EXTRACELLULAR POLYSACCHARIDE-DEGRADING ENZYMES OF POSTIA PLACENTA ISOLATED FROM WOOD OR ARTIFICIAL MEDIA

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INTRODUCTION

A variety of extracellular polysaccharidases produced by brown-rot fungi are thought to play an important role in the wood-rotting process. There is evidence of early removal of hemicellulose, a principal cell wall constituent, by hemicellulases (xylanases, mannanase), which attack and shorten hemicellulose chains sufficiently for further hydrolysis by glycosidases into simple sugars, thus facilitating colonization and penetration of wood (Kirk and Highley, 1973; Kirk and Cowling, 1984; Highley, 1987). The enzyme b-1,4-xylanase, of the brown-rot fungus Postia placenta (Fr.) M. Lars. et Lomb. MAD 698, has been partially purified (Green et al., 1989b) and used as antigen to produce monoclonal antibodies. This glycoprotein typically co-migrates with carboxymethyl-cellulase and has been comprised of as much as 50 percent carbohydrate when extracted from wood.

Highley et al. (1981) qualitatively and quantitatively compared the polysaccharide-degrading enzymes produced by Postia placenta in decayed wood and liquid culture. Quantitative differences were observed in enzyme activities from decayed wood as opposed to liquid media. However, despite these quantitative differences, the authors concluded that the extracellular polysaccharide complex from liquid culture must be structurally similar to that of decayed wood because of similar isoelectric points, electrophoretic properties, and molecular sieving properties,
Culturing brown-rot fungi in artificial liquid medium is a common laboratory practice. The ability to produce large volumes of culture filtrate is an attractive means of procuring enzyme for purification. However, the availability of low molecular weight sugars in artificial media results in excess glucan production, which complicates enzyme purification (C. Clausen, personal observation). We postulated that the increased glucan production noted in liquid cultures of *Postia placenta* MAD 698 (Micales et al., 1990) may alter the type and amount of enzyme produced or disguise antigenic epitopes, thereby altering antibody (Ab) recognition of the antigen, i.e., enzyme. An atypical isolate of *P. placenta*, (ME-20), was also included in this study. As reported by Micales et al. (1990) and Green et al. (1992a), this isolate fails to produce extracellular glucan in liquid culture or to cause weight loss during colonization of wood.

The extracellular polysaccharidases and the role of the glucan sheath were the focus of this study. Our objectives were (1) to analyze differences in enzyme production, glucan composition, and antibody recognition and (2) to determine the differences in brown-rot decay organisms grown on wood as opposed to artificial growth medium.

MATERIALS AND METHODS

Culture Conditions

*Postia placenta* (Fr.) M. Lars., et Lomb. [= *Poria placenta* (Fr.) Cke.] MAD 698 and ME-20 were grown either in 2 percent malt agar (Difco, Detroit, MI), in basal salts solution (Highley, 1973) with 1 percent cellobiose (Sigma, St. Louis, MO), or on southern pine (*Pinus sp.*) in a standard ASTM soil-block method (ASTM, 1971)

Solubilization and Extraction Methods

A 0.1 percent solution of Triton X-100 (Sigma) in glass-distilled water was used to solubilize the mycelial mat of the liquid culture, the culture filtrate, or the decayed wood block. Gentle rocking for 2 hr at 25°C sufficiently solubilized the hyphae and extracted the extracellular enzymes.

Enzyme and Protein Assays

The activities of endo-β-1,4-glucanase, endo-β-1,4-xylanase, endo-β-1,4-mannanase, and endo-β-1,3-glucanase were assayed by the reducing sugar microassay (Green et al., 1989a) using laminarin (Sigma), larch xylan (United States Biochemical Co., Cleveland, OH), guar gum (Nutritional
Biochemical Corp., Cleveland, OH), and carboxymethylcellulose (Hercules, Wilmington, DE), respectively. One unit of enzyme activity was defined as the amount needed to liberate reducing power equivalent to 1 mg glucose per 24 hr at 40°C using D-glucose (Fischer Scientific Co., Fair Lawn, NJ) as standard.

