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Microbial Degradation of Hemicellulosic Materials

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ABSTRACT

Biodegradation of hemicelluloses requires enzyme activities that remove nonxylose substituents from the xylan backbone in addition to endoxylanases and β-xylosidases. Activities removing O-acetyl, arabinose, cinnamic acid-based esters, and uronic acid substituents occurred in *Streptomyces olivochromogenes, Aspergillus niger,* and *Schizophyllum commune.* 4-O-Methylglucuronidase was coinduced with other substituent-hydrolyzing enzymes when appropriate lignocellulosic materials were provided. Fractionation of crude enzymes indicated that the 4-O-methylglucuronidases were polydisperse. The 4-O-methylglucuronidases from *S. commune* and *A. niger* acted on high molecular weight heteroxylans, but those from *S. olivochromogenes* functioned only with small, endo-β-(1,4)-xylanse-solubilized fragments.

Index Entries: 4-O-methylglucuronidase; endo-β-(1,4)-xylanase; hemicellulose; lignocellulose; degradation.

INTRODUCTION

Cellulose hydrolysis into monomeric units requires the action of only three enzymes, endoglucanase, exoglucanase, and β-glucosidase, but hemicellulose degradation is considerably more complicated because of the presence of non-xylose substituents on the xylan backbone. Such sub-

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stituents include O-acetyl groups, arabinose, uronic acids, and cinnamic acid-based esters (*1–3*). Complete degradation of hemicelluloses, therefore, requires the action of substituent-hydrolyzing enzymes in addition to endo-β-(1,4)-xylanase (E.C. 3.2.1.8) and β-xylosidase (E.C. 3.2.1.37) activities. The importance of substituent-hydrolyzing activities in the biodegradation of plant cell wall material is evidenced by the fact that lower endo-β-(1,4)-xylanase-mediated sugar release occurs when they are absent (*4,5*), and that activities such as acetyl xylan esterase (*6*) and α-L-arabinofuranosidase (*5*), enhance the activity of endo-β-(1,4)-xylanases on hemicellulosic substrates. Acetyl xylan esterase and arabinosidase have been the objects of several studies, but little information on α-glucuronidases is available. Crude xylanase preparations from *Trichoderma reesei* have been reported to contain α-glucuronidase activity (*7*), and more recently, Puls et al. (*8*) demonstrated 4-O-methylglucuronidases from *Agaricus bisporus* and *Pleurotus ostreatus* that were active on beechwood 4-O-methylglucurono-D-xylan. In addition, we have recently demonstrated the presence of 4-O-methylglucuronidase in *Streptomyces* spp. (*9*). In some plant cell wall hemicelluloses, some of the arabinosyl substituents are esterified with ferulic and coumaric acids (*10,11*), and, further, it has been suggested that xylans in plant cell walls may be crosslinked or complexed with lignin by diferulate bridges (*12,13*). Recently, an activity that exists as part of the extracellular cellulolytic/xylanolytic enzyme complex from *Streptomyces olivochromogenes* and *Streptomyces flavogriseus* and is capable of releasing ferulic acid from wheat bran was demonstrated (*9,14*). The exact relationship of this enzyme system to endo-β-(1,4)-xylanase is presently unclear, but ferulic acid esterase does play some role in the degradation of hemicellulose.

The following study was undertaken to gain further insight into the nature of substituent-hydrolyzing enzymes from *S. olivochromogenes, A. niger,* and *S. commune,* with particular emphasis on their 4-O-methylglucuronidase systems.

MATERIALS AND METHODS

Organisms and Growth Conditions

Streptomyces olivochromogenes (National Research Culture Collection [NRCC] 2258) and *Aspergillus niger* (NRCC 401127) were grown in a proteose peptone-yeast extract mineral salts medium (1 L/4 L baffled flask) (*15*) at 37 and 30°C, respectively in a gyratory shaker operated at 250 cycles/min. *Schizophyllum commune* (ATCC 38548) (1 L/4 L baffled flask) was grown in the medium described by Desrochers et al. (*16*) at 30°C in a gyratory shaker operated at 150 cycles/min. Avicel PH 105 was supplemented at a final concentration of 1% (w/v). Supernatant fractions were retained for enzyme assay and fractionation.

