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Influence of aromatic compounds on biodegradation of [^{14}C]-labeled xylan and mannan by the white-rot fungus *Phlebia radiata*

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Radiolabeled [^{14}C]arabinoxylan from wheat meal and [^{14}C]galactoglucomannan from red clover meal were prepared by using $^{14}CO_2$ as a precursor. Twice as much mannan was mineralized than xylan after 14 days of incubation with *Phlebia radiata*. Low-molecular-weight phenolic compounds structurally related to lignin increased during mineralization of both hemicellulose fractions. Veratryl alcohol increased degradation of arabinoxylan by approximately 28.5%, whereas veratric acid increased it by only 9.0%. Vanillic acid and ferulic acid also stimulated degradation by 16.6% and 34.7%, respectively. Veratryl alcohol and ferulic acid increased degradation of galactoglucomannan by approximately 75%. Veratraldehyde in both cases repressed the degradation process (23.6% arabinoxylan, 43.8% galactoglucomannan). These results indicate that the degradation of hemicelluloses, e.g., xylan and mannan, by *P. radiata* is enhanced by addition of aromatic compounds.
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Introduction

Basidiomycetous fungi, which cause white rot in wood, degrade lignin, cellulose and hemicellulose from cell walls of angiosperms and gymnosperms [8,30]. The order or proportion in which these wood components are decomposed is not uniform, and a great variation may be found in types of white rot produced. Other researchers have stated that lignin degradation is accelerated in the presence of cellulose or its oligomers [4,14]. The idea of feedback-type interdependence of delignification and carbohydrate degradation processes has been postulated many times since 1974 [3,5,17–19,25,36,37]. This hypothesis is still valid, as the report by Gottlieb *et al* [10] postulating the possibility of mycelium growth on lignin as a sole carbon source has not been confirmed. All enzymes of white-rot fungi can be divided into three groups. The first comprises enzymes attacking the wood constituents directly. This group includes enzymes acting on all ligno–carbohydrate compounds. The second group includes those which cooperate with the first group, but never attack wood on their own. The third group consists of so-called feedback-type enzymes, which play a key role in combining metabolic chains during biodegradation of wood [19].

Hemicellulose, the third main wood component, is present in all layers of the plant cell wall, with a higher concentration in the primary and secondary layers, where they occur closely associated with cellulose and lignin. Reduction of the hemicellulose content in wood is very closely connected with the degradation of lignin during decay by different white-rot fungi [8]. The principal hemicellulose in softwood is galactomannan, whereas in hardwood it is *O*-acetylated-4-*O*-methylglucurono-β-D-xylan, often called glucuronoxylan [8,15].

One of the most efficient degraders of lignin components among the white-rot fungi is *Phlebia radiata* [14]. It is also characterized by various cellulolytic [28] and hemicellulolytic activities [27]. The purpose of this work was to isolate uniformly labeled [^{14}C]galactoglucomannan from red clover and [^{14}C]arabinoglucuronoxylan from wheat, and to study the degradation of the specifically labeled substrates to carbon dioxide under the low-nitrogen and -aeration condition. Additionally, the influence of some aromatic compounds, such as vanillic, veratric and ferulic acids, veratraldehyde and veratryl alcohol on the degradation of hemicellulose, mannan and xylan was investigated.

Materials and methods

Cultivation of the plants

Red clover (*Trifolium rubrum* L., cv. Ulka-super elita) and wheat (*Triticum aestinum* L., cv. Sigma) seeds were obtained from the Department of Plant Cultivation, Agricultural University in Lublin, Poland. Preliminary cultivation of the plants was conducted in a greenhouse for 4 weeks in plastic pots (20×30 cm), each containing a 4-cm layer of garden soil. The growing plants were watered every day with the commercial flower fertilizer Uniflor-BIO (Zabala PENOR, Debno, Poland). After 4 weeks, plant heights were approximately 10 cm.

