Preparation of ^{14}C -radiolabelled lignocelluloses from spring barley of differing maturities and their solubilization by *Phanerochaete chrysosporium* and *Streptomyces cyaneus*

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[U- ^{14}C]Phenylalanine was injected into spring barley plants during 140 d growth. Autoradiography of the plants revealed that, apart from high concentrations around the injection sites, radioactivity was evenly distributed throughout the plants. [^{14}C]Lignin lignocellulose was prepared from plants cropped at 30, 58, 82 and 140 d after sowing, using organic and aqueous solvents followed by enzymic hydrolysis with commercial protease and polysaccharidase preparations. Weight loss due to solvent and enzymic treatments was greatest in the youngest plants, resulting in a preparation of [^{14}C]lignocellulose of specific activity 2–8-fold higher than preparations from more mature plants. When subjected to crude preparations of extracellular protein from either *Phanerochaete chrysosporium* or *Streptomyces cyaneus* the substrate derived from the youngest plants (30 d) was particularly susceptible to solubilization of lignocellulose by polysaccharidases. Substrates prepared from plants after 58 d growth (i.e. fully grown) showed significant levels of lignocellulose solubilization that were not due to the action of polysaccharidases. When used in similar assays the oldest crops (82 and 140 d) were solubilized at very low levels. The desirability of using fully grown plants as substrates in lignin solubilization and biodegradation studies is clearly demonstrated.

Introduction

The biodegradation of lignin and lignocellulose by species of white-rot fungi and actinomycetes is receiving increased attention (Kirk & Farrell, 1987; McCarthy, 1987; Broda *et al.*, 1989). Studies have employed dimeric and polymeric model compounds of lignin (Enoki *et al.*, 1980; Enoki & Gold, 1982; Kirk *et al.*, 1983; Nakatsubo *et al.*, 1981; Kawai *et al.*, 1985; Hammel *et al.*, 1986), and [^{14}C]lignin lignocellulose substrates. The natural substrates have been prepared from cereal grasses (e.g. barley or wheat) grown in the presence of [^{14}C]phenylalanine (McCarthy *et al.*, 1984), by dipping cut stems of woody plants in buffer containing [^{14}C]ferulic acid (Crawford & Crawford, 1978) or [^{14}C]phenylalanine (Benner *et al.*, 1984) and by incubating cuttings of grasses and rushes in sterile water containing [3- ^{14}C]cinnamic acid (Benner *et al.*, 1984). In each case the acids provide a radiolabelled precursor for lignin biosynthesis. The

extraction of non-lignocellulosic materials using aqueous and organic solvents results in a radiolabelled substrate which contains >85% of the radioactivity in the lignin component of lignocellulose (Crawford & Crawford, 1978). This value can be improved to >90% by removal of some residual proteinaceous material using proteases (Odier *et al.*, 1981). The resulting substrates have a specific activity (approximately 10^3 d.p.m. mg^{-1}) sufficient to allow screening of organisms for the ability to degrade lignin (Haider & Trojanowski, 1980).

Analysis of lignocellulose degradation has relied on the measurement of substrate conversion to $^{14}\text{CO}_2$ and/or the appearance of soluble radiolabelled components in culture supernatants. In liquid cultures it has been shown that the substrate is converted to $^{14}\text{CO}_2$, the highest values (30 to 40% after 14 d growth) being obtained from cultures of white-rot fungi (notably *Phanerochaete chrysosporium*) grown on grass lignocelluloses (McCarthy *et al.*, 1984; Agosin *et al.*, 1985). The levels achieved by actinomycetes are much lower; maximal values from some species of *Streptomyces* are typically <10% after 14 d growth (Crawford & Sutherland, 1979; Phelan *et al.*,

Abbreviation: APPL, acid-precipitable polymeric lignin.