Enzyme Substrates

Wheat bran was treated with 0.25% (w/v) potassium acetate at 95°C for 10 min followed with extensive washing with water to remove starch present in the untreated material resulting from contamination with endosperm material. Larchwood xylan (Sigma Chemical Company) was washed several times with methanol to remove colored materials, and was subsequently dialyzed against distilled water and was lyophilized. This material was designated "high molecular weight larchwood xylan." Low molecular weight xylan was obtained as follows: 5 g of high molecular weight larchwood xylan were suspended in 200 mL 10 m*M* potassium phosphate buffer, pH 6.0 containing 0.02% (w/v) sodium azide. After the addition of 2 mL of purified *S. commune* endo-β-(1,4)-xylanase (specific activity = 1,851 U/mg protein), the suspension was incubated at 37°C for 18 h. Following centrifugation of the enzyme digest at 48,000×g for 20 min, the supernatant fraction was incubated at 75°C for 20 min to deactivate endo-β-(1,4)-xylanase. The preparation was then lyophilized and was designated "low molecular weight larchwood xylan."

Enzyme Assays

Endo-β-(1,4)-xylanase, acetyl xylan esterase, L-α-arabinofuranosidase, ferulic acid esterase, and 4-O-methylglucuronidase were measured as described previously (*9,14*). For the first two enzymes, one enzyme unit was defined as the amount of enzyme catalyzing the release of 1 μmole of product per minute at pH 6.0 and 50°C. For remaining enzymes, one enzyme unit was defined as the amount of enzyme catalyzing the release of 1 μmole of product per hour at pH 6.0 and 50°C. Protease was measured with solid Azocoll substrate (Calbiochem), as previously described (*17*).

Analytical Techniques

Protein was measured by the method of Bradford (*18*) using gamma globulin as standard. Mycelial protein was estimated using Benedict's reagent (*19*).

Preparation of Cell-Free Extracts

Cell-free extracts of *S. olivochromogenes* were prepared from lysozyme-EDTA extracts, as previously described (*17*).

Enzyme Fractionations

Culture supernates were concentrated and dialyzed *in situ* against 10 m*M* potassium phosphate buffer, pH 6.0 containing 0.02% (w/v) sodium azide using a Pellicon ultrafiltration apparatus (Millipore Corp.) equipped with 10,000 NMWL membranes. Anion exchange chromatography was

conducted on 2.6×25 cm columns of DEAE Bio-Gel A (Bio-Rad Laboratories) equilibrated with 10 m*M* potassium phosphate buffer, pH 6.0 containing 0.02% (w/v) sodium azide, as previously described (*9*), or by high performance liquid chromatography (HPLC). Twenty mg of protein were applied to 7.5×150 mm DEAE-TSK columns (LKB, Sweden) and samples were eluted at a flowrate of 1 mL/min using the indicated NaCl gradients in the above buffer. Similarly, cation exchange chromatography was performed by HPLC using 7.5×150 mm CM-3SW columns (LKB, Sweden). Hydrophobic interaction HPLC was performed using 7.8×150 mm Phenyl-5-PW columns (LKB, Sweden). Five mg of protein were placed in 10 m*M* potassium phosphate buffer, pH 6.0, containing 1 *M* ammonium sulfate. Samples were eluted at a flowrate of 1 mL/min using a 1-0 *M* ammonium sulfate gradient.

RESULTS

Growth and Production of Hemicellulose-Hydrolyzing Enzymes

The kinetics of growth and enzyme production for *A. niger* grown in IAF medium containing 1% (w/v) wheat bran are depicted in Fig. 1. Growth as measured by the accumulation of mycelial protein continued up to 96 h. Endo-β-(1,4)-xylanase, acetyl xylan esterase, and arabinosidase attained near maximal or maximal levels at 72 h, but 4-O-methylglucuronidase activity peaked at 48 h and then fell continuously during the remainder of growth. By contrast, ferulic acid esterase levels increased during the entire growth cycle. Absolute values varied, but essentially the same patterns of growth and enzyme production were observed with *S. olivochromogenes* under the above culture conditions.

In cultures of *S. commune*, all measured enzyme activities, with the exception of ferulic acid esterase, were readily detectable by the third day of growth (Fig. 2). Extracellular protein peaked at 12 d growth and declined thereafter. Arabinosidase reached maximal levels at d 12, and then diminished slightly, but levels of all other hemicellulose-degrading enzymes rose continuously during growth.

Although all measured hemicellulose-degrading enzyme activities were detectable in basal media lacking lignocellulosic materials, highest levels were obtained when *S. olivochromogenes* and *A. niger* were grown in wheat bran-supplemented media (data not shown). Previous studies indicated that *S. commune* presented highest enzyme levels when grown on the microcrystalline cellulose, Avicel (*20*). Levels of enzyme activity for the three organisms grown as described above are presented in Table 1. Of these three organisms, *S. olivochromogenes* was the poorest source of the hemicellulose-degrading enzymes, both in terms of absolute amount (U/mL) and in specific activity. Although *A. niger* produced high absolute levels of acetyl xylan esterase, endo-β-(1,4)-xylanase, arabinosidase, and