Preparation of radiolabeled hemicelluloses

Red clover and wheat plants were uniformly radiolabeled with ^{14}C by incubation in plastic pots containing the plants in a special hermetic Plexiglas box (wall thickness=8 mm), which also acted as an antiradiation shield. The Plexiglas box also contained a small

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beaker with 5 ml of 1 M $NaH^{14}CO_3$ (740 kBq). Next, 4 ml of 5 M H_2SO_4 was injected through the box by a hermetic valve into the beaker to liberate $^{14}CO_2$. After 8 h of incubation in the dark, the box was aerated (10 vol of the box). Nonmetabolized $^{14}CO_2$ was absorbed in a glass washer connected to the Plexiglas box *via* a hermetic valve, which contained 300 ml of 20% KOH. Subsequently, the plastic pots with plants were placed under the Aquarelle fluorescent lamp (Philips, Eindhoven, The Netherlands) for 16 h. The procedure was repeated for the next 21 days. The stalk portions of the plants were harvested, dried at 55°C and ground to 0.25–0.50-mm sizes by an Atomizer (MSE, London, England).

Preparation of holocellulose

The straw or red clover meal was extracted with benzene–ethanol solution (2:1 vol/vol) for 12 h in a Soxhlet extractor, then with ethanol for 24 h and air-dried at room temperature. The extractive-free straws were used to prepare holocellulose [2]. The delignification procedure was conducted using 50 g of straw, which was suspended in 2 dm^3 of water with stirring at 75°C. When the mixture reached the proper temperature, glacial acetic acid (2.8 cm^3) and sodium chlorite (35 g) were added with stirring. This procedure was repeated three times at 1-h intervals. The mixture was then cooled rapidly to 25°C, filtered, washed with ethanol and dried.

Preparation of galactoglucomannan

Red clover holocellulose (19.6 g) was suspended in 150 cm^3 of 24% KOH (wt/wt) in a round-bottomed flask and shaken at 150 rpm for 8 h [34]. The air in the flask was displaced with nitrogen, which was continuously passed during extraction. The solid residues were filtered and washed with 100 cm^3 of water. The extracts and washings were mixed with 4 vol of ethanol containing 75 cm^3 of acetic acid. The precipitates were collected by centrifugation at 4000 rpm for 15 min, and washed in succession with 70% ethanol, 96% ethanol and ether. The final materials were dried in a vacuum desiccator. The mixture of polysaccharides was then dissolved in 50 cm^3 of water, mixed with 50 cm^3 of 20% KOH and precipitated with 200 cm^3 of 5% $Ba(OH)_2$, which was added dropwise [23]. The precipitate containing galactoglucomannan was centrifuged at 12,000 rpm for 10 min, and washed three times with water and ethanol.

Preparation of arabinoxylan

The prepared holocellulose (35 g) from wheat was dissolved in 750 cm^3 of 5% KOH at 25°C for 8 h [1]. The extraction apparatus consisted of a 2-dm^3 glass flask fitted with a KPG stirrer. Additional valves of the flask were used as intake and exhaust ports for nitrogen gas continuously passed during the extraction period. The extract was removed from the flask and the procedure was repeated twice. The clarified solution was then neutralized with cold HCl, concentrated 10 times in a rotary evaporator at 40°C, dialyzed in the presence of distilled water and mixed with 3 vol of ethanol. The precipitate was successively washed with 75% and 95% ethanol and ether, respectively.

Structural analysis of hemicelluloses

Acidic and neutral sugars were determined in [^{14}C]arabinoxylan and [^{14}C]galactoglucomannan according to the modified procedure of York *et al* [38]. Methanolysis of these polysaccharides was performed with 1 M HCl in MeOH at 80°C for 16 h. The resulted methyl glycosides were then dried under a stream of nitrogen (traces of acid were removed by coevaporation of HCl with MeOH). The products were carboxyl-reduced with $NaBD_4$ in $MeOH/D_2O$, 1:1 by volume, at 4°C for 48 h. Hydrolysis was performed with 2 M trifluoroacetic acid (TFA) at 120°C for 2 h. Afterwards, conventional acetylation was carried out with acetic anhydride/pyridine, 1:1 by volume, at 100°C for 30 min. The resulting mixtures of deuterium-reduced alditol acetates of neutral sugars and uronic acid derivatives dideuterated at C_6 were analyzed by GC-MS using a Hewlett-Packard 5890 chromatograph, coupled to a Hewlett-Packard 5970 mass spectrometer equipped with a glass capillary column HP 5971 (30 m×0.25 mm) using a temperature program of 150°C (5 min) to 310°C at 5°C/min [12,28,31].