1979; McCarthy & Broda, 1984). However, it has been shown that actinomycetes can solubilize up to 30% of the lignin in lignocellulose (Phelan *et al.*, 1979; McCarthy & Broda, 1984). Using several strains of *Streptomyces*, Phelan *et al.* (1979) have shown that degradation of the lignin component is incomplete and directed more towards decomposition of the ring structures rather than the side-chain components of lignin. The degradation of the carbohydrate component occurs simultaneously with lignin degradation but is more complete and accounts for the substantial weight losses observed when cultures are grown on lignocellulose (Phelan *et al.*, 1979). This ability of actinomycetes to solubilize lignocellulose has been demonstrated not only for liquid cultures but also for supernatants from cultures (McCarthy *et al.*, 1986; Mason *et al.*, 1988).

Studies using radiolabelled natural substrates have failed to reveal a correlation either between the amount of $^{14}\text{CO}_2$ and lignin peroxidase activity in cultures of *P. chrysosporium* (Kirk *et al.*, 1986; O. M. Birch & P. Broda, unpublished results) or between the solubilization of radiolabelled components and the levels of acid-precipitable polymeric lignin (APPL) in cultures of *S. viridosporus* and *S. cyaneus* (Deobald & Crawford, 1987; Mason, 1988). The levels of lignin peroxidase activity and of APPL production are, however, taken to be evidence of ligninolytic activity by *P. chrysosporium* and *Streptomyces* spp. respectively. Most recently, in an exhaustive study of twenty actinomycete strains chosen as a representative collection (Ball *et al.*, 1989), it was demonstrated that there is a poor correlation between the ability to utilize synthetic substrates and the solubilization of lignin from lignocellulose. These studies indicated that the conversion of lignin to $^{14}\text{CO}_2$ and the solubilization of lignin require enzymic activities additional to ligninolytic activity. This is consistent with the observation that the molecular sizes of the radiolabelled products obtained as a result of actinomycete degradation of [^{14}C]lignocellulose are not the same as those of the APPL components found when the organism is grown on ball-milled straw (Mason, 1988). Furthermore, a wide range of values for the solubilization of substrates by actinomycetes have been obtained, particularly when different batches of substrate have been used (unpublished observations).

The object of this study was to prepare from cereal grasses sources of [^{14}C]lignocellulose that are suitable for studying the effect of plant maturity on the susceptibility of lignocellulose to biodegradation by preparations of extracellular enzymes from *P. chrysosporium* and *S. cyaneus*. We show that preparations from plants of different ages can be used to distinguish the lignin-solubilizing effects of polysaccharidases from other activities.

Methods

Preparation of [^{14}C]lignocellulose. Of 175 spring barley seeds (variety Golden Promise) sown, 158 had germinated after 20 d. When the plants were approximately 20 cm high, 4 μl [$0.2\ \mu\text{Ci}$ (7.4 kBq)] of [^{14}C]phenylalanine was injected into the plant stem 2–4 cm above soil level. Subsequent injections of the radiolabelled amino acid were made approximately every 4–6 d until 80 d. Plants were cropped at 30, 58, 82 and 140 d after sowing and the distribution of the radiolabel was determined by autoradiography (the dried plants were exposed to X-ray film for 2 d). The crops were stored at $-80\ ^\circ\text{C}$ prior to extraction using the methods described by Crawford & Crawford (1978). The plants were cut into 1–2 cm strips and were then homogenized (Polytron) in distilled water (15 ml per g of plant material). The suspension was heated to $80\ ^\circ\text{C}$ for 6 h, filtered and the distilled water extraction was repeated. The residue was then transferred to a Soxhlet apparatus in which the material was extracted for $2 \times 4\ \text{h}$ each with toluene/ethanol (1:1, v/v) and ethanol. The resulting extractive-free lignocellulose was resuspended in distilled water at $80\ ^\circ\text{C}$ for 6 h and was then filtered and lyophilized.