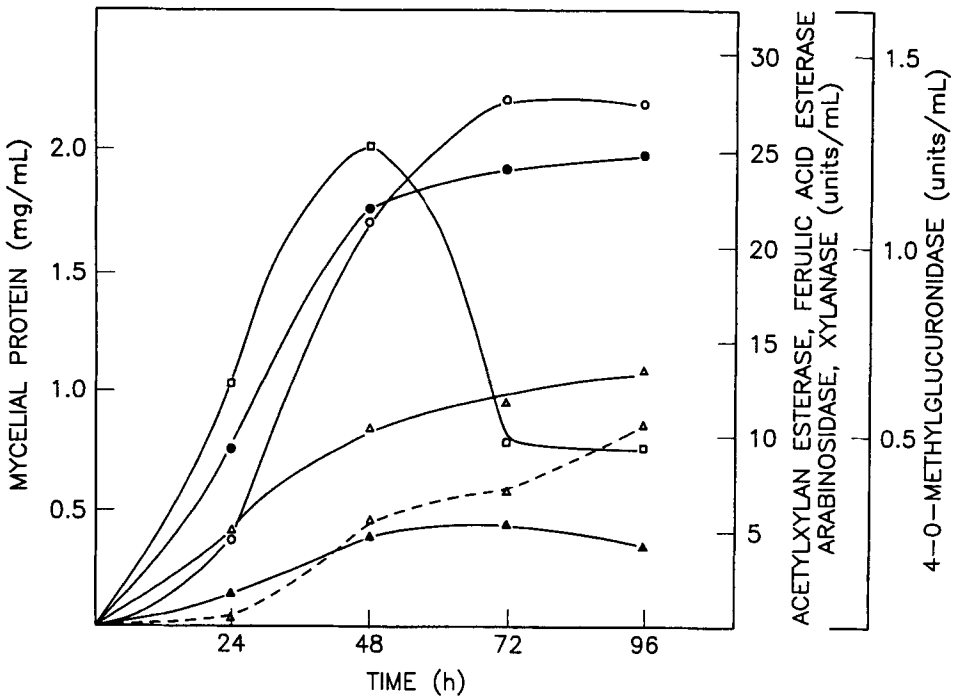

Fig. 1. Production of hemicellulose-degrading enzymes by *Aspergillus niger* grown in wheat bran-supplemented cultures. Mycelial protein (●——●); endo-β-(1,4)-xylanase (○——○); 4-O-methylglucuronidase (□——□); acetyl xylan esterase (△——△); ferulic acid esterase (△------△); arabinosidase (▲——▲).

ferulic acid esterase, the high specific activities observed were a result of low extracellular protein concentrations which rarely exceeded 0.1 mg/mL of culture. In all respects, *S. commune* was a superior source of acetyl xylan esterase, endo-β-(1,4)-xylanase, and 4-O-methylglucuronidase.

Studies with Crude *Streptomyces olivochromogenes* Hemicellulose-Degrading Enzymes

Fractionation of extracellular concentrates from wheat bran-supplemented cultures of *S. olivochromogenes* by cation exchange chromatography disclosed the existence of one neutral 4-O-methylglucuronidase accounting for approximately 35% of input activity, and three cationic forms of the enzyme (Fig. 3). In this instance, 4-O-methylglucuronidase was measured with high molecular weight larchwood xylan. The three cationic 4-O-methylglucuronidases coeluted with endo-β-(1,4)-xylanase activities with 0.27, 0.42, and 0.6 *M* NaCl, respectively.

Since all of the above subfractions contained both 4-O-methylglucuronidase and endo-β-(1,4)-xylanase activities, intracellular material was examined to determine if it were possible to obtain endo-β-(1,4)-xylanase-free 4-O-methylglucuronidase. Intracellular components from a 1-L culture

