoid) containing agar (1.8% w/v) adjusted to pH 8.0. The inocula were obtained from aqueous suspensions of sporulating growth. The organisms were cultured in 1l of basal medium (M6) containing 0.1% (w/v) yeast extract (McCarthy and Broda, 1984). Flasks (2l) were incubated at 37° C with shaking (150 rpm) for up to 17 days. In addition the medium contained either 0.1% (w/v) glucose, 0.1% (v/v) glycerol, 0.1% (w/v) mannitol, or 0.5% (w/v) ball-milled barley straw (mean particle size 2 μm in diameter). At the end of experiments, portions of the culture medium were examined microscopically and also by subculturing on nutrient media, in order to confirm that the cultures were axenic.

Isolation and characterisation of extracellular proteins. During growth aliquots of the cultures were filtered and ammonium sulphate was added to the filtrates to a final concentration of 50%. The solutions were left to stand at 4° C for 12 h and the precipitates recovered by centrifugation at 4000x*g* for 30 min at 4° C. Protein determinations of filtrates prior to precipitation by ammonium sulphate were performed according to the method of Bradford (1976). For SDS gel electrophoresis acetone (3:1 v/v) was added to the filtrate (5 to 10 ml) and the precipitate was recovered by centrifugation (10,000 *g* for 30 min at 4° C). The precipitate was dried under vacuum and prior to electrophoresis dissolved in 20—50 μl of 0.01M NaOH. SDS polyacrylamide gel electrophoresis was performed according to the methods described by Laemmli (1970). Extracellular proteins were separated on isoelectric focussing gels following the methods described by Righetti and Grianazza (1980).

For the FPLC purification (Pharmacia) of the enzyme responsible for the solubilisation of lignocellulose, crude preparations of extracellular proteins were dissolved in 0.1 M Tris/HCl buffer pH 7.0 (10 mg/ml) and aliquots (0.5 ml) gel filtered on columns of Superose 12 (Pharmacia). Absorbance of the eluate (0.6 ml fractions) was measured at 280 nm. Aliquots (200—300 μl) of the eluate were used for assays of [^{14}C] lignocellulose solubilisation and cellulase activity.

Assay for [^{14}C] lignin solubilization. Preparation of [^{14}C] lignocellulose from wheat has been previously described (McCarthy et al. 1984). This material had a specific activity of 3700 dpm per mg of substrate. ^{14}C-labelled lignocellulose was suspended in 20 mM Tris HCl pH 7.0 containing 0.1% Triton X-100 sonicated for 30 s and recovered by centrifugation (1,500 *g* for 10 min at 4° C). The substrate was washed again before a final dilution to 5 mg/ml. For assay, 50 μl of the substrate suspension (corresponding to 0.25 mg) was added to solutions (200 μl) of enzyme (100 μg to 1 mg/ml). After incubation at 37° C overnight assay tubes were centrifuged and 50 μl of the supernatant was added to 4 ml of scintillation fluid (LKB, Optiphase Safe) and the radioactivity was measured.

Assays for cellulase activity. Cellulase assays were performed using the cellulose-azure method adapted from Rinderknecht et al. (1967). Aliquots (300 μl) of the eluate from columns of Superose 12 were added to 1 ml of 0.02M sodium phosphate buffer pH 7.0 containing 0.05 M NaCl and 2% (w/v) azure-cellulose (Sigma). The solutions were incubated at 37° C overnight, centrifuged at 1,500 *g* for 10 min and the absorbance of the supernatant measured at 595 nm. Standard curves were obtained using commercial preparations of cellulase (Sigma). Cellulase assays were also performed using the agar diffusion method as described by Tansey (1971). Supernatants (50 μl) were incubated in plates of agar (2% w/v) containing ammonium oxalate (0.5% w/v), carboxymethyl cellulose (1.2% w/v) and sodium phosphate (0.1 M pH 7.0) overnight at 30° C. The plates were washed with an aqueous solution of copper acetate (10% w/v).