For enzymic treatment of the lignocellulose, the material was resuspended in 0.2 M-sodium phosphate buffer, pH 7.4 (1%, w/v) containing sodium azide (0.02%) and Pronase E (Sigma; 0.06%). The suspension was incubated at $30\ ^\circ\text{C}$ overnight and was then filtered and washed with distilled water. The procedure was repeated until no more radioactivity was released. The lignocellulose was treated sequentially in a similar way with two cellulase preparations (*Penicillium funiculosum*, *Trichoderma viride*; Sigma; 0.06%), washed with distilled water, lyophilized and stored at $-20\ ^\circ\text{C}$.

The specific activity of the [^{14}C]lignocellulose was determined by combustion in a tube furnace (Carbolite). Samples of the lignocellulose (10–15 mg) were heated to $900\ ^\circ\text{C}$ under a stream of dry air. The released $^{14}\text{CO}_2$ was trapped by bubbling into two vials (4 ml) of scintillation fluid (POPOP, 0.192 g l^{-1} ; PPO, 3 g l^{-1} ; toluene/ethanol-amine/methanol, 60:18:22, by vol.); the radioactivity was then measured. Klason lignin determinations were done according to methods described by Effland (1977).

Preparation of extracellular enzymes from *P. chrysosporium* and *S. cyaneus*. Fungal and bacterial strains were maintained as suspensions of spores and hyphae as described previously (McCarthy *et al.*, 1984; Brown *et al.*, 1988). Cultures of *P. chrysosporium* ME446 (ATCC 34541) were grown on 0.01 M-sodium acetate buffer, pH 4.5 (25 ml) containing (g l^{-1}) KH_2PO_4 (0.2), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.05), $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (0.013), thiamine (0.001), glucose (1), $\text{NH}_4\text{H}_2\text{PO}_4$ (0.276), veratryl alcohol (0.068) and 7 ml mineral salts soln l^{-1} containing (g l^{-1}) nitrilotriacetate (1.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.5), NaCl (1), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.1), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.082), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1), $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (0.01), H_2BO_3 (0.01), $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ (0.01), CuSO_4 (0.05), in 250 ml Erlenmeyer flasks and were maintained at $37\ ^\circ\text{C}$ for 5 d. A crude extract of extracellular proteins was obtained following filtration (glassfibre GF/A) and lyophilization of the filtrate. The crude extract was stored at $-20\ ^\circ\text{C}$ prior to use in assays.

S. cyaneus (MT813) was cultured on 1 litre of basal medium (M6) containing yeast extract (1 g l^{-1}) and ball-milled barley straw (0.5%, w/v; McCarthy & Broda, 1984; Mason *et al.*, 1988). Flasks (2 litre) were incubated at $37\ ^\circ\text{C}$ with shaking at 150 r.p.m. for 6 d. Cultures were filtered and the filtrate was lyophilized. The crude extract of extracellular proteins was stored at $-20\ ^\circ\text{C}$ under nitrogen prior to use in assays.

Assay for [^{14}C]lignocellulose solubilization. Crude extracts of extracellular proteins from both organisms were dissolved in 0.05 M-Tris/HCl buffer, pH 7.4 (0.4 mg ml^{-1}) and were then added to suspensions of

radiolabelled substrates (100 µl containing 0.25–0.5 mg). After incubation at 37 °C overnight the assay tubes were centrifuged and 100 µl of the supernatant was added to 4 ml of scintillation fluid (LKB Optiphase Safe); the radioactivity was then measured.

Assay for [^{14}C]lignocellulose mineralization to $^{14}\text{CO}_2$. The production of $^{14}\text{CO}_2$ was measured from liquid cultures of *P. chrysosporium* grown on [^{14}C]lignocellulose (3000 d.p.m.) and ball-milled straw (0.25%, w/v). Cultures were grown on the medium (25 ml) used for the preparation of extracellular proteins but with glucose omitted. After incubation at 37 °C the flasks were flushed with O_2 and the free $^{14}\text{CO}_2$ was collected into two vials of scintillation fluid (4 ml) as described above for the determination of specific activities.