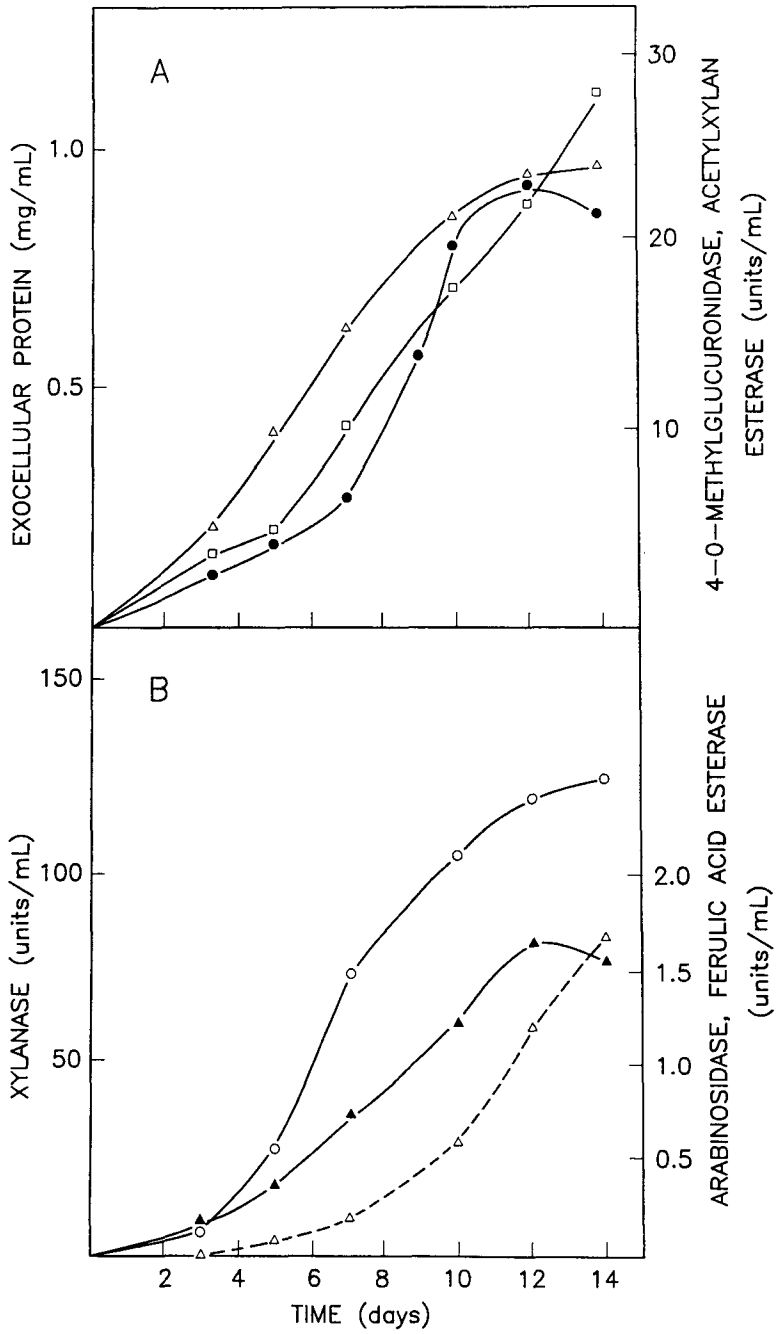

Fig. 2. Production of hemicellulose-degrading enzymes by *Schizophyllum commune* grown in Avicel-supplemented cultures. Mycelial protein (●——●); endo-β-(1,4)-xylanase (○——○); 4-O-methylglucuronidase (□——□); acetyl xylan esterase (△——△); ferulic acid esterase (△------△); arabinosidase (▲——▲).

Table 1
Hemicellulose-Hydrolyzing Enzymes from *Streptomyces olivochromogenes*, *Aspergillus niger*, and *Schizophyllum commune*

Organism	Enzyme activity[a]	U/mL	Specific activity[b]
S. olivochromogenes	Acetyl xylan esterase	4.98	12.34
	Arabinosidase	1.91	4.74
	Endo-β-(1,4)-xylanase	11.59	28.75
	Ferulic acid esterase	1.20	2.97
	4-O-Methylglucuronidase	1.73	4.29
A. niger	Acetyl xylan esterase	14.04	175.50
	Arabinosidase	5.98	74.75
	Endo-β-(1,4)-xylanase	27.07	338.38
	Ferulic acid esterase	10.58	132.25
	4-O-Methylglucuronidase	1.02	12.75
S. commune	Acetyl xylan esterase	22.50	22.28
	Arabinosidase	1.65	1.79
	Endo-β-(1,4)-xylanase	121.50	131.52
	Ferulic acid esterase	1.20	1.30
	4-O-Methylglucuronidase	22.00	23.91

[a]All enzyme levels were obtained from 96 h cultures of *S. olivochromogenes* and *A. niger* except for 4-O-methylglucuronidase, which was determined from 48 h cultures. Enzyme levels for *S. commune* are from 12-d cultures.
[b]Specific activity is expressed as U/mg protein.