The b-D-galactosidase and a-D-galactosidase activities were assayed by determining the liberation of p-nitrophenol from respective p-nitrophenol substrates (Agrawal and Bahl, 1968). A unit of enzyme activity was defined as the amount needed to liberate 1 mM of p-nitrophenol per hour.

Total protein was determined by the BCA microassay (Pierce, Rockford, IL) using bovine serum albumin (Sigma) as a standard.

**Carbohydrate Determination**

Carbohydrate content of individual extracts was determined by HPLC with an Aminex HPX-87P (Biorad, Rockville Centre, NY) (Pettersen and Schwandt, 1991).

**Gel Filtration**

Molecular weight estimates were determined on a 90- by 26-cm Fractogel TSK HW-55(F) (EM Science, Gibbstown, NJ) column equilibrated with 0.1 M acetate buffer, pH 4.0. Gel filtration molecular weight markers were cytochrome C, carbonic anhydrase, and bovine albumin (Sigma).

**Monoclonal Antibody**

BALB/c mice were immunized with the xylanase fraction of R. placenta-decayed wood that had been partially purified by column chromatography (Green et al., 1989b). Antigen was mixed 1:1 with Freund’s complete adjuvant and mice were boosted at 4-week intervals (Stahli et al., 1983). Hybridomas were produced by conventional HAT selection techniques (Galfre and Milstein 1981). Polyethylene glycol fusion of NS-1 myeloma cells to mouse spleen cells yielded hybridomas that were cloned by dilution cloning and characterized for xylanase specificity (Clausen et al., 1992).

**Enzyme-linked Immunosorbent Assay (ELISA)**

Polystyrene 96-well ELISA plates were coated overnight at 25°C with antigen (protein extracts) in 0.1 M Tris buffer with 0.15 M NaCl, pH 9.0, rinsed five times with coating buffer, and blocked with PBS-Tween 20 (Sigma) for 15 min. A 1:1000 dilution of monoclonal antibody (F I ) to P. placenta xylanase was added to each well and incubated for 2 hr. The plate was washed with PBS-Tween and incubated with secondary antibody (horseradish peroxidase
labeled goat anti-mouse IgG) (Hyclone, Logan, UT) for 2 hr. Plates were thoroughly washed with water, developed with o-phenylene diamine, and read at optical density (OD₄₉₀).

**Western Blot**

Protein extracts and prestained molecular weight standards (Biorad) were separated by SDS-PAGE using precast 12-percent gels (Biorad) and transferred to nitrocellulose paper using a Biorad Transblot apparatus. The transfer was conducted in 25 mM Tris-HCl, 192 mM glycine, 20 percent methanol, pH 8.3 at 100 V for 2 hr. The blot was then blocked for 1 hr in Tris buffered saline (TBS) (0.02 M Tris, 0.5 M NaCl, ph 7.5) with 3 percent gelatin, rinsed briefly with distilled water, and incubated with a 1:1000 dilution of monoclonal antibody (MAb) for 2 hr at 25°C. The blot was washed with TBS (with 0.05 percent Tween 20 (TBS-Tween)) and incubated with horseradish peroxidase conjugated goat anti-mouse IgG in TBS with 1 percent gelatin for 2 hr. The was again rinsed with TBS-Tween and developed in 60 mg 4-chloro-1-naphthol (Sigma) in 20 ml methanol and 60 ml hydrogen peroxide in 100 ml TBS (Towbin et al., 1979).

**Affinity Chromatography**

Monoclonal antibody specific for xylanase of *P. placenta* was coupled to CNBf-activated Sepharose 4B (Pharmacia, Piscataway, NJ) per Pharmacia Principles and Methods. Gel swelled in 1 mM HCl was washed and coupled to the MAb at 4°C for 18 hr. Extracts were applied to the column in 50 mM phosphate buffer, 0.15 M NaCl, and 0.1 percent Tween 20. Unbound enzyme was washed from the column, and bound enzyme was eluted in 100 mM glycine-HCl, pH 2.6, 0.15 M NaCl, and 0.01 percent Tween 20, and neutralized with 1 M phosphate buffer, pH 7.5. The eluted protein peak was assayed for polysaccharidases as previously described (Green et al. 1989a).