Assay for xylanase activity. Xylanase activity was assayed by incubating enzyme solutions with xylan and measuring the amounts of reducing sugars liberated from the substrate. The xylan substrate was prepared following methods described by Gruninger and Fiechter (1986). Reducing sugars were determined following methods described by Mopper and Gindler (1973), Sinner and Puls (1978). Absorbances of solutions were measured at 562 nm and standards prepared using xylose.

Results

The amounts of extracellular proteins present in the supernatants of the three strains, grown in the presence of either ball-milled straw or glucose, were measured during time course experiments over 17 days (Figs. 1a, b and c). The amount of protein present in the supernatants from *S. cyaneus* (Fig. 1c) reached a maximum level after 2—4 days of growth and then declined slowly, whereas the levels of protein in the supernatants of cultures of *T. mesophila* and *Actinomadura* gradually increased over the 17 day growth period. The extracellular proteins, isolated from the supernatants of organisms grown on the two types of medium at 2, 6 and 9 day periods were submitted to SDS gel electrophoresis (Fig. 2). In all cases the

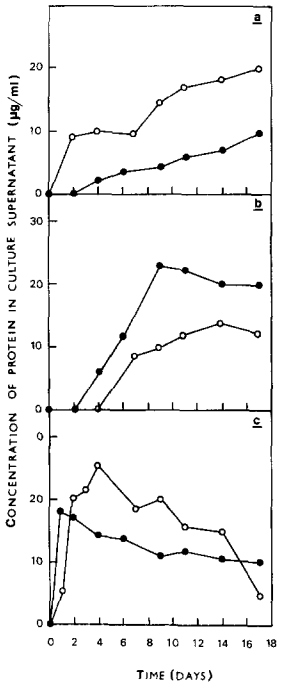

Fig. 1. Protein concentrations present in the supernatants obtained from culture media containing either 0.1% glucose (○) or 0.5% ball-milled barley straw (●). Supernatants were collected from cultures of *Actinomadura* (**a**), *Thermomonospora mesophila* (**b**) and *Streptomyces cyaneus* (**c**) over a 17 day time course. Protein concentrations were determined utilising protein-dye binding as reported by Bradford (1976)

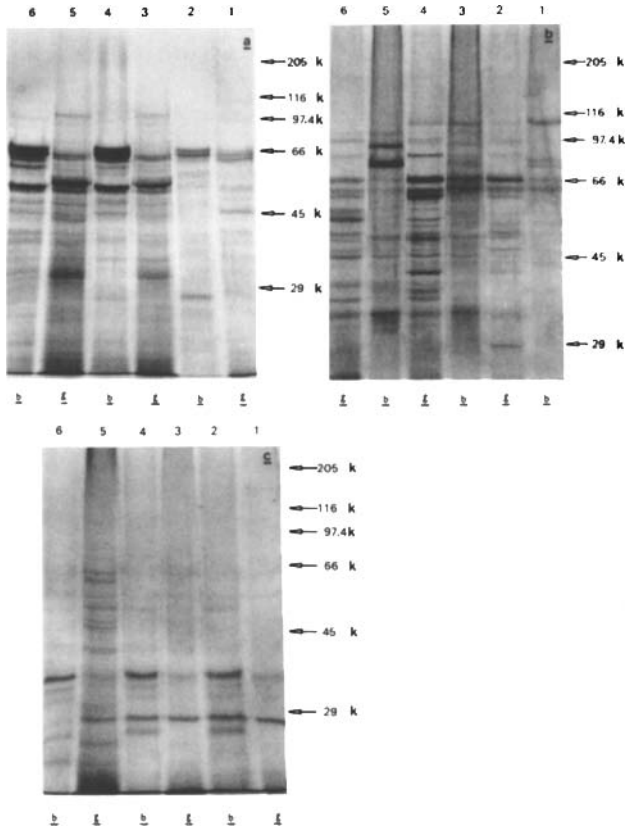

Fig. 2. SDS gel electrophoresis of extracellular proteins present in the supernatant of culture media which contained either 0.1% glucose (labelled *g*) or 0.5% ball-milled straw (labelled *b*). Supernatants were collected from cultures of *Actinomadura* (**a**), *Thermomonospora mesophila* (**b**) and *Streptomyces cyaneus*. (**c**) Samples applied to the electrophoresis gels were aliquots from cultures taken after 2 (*tracks 1 and 2*) 6 (*tracks 3 and 4*) and 9 days (*tracks 5 and 6*)

proteins ranged in size from about 10000 to 100000. Some of the extracellular proteins were present under both growth conditions, but others were specific to the type of medium.