of *S. olivochromogenes* grown for 48 h in IAF basal medium supplemented with 1% (w/v) wheat bran were prepared as described in Materials and Methods. Analysis of intracellular material indicated that under these growth conditions, approximately 50% of the total culture 4-O-methylglucuronidase activity was cell-associated, whereas more than 98% of the total endo-β-(1,4)-xylanase was extracellular. Results of different treatments on the intracellular 4-O-methylglucuronidase from *S. olivochromogenes* are presented in Table 2. Independently, neither enzyme preparation released 4-O-methylglucuronic acid from high molecular weight larchwood xylan. Simultaneous addition of endo-β-(1,4)-xylanase to 4-O-methylglucuronidase reaction mixtures, however, resulted in release of 4-O-methylglucuronic acid. Activity was elevated more than threefold when the substrate was preincubated with the endo-β-(1,4)-xylanase. Thermal deactivation of endo-β-(1,4)-xylanase after 30 min pre-incubation prior to addition of 4-O-methylglucuronidase resulted in a significant reduction of activity. These data clearly indicate that intracellular 4-O-methylglucuronidase from *S. olivochromogenes* requires low molecular weight substrate. For this reason, low molecular weight larchwood xylan was used as substrate in the following experiments unless otherwise specified.

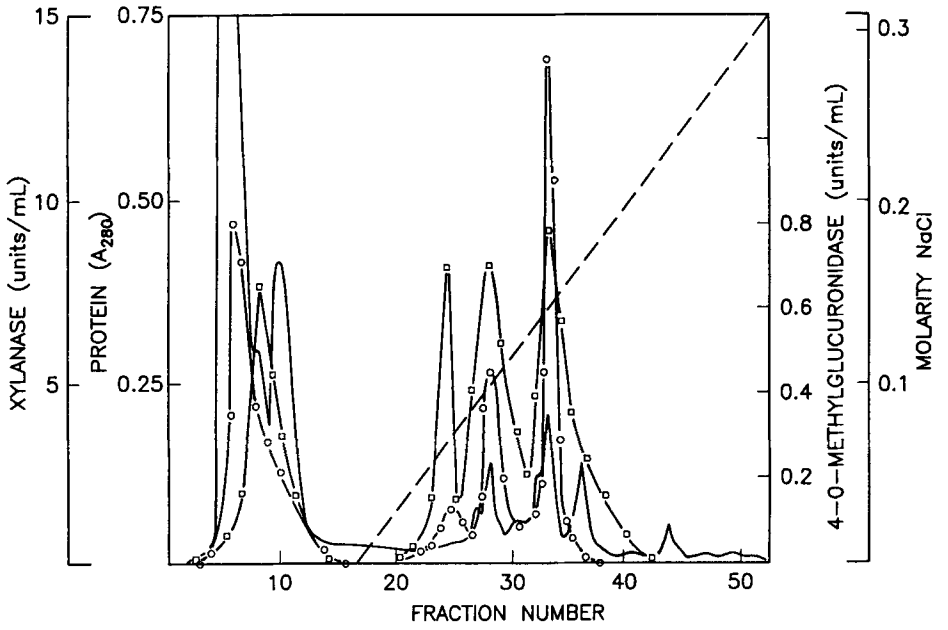

Fig. 3. Fractionation of *Streptomyces olivochromogenes* 4-O-methylglucuronidase and endo-β-(1,4)-xylanase by HPLC. Two mg protein were fractionated on a CM-3SW column. Molarity NaCl (----------); protein (———); 4-O-methylglucuronidase (□——□); endo-β-(1,4)-xylanase (○——○).

Fractionation of 4-O-Methylglucuronidase from *Aspergillus niger*

The elution profile of crude ultrafiltrates from *A. niger* fractionated by HPLC on anion exchange columns as described in Materials and Methods appears in Fig. 4. Three neutral 4-O-methylglucuronidase activities and five anionic activities eluting with 0.05, 0.18, 0.24, 0.38, and 0.48 *M* NaCl, respectively, were observed. Neither the input material nor effluent fractions contained detectable protease activity. Enzyme activity in fractions 17–21 inclusive, and in fractions 30–33 inclusive were pooled, concentrated, and dialyzed *in situ* using an Amicon ultrafiltration cell equipped with YM-2 membranes, and were designated "*A. niger* 4-O-methylglucuronidase 1" and "*A. niger* 4-O-methylglucuronidase 2," respectively. Neither of these fractions contained detectable endo-β-(1,4)-xylanase activity.