Results

Preparation of [^{14}C]lignocellulose from spring barley of differing maturities

During growth of the spring barley plants, injections of [^{14}C]phenylalanine maintained a constant level of radioactivity in the plants [approximately $0.3 \mu\text{Ci g}^{-1}$ (11.1 kBq g^{-1})]. Plants cropped at 30, 58, 82 and 140 d after sowing represent quite different stages of plant development: crop 1 (30 d) were immature green plants; crop 2 (58 d) were almost full grown seedless green plants; crop 3 (82 d) were full grown green–yellow plants with an ear of barley; and crop 4 (140 d) were dried yellow plants. Autoradiograms of the plants at the different growth stages revealed heavy concentrations of radiolabel around the injection sites and scar tissue; otherwise, the radioactivity was evenly distributed throughout the plant tissue. Furthermore, radiolabel was not observed in the seeds of older plants (82 d and 140 d). Between 37–47% of the radioactivity injected was recovered from the crops; presumably the balance was lost through respiration, deposition of the label in the roots and soil, and/or mechanical loss during injections. Recoveries of plant tissue following solvent extraction and enzymic hydrolysis varied from 0.5% for the

Table 2. Radioactivity recovered during solvent extractions and enzymic treatments of [^{14}C]lignocellulose preparations from crops 1, 2, 3 and 4

	Radioactivity (expressed as a percentage of the total):		
	Extracted by solvents (%)	Extracted during enzymic treatments* (%)	Insoluble [^{14}C]- lignocellulose (%)
Crop 1 (30 d)	21	55	24
Crop 2 (58 d)	45	17	38
Crop 3 (82 d)	45	17	38
Crop 4 (140 d)	35	14	51

* Commercial preparations of proteases and polysaccharides.

youngest crop to 12.3% for the oldest; thus the younger crops were more susceptible to the extraction procedures and to enzymic digestion (Table 1). The enzymic hydrolysis caused by the commercial cellulase preparation is most likely to be due to a number of polysaccharidase activities since the preparations contain, in addition to cellulase, contaminating activities such as xylanase. No attempt has been made in this study to distinguish between the various types of polysaccharidases and their individual contributions to the overall solubilization and degradation of the substrate.

Recoveries of radioactivity during solvent extraction suggested that less radiolabel had been incorporated into the lignocellulose fraction of the older crops than of crop 1. However, when the plant tissue was subjected to enzymic hydrolysis, material from crop 1 was more susceptible to degradation by protease and polysaccharidases (55%) than were crops 2, 3 and 4, which gave values of between 17 and 14% (Table 2). The large weight loss during the enzymic treatments of crop 1 was reflected in the high specific activity of [^{14}C]lignocellulose of this preparation compared to those from the other

Table 1. Recovery of plant tissue and radioactivity during the preparation of [^{14}C]lignocellulose

	Amount of [^{14}C]- phenylalanine injected [$\mu\text{Ci g}^{-1}$ (kBq g^{-1})]*	^{14}C recovered in plant (%)	Recovery of plant tissue after solvent extraction (% weight)	Specific activity after solvent extraction (d.p.m. mg^{-1})†	Recovery of plant tissue after enzymic treatments (% weight)‡	Specific activity after enzymic treatments (d.p.m. mg^{-1})‡
Crop 1 (30 d)	0.29 (10.7)	36.9	4.3	4253 ± 514	0.5	10168 ± 689
Crop 2 (58 d)	0.33 (12.2)	40.2	7.3	2432 ± 183	3.1	4169 ± 816
Crop 3 (82 d)	0.29 (10.7)	48.9	14.7	1323 ± 60	7.0	2004 ± 250
Crop 4 (140 d)	0.52 (19.2)	46.4	24.0	1083 ± 77	12.3	1529 ± 84

* Expressed as radioactivity per g wet weight of plant in the case of crops 1, 2 and 3. For crop 4 the value is with respect to dry plant weight.