Fungus and culture conditions

P. radiata Fr. strain no. 79 (ATCC 64658) was isolated at the Department of Microbiology, University of Helsinki, Finland [14]. The fungus was maintained on 2% (wt/vol) malt agar slants. For inoculum, fungal agar plugs (*ca.* 0.5 cm^2) were grown in ADMS medium containing 2.0 mM nitrogen (LN) and 56 mM glucose in nonagitated conical flasks for 6 days at 28°C. The mycelial mats were collected and homogenized in a Waring blender. After inoculation with 4% (vol/vol) of the homogenates, 100-ml conical flasks, each containing 10 ml of ADMS-LN medium supplemented with 1% (wt/vol) galactoglucomannan or arabinoglucuronoxylan as a carbon source, were incubated stationary at 28°C. About 1 kBq of [^{14}C]galactoglucomannan from red clover, or [^{14}C]arabinoxylan from wheat straw, was added to each inoculated flask at the beginning of cultivation. In several cases, nonlabeled aromatic compounds, vanillic (4-hydroxy-3-methoxybenzoic) acid, veratric (3,4-dimethoxybenzoic) acid, veratraldehyde (3,4-dimethoxybenzaldehyde), veratryl (3,4-dimethoxybenzyl alcohol) and ferulic (4-hydroxy-3-methoxycinnamic) acid, were added 3 days after the inoculation to make a final concentration of 1 mM. All

Table 1 Characterization of ^{14}C-labeled hemicellulose preparations

Species	Stages of preparation	Amount of preparates [%]	Specific radioactivity [kBq/g]	^{14}C yield [%]
Trif. rubrum	Straw meal	27.0	2096.5	100
	Holocellulose	19.6	967.1	33.5
	Hemicellulose	4.62	481.5	3.93
	Mannan	1.28	239.0	0.54
Trit. aestinum	Straw meal	69.8	1324.6	100
	Holocellulose	50.0	639.9	34.6
	Xylan	15.0	309.6	5.02

Table 2 Sugar composition of ^{14}C-labeled xylan and mannan

Hemicellulose	Sugar composition	Relative content [mol%]	Chemical formula
Trit. aestinum xylan	Arabinose	8.9	4-*O*-Me-glucuronoglucoarabinoxylan
	Xylose	50.3	
	4-*O*-methylglucuronic acid	0.8	
	Glucuronic acid	1.9	
	Glucose	8.3	
	Mannose	–	
	Galactose	–	
	Disaccharide alditols	29.8	
Trif. rubrum mannan	Arabinose	1.2	Arabinogalactoglucomannan
	Xylose	–	
	4-*O*-methylglucuronic acid	–	
	Glucuronic acid	–	
	Glucose	1.9	
	Mannose	69.0	
	Galactose	14.0	
	Disaccharide alditols	13.9	

aromatic compounds were reagent grade. Veratryl alcohol was vacuum-distilled prior to use. At least three parallel flasks were used in every cultivation.

Radiorespirometry

The release of $^{14}CO_2$ was measured daily after absorption in KOH as described by Haider and Trojanowski [11] in a DIMILUME scintillator (Packard, Frankfurt, Germany). Uninoculated flasks, each containing one of the labeled compounds, served as the control. Culture liquors were filtered through Whatman no. 4 filter paper on a glass filter (Shot no. 4, Duran, Germany). The filter paper plus mycelium after washing and solubilization was used to determine the residual ^{14}C activity. Radioactivity in the growth liquid plus washings was determined according to Hatakka and Uusi-Rauva [14]. Radioactivity was measured in a liquid scintillation counter (Beckman 5000) using a [^{14}C–O] internal standard for the standardization of the samples (LKB Wallac, Oy, Finland).

Results and discussion

Preparation and chemical structure of hemicellulosic fractions

Hemicellulose, in terms of its significance in the biomass, is the most abundant plant constituent after cellulose and lignin. It is synthesized in the plant cell within the Golgi apparatus from which, *via* vesicles, it is secreted to the cell wall through fusion with the plasma membrane [6]. This polysaccharide is synthesized from UDP glucose and GDP glucose by the action of a variety of enzymes, e.g., dehydrogenase, decarboxylase and different epimerases, producing hemicellulose precursors [33]. Hemicelluloses are composed of both linear and branched heteropolymers of D-glucose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. These individual sugars may be acetylated and/or methylated [8].

During the first stage of the experiment, we tried to obtain the main ^{14}C-labeled hemicellulose fractions, i.e., mannan and xylan. Table 1 demonstrates the efficiency and specific activity of preparations during the purification of mannan from red clover and xylan from wheat. In the case of mannan, the separation procedure falls into three stages, giving about 4.7% efficiency with the specific activity 239 kBq/g. The total ^{14}C amount incorporated into mannan was approximately 0.5% of its initial content in red clover meal. The ^{14}C-labeled xylan was obtained during two stages of separation with 20% yield and specific activity 309.6 kBq/g. The ^{14}C amount incorporated into this preparation equals approximately 5% of initial radioactivities included in wheat plant meal.