The ability of the supernatant protein preparations to solubilise ^{14}C-labelled lignocellulose is shown in Fig. 3. Less than 6% of the total ^{14}C-labelled lignocellulose was solubilised by any of the preparations obtained from the cultures grown on glucose, whereas values of 20%—30% were achieved with the preparations obtained from cultures grown on medium containing ball-milled straw. With the latter preparations there was a correlation between the amounts of extracellular protein (Fig. 1) and solubilising activity. When the organisms were grown on medium containing either glycerol or mannitol there was little (<6%) solubilising activity found in the supernatants. Additionally, only samples from cultures grown on ball-milled straw contained cellulase or hemicellulase activities, when tested in assays.

Material from *S. cyaneus* was chosen for further study since this organism produced the highest levels of activities (Fig. 3). When the ammonium sulphate was added to the culture supernatants, following filtration to remove *inter alia* bacterial cells, a large amount of soluble by-products of straw degradation were precipitated along with proteins. The presence of the by-products caused a high background staining on SDS electrophoresis gels (Fig. 2). It was found that they could be removed by adjusting the pH of the culture media to 3.0, leaving the ectracellular enzymes in solution, but, at this pH the enzymes were irreversibly inactivated. When the extracellular proteins were subjected to isoelectric focussing via gel electrophoresis it was found that their isoelectric points were in the range 5—6.5.

Crude extracts of the extracellular proteins were subjected to gel filtration on columns of Superose 12 (which separates proteins in the molecular weight range 1×10^3 to 3×10^5) using FPLC (Pharmacia). Absorbance of the eluate at 280 nm demonstrated material throughout the molecular weight range. When aliquots of the fractions were incubated with ^{14}C-labelled lignocellulose, it was

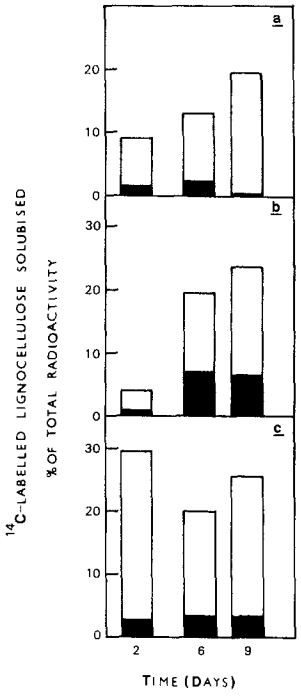

Fig. 3. Solubilisation of ^{14}C-labelled lignocellulose by extracellular proteins from the supernatants of media obtained from cultures of *Actinomadura* (**a**), *Thermomonospora mesophila* (**b**) and *Streptomyces cyaneus* (**c**). Supernatants were collected from culture media which contained either ball-milled barley straw (*open bars*) or glucose (*shaded bars*) after 2, 6 and 9 days growth. Radioactivity solubilised as a result of incubating radiolabelled lignocellulose with extracellular proteins is expressed as a percentage of the total radioactivity that was added to assay tubes