† The mean $\pm$ SD for the data shown were calculated from five determinations.

‡ Weight and specific activity determined after treatment with commercial protease and polysaccharidases.

Fig. 1. Radioactivity (expressed as a percentage of the total added to assay tubes) solubilized and recovered from assay supernatants from lignocellulose samples that had not been treated with polysaccharidases. Extracellular enzymes from *P. chrysosporium* (a) and *S. cyaneus* (b) obtained from lyophilized culture supernatants were added to crops 1 (○), 2 (●), 3 (△) and 4 (▲). Values are means of duplicate determinations.

Fig. 2. Radioactivity solubilized and recovered from assay supernatants containing lignocellulose substrates that had been exhaustively pretreated with Pronase and polysaccharidases. Extracellular enzymes from *P. chrysosporium* (a) and *S. cyaneus* (b) obtained from lyophilized culture supernatants were added to crops 1 (○), 2 (●), 3 (△) and 4 (▲). Values are means of duplicate determinations.

crops. When Klason lignin determinations were done on the lignocellulose fractions from each of the crops it was found that >90% of the radiolabel was in the acid-insoluble component, corresponding to Klason lignin.

Substrate solubilization and conversion to CO₂

When the extracellular preparations of cultures from *P. chrysosporium* and *S. cyaneus* were incubated with [¹⁴C]lignocellulose preparations obtained after solvent extractions and protease treatments, it was found that as the age of the crop increased the resulting lignocellulose substrates were more resistant to enzymic degradation (Fig. 1). Maximal levels of solubilization, 29 and 20%, were achieved for *P. chrysosporium* and *S. cyaneus* respectively when substrate prepared from the youngest crop was used. However, levels fell to about 6% for both organisms when the oldest crop was used. When enzyme preparations from the two organisms were incubated with [¹⁴C]lignocelluloses prepared from both crops 1 and 2 which had been pretreated with both protease and polysaccharidases, 8–10% of the radioactivity was solubilized (Fig. 2). However, crop 3 was more resistant

Fig. 3. Radioactivity released as ¹⁴CO₂ from liquid cultures of *P. chrysosporium* grown on media containing crops 1 (○), 2 (●), 3 (△) and 4 (▲) over a 36 d growth period. Values are means of triplicate determinations.

to enzymic degradation, resulting in much lower levels (0–1%), while crop 4 remained totally undegraded under similar culture conditions.

When cultures of *P. chrysosporium* were grown in the presence of ball-milled straw and [¹⁴C]lignocellulose was prepared from crops 1–4, the percentage of radioactivity released as ¹⁴CO₂ rose to maximal levels of 5–6% after about 16 d in all cases (Fig. 3).

Discussion

Previous studies on the biodegradation of [^{14}C]lignocellulose have utilized methodologies employing various combinations of radiolabelled precursors (e.g. ferulic acid, cinnamic acid and phenylalanine), durations of labelling and types of plant (e.g. cereal grasses and woody plants). It has therefore been difficult to assess and compare the abilities of different organisms (e.g. brown-rot fungi, white-rot fungi and actinomycetes) to solubilize lignocellulose or to degrade the lignin component and indeed widely differing values have been reported. The situation is aggravated by the complexity of the lignocellulose substrate, which is susceptible to many types of enzymic attack. For example, during investigations (Mason *et al.*, 1988, 1990) of the activities of culture supernatants of *S. cyaneus* it was found that the actions of cellulase, xylanase and a previously unidentified lignocellulose-solubilizing activity accounted for the solubilization of [^{14}C]lignocellulose. However, if the substrate was pretreated with commercial cellulases the subsequent solubilization of substrates was solely due to an activity unconnected with polysaccharidases.