The hemicelluloses isolated from wheat and red clover were subjected to chemical studies. Arabinoxylans have been found primarily in certain grains and grasses [32]. L-arabinofuranose units are linked as individual side chains directly to the linear or singly branched xylan skeleton [9]. Sugar analysis of [^{14}C]xylan revealed xylose to be the main component of this

Table 3 ^{14}C distribution measured 14 days after inoculation of *P. radiata* on the substrates with 1% arabinoxylan (including ^{14}C labeling) and 1 mM aromatic compounds

Substrates	$^{14}CO_2$ evolved [%]	^{14}C residue/mycelium [%]	^{14}C growth liquid [%]*	^{14}C total [%]
1% Wheat xylan	15.7±1.33	55.7±2.41	25.0±1.18	96.4±3.86
1% Wheat xylan+1 mM vanillic acid	18.3±1.16	64.8±2.99	11.3±0.98	94.3±4.34
1% Wheat xylan+1 mM veratric acid	17.1±0.94	45.1±1.61	34.9±2.79	97.2±2.94
1% Wheat xylan+1 mM veratraldehyde	12.0±0.91	47.9±2.89	32.3±3.06	92.2±4.72
1% Wheat xylan+1 mM veratryl alcohol	20.2±1.19	70.6±4.04	4.90±0.26	95.7±5.23
1% Wheat xylan+1 mM ferulic acid	21.1±1.02	65.4±2.22	6.97±0.44	93.5±4.76

*The right side values indicate the standard deviation at significant level, $P>0.05$.

Table 4 ^{14}C distribution measured 14 days after inoculation of *P. radiata* on the substrates with 1% galactoglucomannan (including ^{14}C labeling) and 1 mM aromatic compounds

Substrates	$^{14}CO_2$ evolved [%]	^{14}C residue/mycelium [%]	^{14}C growth liquid [%]*	^{14}C total [%]
1% Red clover mannan	29.7±1.39	17.9±1.08	44.5±3.06	92.2±4.62
1% Red clover mannan+1 mM vanillic acid	32.1±1.71	11.7±0.79	47.8±3.71	91.7±4.92
1% Red clover mannan+1 mM veratric acid	18.4±1.16	21.4±1.42	50.9±2.48	90.7±5.06
1% Red clover mannan+1 mM veratraldehyde	16.7±1.45	13.1±0.65	62.0±4.98	91.1±5.23
1% Red clover mannan+1 mM veratryl alcohol	52.1±3.74	21.3±1.49	16.4±1.11	89.7±5.62
1% Red clover mannan+1 mM ferulic acid	52.0±2.68	15.5±1.22	23.3±1.93	90.8±4.99

*The right side values indicate the standard deviation at significant level, $P>0.05$.

polysaccharide. Additionally, arabinose, glucuronic acid, 4-*O*-methylglucuronic acid and glucose were determined (Table 2). In wheat flour, arabinoses is linked to the C_3 position of xylose, but some xylose units are also substituted to arabinose in both C_2 and C_3 positions [32]. The ratio of xylose to arabinose varies in wheat from 2:1 to 1:2.4 [24]. The basic skeleton of xylans found in the tissue of all land plants is a linear backbone of 1,4-anhydro-β-L-xylopyranose.

In the case of [^{14}C]mannan, the main components such as mannose, galactose, glucose as well as small amounts of arabinose are summarized in Table 2. These data show that xylan isolated from wheat was a rather pure fraction of arabinoxylan containing no mannose. The same applies to mannan obtained from red clover, which has a structure of galactoglucomannan without xylose units. Most of the mannose found in wood is present in the form of a glucomannan with relatively low molecular weight. The reported average degree of polymerization varies from 40 to 100 [7,9]. Softwoods contain about 25% mannan with a glucomannan backbone to which acetyl groups and galactose residues are attached. The portion of galactose units joined by β-(1,6) linkages is different in mannans isolated by water and by alkali extraction [35]. The acetyl groups seem to be distributed equally on the C_2 and C_3 of mannose units [21]. The mannans are also widespread in the vegetable kingdom where their structure is very similar to those found in softwood [9]. They are the main components of seeds, particularly important as a reserve nutrient during the germination of seeds [16]