Fractionation of 4-O-Methylglucuronidase from *Schizophyllum commune*

Initial fractionation of crude extracellular ultrafiltrates from *S. commune* (1 g protein) was achieved by anion exchange chromatography on DEAE Bio-Gel A (Fig. 5). Most of the endo-β-(1,4)-xylanase activity eluted

Table 2
Effect of Various Treatments on Intracellular 4-O-Methylglucuronidase from *Streptomyces olivochromogenes*[a]

Treatment	Activity, μmols/h/mg protein	Relative percent activity
4-O-Methylglucuronidase alone	0	0
Endo-β-(1,4)-xylanase alone	0	0
4-O-Methylglucuronidase + endo-β-(1,4)-xylanase[b]	3.86	100
30 Min pre-incubation with endo-β-(1,4)-xylanase, followed by addition of 4-O-methylglucuronidase	12.46	323
60 Min pre-incubation with endo-β-(1,4)-xylanase, followed by addition of 4-O-methylglucuronidase	13.83	358
30 Min pre-incubation with endo-β-(1,4)-xylanase, 5 min at 100°C, followed by addition of 4-O-methylglucuronidase	7.27	199

[a]High molecular weight larchwood xylan (2% w/v) was used as substrate throughout using 0.46 mg 4-O-methylglucuronidase per assay.
[b]Endo-β-(1,4)-xylanase (50 U per test) was a purified preparation from *S. commune*.

in the wash fraction, but a small amount of activity was co-eluted with arabinosidase and 4-O-methylglucuronidase by 0.18 *M* NaCl. Four minor 4-O-methylglucuronidase activities eluting with 0.07, 0.14, 0.18, and 0.32 *M* NaCl, respectively, and one major activity eluting with 0.24 *M* NaCl were observed. After pooling, dialyzing, and concentrating the major 4-O-methylglucuronidase activity found in fractions 308–318 inclusive (designated "*S. commune* 4-O-methylglucuronidase 1"), the material was subjected to further fractionation by hydrophobic interaction chromatography, as described in Material and Methods. As the data in Fig. 6 indicate, six hydrophobic forms of 4-O-methylglucuronidase activity were distinguishable. Of these, only one activity eluting with 0.1 *M* $(NH_4)_2SO_4$ was clearly associated with a protein peak. After pooling of fractions 33–36 inclusive, and concentration and dialysis, as previously described, this material was designated "*S. commune* 4-O-methylglucuronidase 2." This particular subfraction was devoid of detectable endo-β-(1,4)-xylanase activity.

Activity of 4-O-Methylglucuronidases with High and Low Molecular Weight Larchwood Xylan

Various enzyme preparations, as designated below, were tested for their ability to release 4-O-methylglucuronic acid from high and low mo-

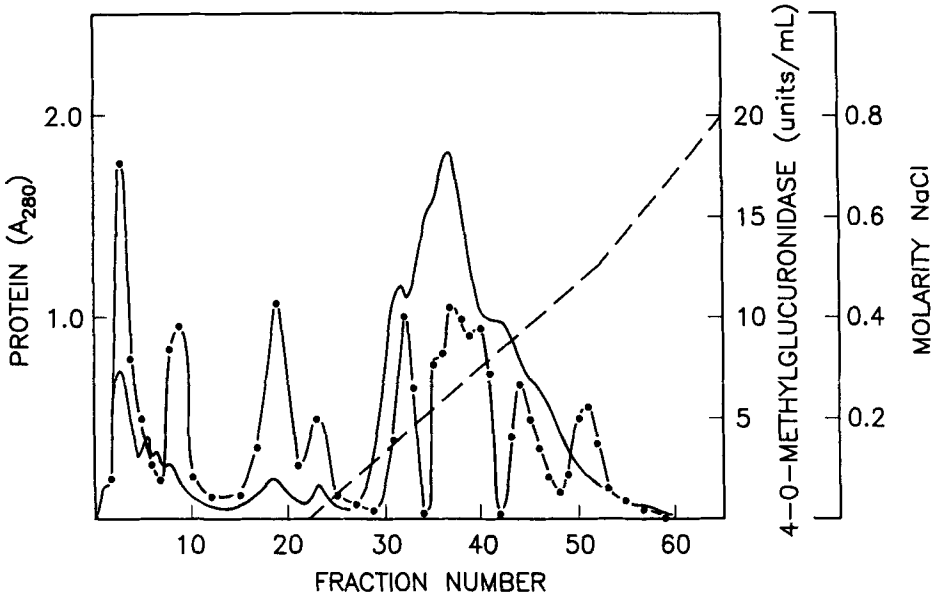

Fig. 4. Fractionation of *Aspergillus niger* 4-O-methylglucuronidase by HPLC. Twenty mg protein were fractionated on a TSK-DEAE column. Molarity NaCl (----------); protein (————); 4-O-methylglucuronidase (●——●).

lecular weight larchwood xylan in the presence and absence of purified *S. commune* endo-β-(1,4)-xylanase. For *S. olivochromogenes* only the intracellular 4-O-methylglucuronidase was used. Appropriate dilutions were used such that the small amounts of contaminating endo-β-(1,4)-xylanase activity were below levels of detection. For *A. niger* and *S. commune*, respective 4-O-methylglucuronidases 1 and 2 as described above served as enzymes. Results are presented in Table 3. In the absence of endo-β-(1,4)-xylanase, intracellular 4-O-methylglucuronidase was the sole activity unable to function with high molecular weight substrate, although the enzyme would degrade the low molecular weight substrate. The 4-O-methylglucuronidases from *A. niger* and *S. commune* were active on both substrates, but clearly preferred the high molecular weight larchwood xylan. In all cases, addition of endo-β-(1,4)-xylanase enhanced 4-O-methylglucuronidase activity.