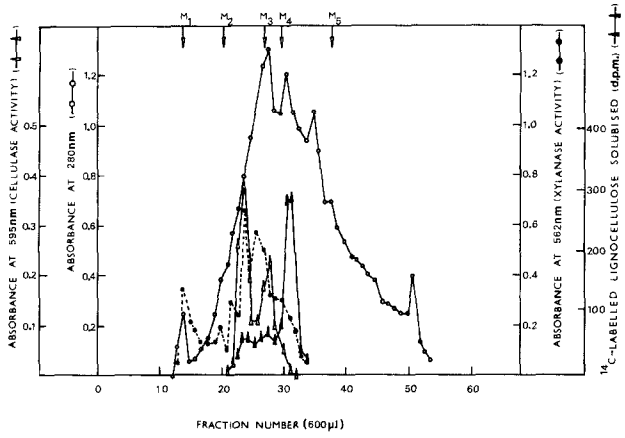

Fig. 4. Gel filtration, on columns of Superose 12, of extracellular proteins from cultures of *Streptomyces cyaneus* grown on media containing 0.5% (w/v) ball-milled barley straw. Absorbance of the eluate (600 µl fractions) is represented by the symbols, (o), and radioactivity solubilised as a result of incubating ^{14}C-labelled lignocellulose with aliquots of the eluate represented by the symbols, (▲). Cellulase activity is represented by symbols, (△) and hemicellulase activity is represented by symbols, (●). Additionally when fractions were incubated with cellulose on agar plates only fraction number 25 exhibited cellulase activity as indicated by clearing zones (see text). The column was calibrated with the molecular weight markers *M1*, Blue Dextran; *M2*, albumin; *M3*, carbonic anhydrase; *M4*, chymotrypsinogen A; *M5*, cytochrome C

found that the material with the ability to solubilise the ^{14}C-labelled substrate was eluted from the column as a single peak with an apparent molecular weight of 20000. Additionally when samples from the column eluates were incubated with carboxymethyl cellulose on agar plates, cellulase activity (as indicated by clearing zones) was detected only in the fraction that contained material of molecular weight about 45000. The same fraction also showed cellulase activity when incubated with cellulose-azure (Fig. 4). This latter test also revealed a smaller amount of activity (see Fig. 4) in the fractions corresponding to molecular weight of about 30000. When eluate fractions were tested for the presence of xylanase, the major peak of activity occurred only in those fractions corresponding to an apparent molecular weight of about 45000.

Discussion

A previous paper (McCarthy and Broda 1984), has described the degradation of ^{14}C-labelled cereal lignocellulose and its conversion into $^{14}CO_2$ and soluble products by actinomycetes. On the basis of a screening programme for actinomycetes that may degrade lignin we chose three strains, *S. cyaneus, T. mesophila* and *Actinomadura* sp. for further study. We have expanded our studies in order to develop a cell-free system to investigate the biodegradation of lignocellulose and identify the enzyme(s) involved.

We have demonstrated that while the amount of extracellular proteins is similar for the organisms grown on medium containing either ball-milled straw or glucose, those cultures grown on straw had a greater ability to solubilise lignocellulose. The presence of ball-milled straw results in the increased production of the enzyme(s) responsible for the degradation of lignocellulose, since growth of the organisms on media containing either mannitol, glycerol, or glucose had little or no activity. Although the rate of growth of the organisms on medium containing ball-milled straw differed, the levels of extracellular proteins corresponded with the ability to solubilise ^{14}C-labelled lignocellulose in each case.

The presence of by-products of straw lignocellulose degradation in the culture supernatants made

further purification of extracellular enzymes difficult. The crude material was unsuitable for ion exchange chromatography (the resultant elution profiles were not reproducible and consisted of broad unresolved peaks). However, a partial purification of the crude material was achieved by using gel filtration columns of Superose 12. Material responsible for the solubilisation of radiolabelled lignocellulose had an apparent molecular weight of 20000. This material was quite separate from cellulase (45000 and 30000 molecular weight) and hemicellulase (45000 molecular weight) and is solely responsible for the solubilisation of lignocellulose in *S. cyaneus*.

Most of the studies on the lignolytic enzymes of white-rot fungi have utilised synthetic substrates and model compounds. In this study, in common with some others we have used a ^{14}C-labelled lignocellulose which has been shown to have the lignin component specifically radiolabelled. However, while the use of this substrate has conferred the advantage of modelling a system that more closely resembles that found in the natural state, the result of the solubilisation of this complex substrate is less easily interpreted. The results reported here, obtained by utilising a cell free system do not reveal information as to the nature of the lignin derived products of solubilisation. Further studies are in progress on the chemical nature of the solubilised lignin in order to reveal the extent of lignin degradation, if any. A complete purification and characterisation of the enzyme activity described in this study combined with further studies on the degradation products of lignocellulose would establish the extent of lignin degradation by actinomycetes.

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