Biodegradation of grass hemicelluloses

The white-rot fungi are the most efficient degraders of lignocellulosic components. *P. radiata*, an efficient degrader of lignin [13], was also characterized as a hemicellulolytic enzyme producer [27]. Degradation of ^{14}C-labeled wheat arabinoxylan by *P. radiata* growing on medium with low nitrogen concentration (2 mM) and the influence of some aromatic compounds on the process are shown in Table 3. Veratryl alcohol increased degradation of arabinoxylan by approximately 28.5%, whereas veratric acid increased degradation by only 9.0%, but the statistical analysis of this result shows no significant difference from reference data. Vanillic acid (16.6%) and ferulic acid (34.7%) also stimulated degradation by 16.6% and 34.7%, respectively. The addition of veratraldehyde resulted in about 23.6% suppression of carbon dioxide released in comparison to the control.

A similar tendency was observed in the case of ^{14}C-labeled red clover galactoglucomannan degradation (Table 4). Veratryl alcohol and ferulic acid increased degradation of mannan by approximately 75%. Veratric acid repressed $^{14}CO_2$ production by 38% as compared to the control. In the case of veratraldehyde, hemicellulose degradation was depressed by 43.8%. These low-molecular-weight aromatic compounds can increase the production of laccase, lignin peroxidase, manganese-dependent peroxidase and feedback-type enzymes such as glucose oxidase, cellobiosoquinone oxidoreductase and glyoxal oxidase (ligninolytic enzymes) in cultures of the white-rot fungus, *P. radiata* [29]. All the compounds tested increased production of laccase regardless of their nonphenolic or phenolic structure. Veratryl alcohol and veratraldehyde were the most potent stimulators of lignin peroxidase, whereas MnP was most effectively triggered by veratraldehyde supplementation [26]. According to a recent assumption, the molecular size of enzymes involved in wood decay does not permit complete penetration of wood. In the case of fungal cells possessing high-enough redox potential, the low molecular mediators of internal or external origin migrate to some distance from the enzymes and oxidize lignin [20]. A similar effect of several aromatic compounds was observed in the β-1,4-glucosidase activity, whereas exo-β-1,4-glucanase and endo-β-1, 4-glucanase activities significantly decreased in *Trametes versicolor* and *P. radiata* [22,28].

The dynamic rates of $^{14}CO_2$ evolution from arabinoxylan and galactoglucomannan during cultivation are presented in Figure 1.

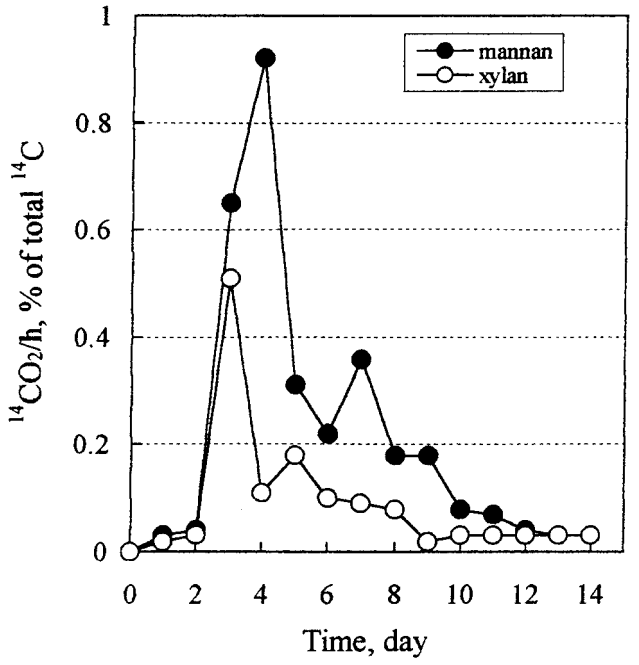

Figure 1 Degradation of arabinoxylan and galactoglucomannan by *P. radiata*.

The degradation processes for ^{14}C-labeled arabinoxylan and galactoglucomannan show slight similarities. In both cases, a single major maximum of carbon dioxide evolution was observed. Maximum arabinoxylan degradation was observed a little earlier than that of galactoglucomannan. As a conclusion, the degradation of hemicelluloses, e.g., mannan and xylan, by *P. radiata* is influenced by addition of aromatic compounds.

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