DISCUSSION

Given the premise that biodegradation of hemicellulose is heavily dependent on the presence of substituent-hydrolyzing activities, all three organisms studied here are well equipped to mediate such processes. Although the growth cycle of *S. commune* is much longer than for *S. olivochromogenes* and *A. niger*, this system offers a number of advantages: high

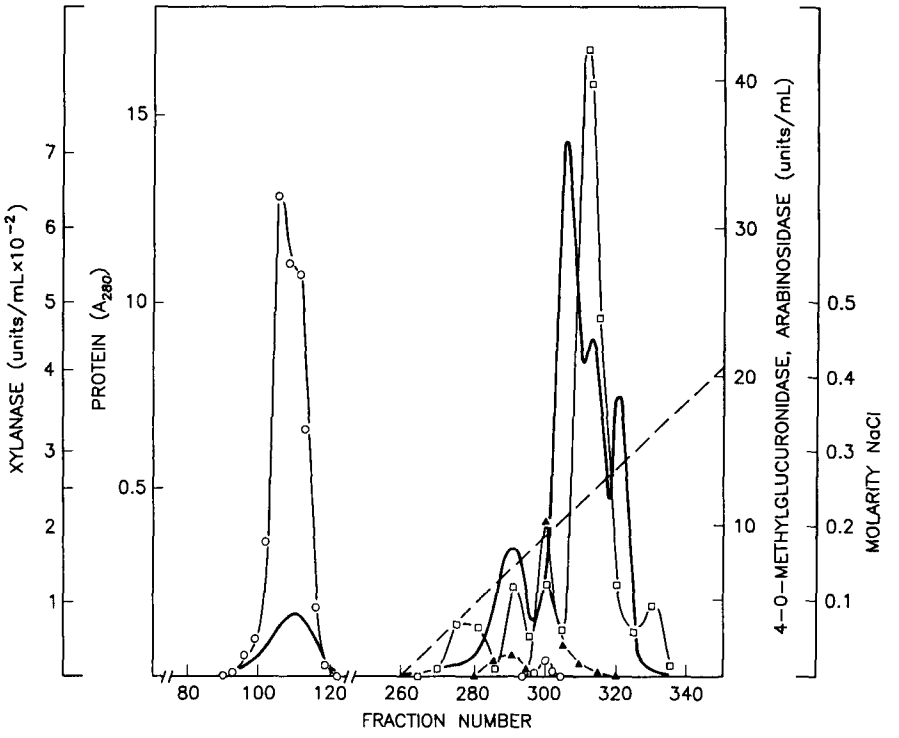

Fig. 5. Fractionation of *Schizophyllum commune* hemicellulose-hydrolyzing enzymes by DEAE Bio-Gel A chromatography. Molarity NaCl (----------); protein (————); arabinosidase (▲——▲); endo-β-(1,4)-xylanase (○——○); 4-O-methylglucuronidase (□——□).

levels of acetyl esterase, endo-β-(1,4)-xylanase, and 4-O-methylglucuronidase (Table 1), lack of contaminating extracellular pigments encountered in the other systems, relatively high levels of extracellular protein, and lack of detectable proteases. However, *A. niger* provides an excellent source of ferulic acid esterase and arabinosidase where levels were 10- and threefold greater, respectively, than in the other two systems.

Data presented in Table 2 clearly indicate that the intracellular 4-O-methylglucuronidase from *S. olivochromogenes*, like the α-glucuronidase from *A. bisporus* (*8*), preferred low molecular weight substrate. Pre-incubation of substrate with endo-β-(1,4)-xylanase rather than simultaneous addition to reaction mixtures resulted in a threefold increase of 4-O-methylglucuronidase activity. The observed difference between 4-O-methylglucuronidase activity in tests where the endo-β-(1,4)-xylanase was heat-deactivated is likely a measure of the synergistic effect of endo-β-(1,4)-xylanase on 4-O-methylglucuronidase activity. Unlike the intracellular activity from *S. olivochromogenes*, the extracellular 4-O-methylglucuronidases from *A. niger* and *S. commune* were active on high molecular weight larchwood xylan (Table 3). To our knowledge, this is the first report of such ac-