This study has utilized radiolabelled substrates derived from spring barley plants of differing maturities. As the crop matures, the anatomical and morphological changes, plant metabolites and environmental factors (Glasser & Kelley, 1987) all affect the degree of cell-wall lignification. In addition, environmental conditions may affect the polymerization of lignin (Glasser & Kelley, 1987) resulting not in a uniform copolymerization of the aromatic monomers (coniferyl, sinapyl and *p*-coumaryl alcohols) (Koukol & Conn, 1961) but, as is the case for hardwood lignins (Higuchi, 1980), in a heterogeneous population of polymers of varying solubilities. The increasing age of the crops results in substrates which become initially more resistant to the actions of polysaccharidases and then also to other lignocellulose-solubilization activities, at least in culture supernatants. The complexity of the grass lignocelluloses, particularly those derived from the more mature cereal grasses, has afforded us with the opportunity to use a substrate (derived from the 58 d crop) which can distinguish between the actions of polysaccharidases and other lignocellulose-solubilizing activities.

Previous studies (McCarthy & Broda, 1984; McCarthy *et al.*, 1984; Mason *et al.*, 1988) have utilized crops of young plants for substrate preparation equivalent to the 30 d crop used in this study, or in some cases even younger. From this study it appears that material that is about 58-d-old, i.e. from fully grown but still green plants, may be the best choice of substrate since this crop gave near maximal solubilization of lignin (Fig. 2) with the least susceptibility to polysaccharidases (Fig. 1).

However, when the contribution of polysaccharidases is removed, either by extensively pretreating the substrate with commercial preparations of polysaccharidases or, preferably, by the choice of a suitable plant crop, the level of solubilization is reduced to approximately 10%. These levels of solubilization, although reproducible, fall uncomfortably close to the values of 5–6% reported by most groups when preparing substrates from cereal grasses (either treated or not treated with polysaccharidases) that are not accountable as radioactivity present in the lignin component as determined from Klason lignin determinations. Therefore, it becomes difficult using the above methodology to quantify the true amount of lignin solubilization or degradation occurring during an organism's attack on lignocellulose that is distinct from the actions of polysaccharidases.

Neither organism used in this study gave extracellular preparations capable of degrading the older yellow plant material (crops 3 and 4) to any significant extent, although the $^{14}\text{CO}_2$ release experiment (Fig. 3) shows that *P. chrysosporium* can do this *in vivo*. It would be of immediate interest if enzyme preparations from any of the organisms reported in previous studies to be capable of degrading lignin were able to attack the older cereal crops, or if changing the culture conditions were to yield preparations that gave increased degradation of lignocellulose, since most of the previous studies were optimized for younger substrates. For example, we chose 0.05 M-Tris/HCl buffer, pH 7.4, because of its ability to dissolve the crude preparations of extracellular enzymes from *P. chrysosporium* and *S. cyaneus* (mostly consisting of buffer salts and metabolites); more acidic buffers might have provided a solution closer to the pH optimum of the polysaccharidases, at least in the case of *P. chrysosporium* (Eriksson & Pettersson, 1975).

This study has not just provided a substrate that is suitable for studying lignin solubilization from lignocellulose by micro-organisms while minimizing the effects of polysaccharidases. Although we did not attempt to optimize the conditions for the extracellular enzyme activities, when material resistant to the actions of polysaccharidases (i.e. 58 d or older) was subjected to extracellular enzymes from *P. chrysosporium* and *S. cyaneus*, the lignin-solubilization values were similar. Earlier studies using natural substrates, primarily based on $^{14}\text{CO}_2$ liberation, have suggested that *P. chrysosporium* is a more efficient organism for lignin solubilization than are actinomycetes; however, this may only be the case for substrates extracted from immature crops (for example 30 d or younger) and therefore susceptible to polysaccharidases. Our results suggest that both organisms have *in vitro* enzymic activities capable of lignin solubilization that are unlikely to be due to lignin peroxidases, since these experiments were done in the absence of H_2O_2 .

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