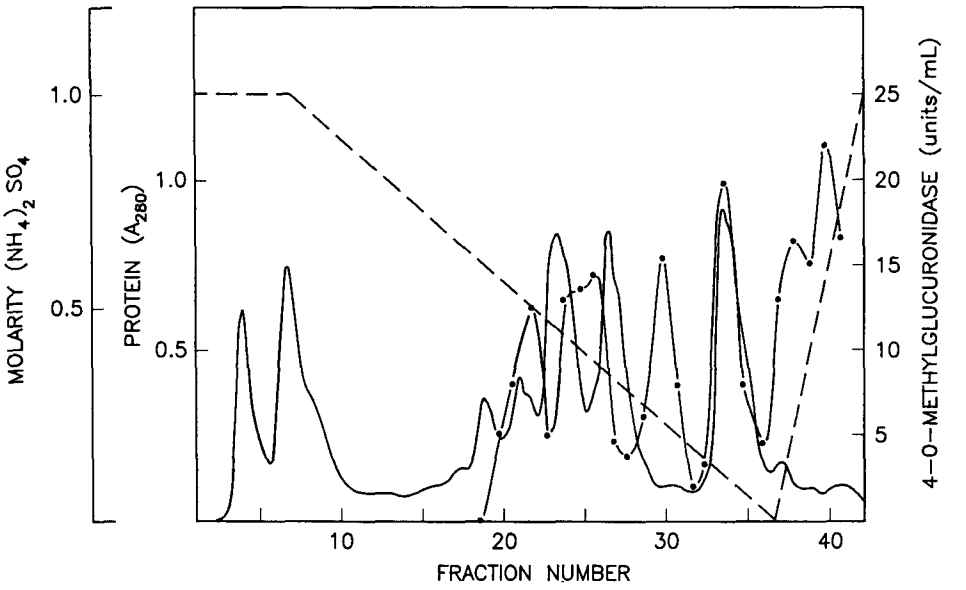

Fig. 6. Fractionation of *Schizophyllum commune* 4-O-methylglucuronidase by hydrophobic interaction chromatography. Five mg of protein from the major fraction of 4-O-methylglucuronidase activity obtained from DEAE Bio-Gel A chromatography (*see text*) were fractionated on a phenyl-5-PW column. Molarity $(NH_4)_2SO_4$ (----------); Protein (————); 4-O-methylglucuronidase (●——●).

Table 3
Activity of Various 4-O-Methylglucaronidases on High and Low Molecular Weight Larchwood Xylan[a]

	Activity, μmol/h/mg protein			
	−Endo-β-(1,4)-xylanase[b]		+Endo-β-(1,4)xylanase	
Enzyme	High LWX	Low LWX	High LWX	Low LWX
S. olivochromogenes intracellular mGuAse	0	3.9	3.1	4.6
A. niger mGuAse 1	156.8	96.4	166.8	143.2
A. niger mGuAse 2	468.2	165.3	519.5	182.2
S. commune mGuAse 1	3,482.4	237.4	8,313.6	414.7
S. commune mGuAse 2	45.5	13.0	160.9	42.5

[a]Abbreviations used are: High LWX, high molecular weight larchwood xylan; Low LWX, low molecular weight larchwood xylan; mGuAse, 4-O-methylglucuronidase.
[b]Endo-β-(1,4)-xylanase (24 U/test) was a purified preparation from *S. commune*.

tivity on high molecular weight glucurono-D-xylans. Moreover, although endo-β-(1,4)-xylanase was not required for 4-O-methylglucuronidase activity, its addition to the endo-β-(1,4)-xylanase-free subfractions resulted in increased liberation of 4-O-methylglucuronic acid in every case. These findings support the hypothesis that the synergy known to occur between acetyl xylan esterase and endo-β-(1,4)-xylanase (*3,4,6*) is operative in the 4-O-methylglucuronidase system, and may have equally great importance in the degradation of lignocellulosic materials.

Fractionation studies of the 4-O-methylglucuronidase preparations from each of the organisms provided some insight into the complexity of the xylanolytic enzyme systems. In each case, multiple forms of 4-O-methylglucuronidase activity were observed. Possibly, multiple enzymes in the *S. olivochromogenes* system may arise from proteolysis since copious amounts of protease are co-excreted with xylanolytic proteins. However, quantitative maintenance of *S. olivochromogenes* enzyme activities during purification militates against proteolysis as a major factor in the generation of such polydispersity. Enzyme preparations from *A. niger* and *S. commune* did not contain detectable protease activity. Hence, the presence of multiple 4-O-methylglucuronidase activities in such genetically diverse organisms suggests some commonality resulting from either the excretion process, or perhaps from differences in substrate specificities of the various isoenzymes. Their purification to molecular homogeneity and determination of physicochemical and kinetic properties will hopefully provide further insight.

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