**Deglycosylation**

Liquid culture supernatant of MAD 698 was treated with N-Glycosidase F (PGNase F, Boehringer-Mannheim, Indianapolis, IN) to determine the affect of deglycosylation on antigen binding to the MAb affinity column. Twenty units of PGNase were incubated for 17 h at 37°C in 100 mM sodium acetate buffer, pH 5.0, before binding to the affinity column. An untreated sample served as a control. ELISA as previously described was also performed on treated and untreated samples,
RESULTS

Enzyme extracts were prepared from \textit{P. placenta} MAD 698 and ME-20 grown on wood, on agar, or in liquid culture. For isolate MAD 698, the wood extract exhibited the highest protein to carbohydrate ratio (0.116) compared with culture supernatant, which had the lowest ratio (0.029). The extracted mycelial mat from the MAD 698 liquid culture had the highest galactosidase and hemicellulase activities. The water extract from ME-20-decayed wood contained galactosidase activities similar to that of the water extract from MAD 698-decayed wood. These extracts also contained similar carboxymethylcellulase (CMCase) activity and high laminarinase activity. Mannanase was low or not detectable in all the extracts.

ELISA results using a MAb to xylanase of \textit{P. placenta} MAD 698 demonstrated high readings (\text{OD}_{490}) for all MAD 698 extracts regardless of growth substrate (0.72-1.60) (Table 1). The ME-20 extract, however, was not recognized by the MAb in ELISA (\text{OD}_{490} = 0.05-0.51). Likewise, all extracts of MAD 698 bound to two bands on a western blot at 32 and 36 kDa molecular weight (Clausen et al., 1992). Gel filtration chromatography estimates the molecular weight of MAD 698 xylanase at 32 kDa.

Total carbohydrate was highest in the supernatant of MAD 698 liquid culture (21.54 mg/ml) compared to the wood extract (9.40 mg/ml) (Table 2). Sugar composition varied for each extract. Sugars from the ME-20 water extract were predictably negligible. Sugar content was also low in the MAD 698 water extract, but glucose and mannose were measurable. Samples solubilized with Tritox X-100 had markedly higher carbohydrate content. The glucose, galactose, xylose, and mannose content of the MAD 698 culture was twice that of the MAD 698 wood extract. The MAD 698 agar extract contained high amounts of galactose and a negligible amount of mannose.

The results of affinity chromatography showed that the MAD 698 wood extract exhibited 10.7 times more xylanase and 2.1 times more CMCase binding specificity than did the liquid culture filtrate (Table 3). Laminarinase activity was detected in the affinity eluant. However, laminarinase binding appeared to be nonspecific. Only 10 percent of the total laminarinase activity was detected in the eluant, but 94 percent of the total xylanase activity per milliliter was bound to the affinity column. There was no relationship between laminarinase activity and ELISA binding for MAD 698 chromatography fractions (Fig. 1).
Table 1. Comparison of Enzyme Preparations From Cultures of *P. placenta* Grown on Various Substances

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein (mg/ml)</th>
<th>Total CHO (mg/ml)</th>
<th>a-GAL (mM p-nitro/ ml)</th>
<th>b-GAL (mM p-nitro/ ml)</th>
<th>ELISAOD&lt;sub&gt;490&lt;/sub&gt;</th>
<th>Polysaccharidase&lt;sup&gt;a&lt;/sup&gt; (mg glucose/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Xylanase</td>
</tr>
<tr>
<td>0.1% Triton X-100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAD 698-wood</td>
<td>14.42</td>
<td>9.40</td>
<td>0.18</td>
<td>0.13</td>
<td>0.72</td>
<td>571</td>
</tr>
<tr>
<td>ME-20-wood</td>
<td>1.01</td>
<td>ND</td>
<td>0.72</td>
<td>0.60</td>
<td>0.05</td>
<td>566</td>
</tr>
<tr>
<td>MAD 698-agar</td>
<td>12.25</td>
<td>14.90</td>
<td>1.46</td>
<td>2.95</td>
<td>1.40</td>
<td>2,382</td>
</tr>
<tr>
<td>MAD 698-mat&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.95</td>
<td>12.47</td>
<td>4.13</td>
<td>4.14</td>
<td>1.60</td>
<td>3,511</td>
</tr>
<tr>
<td>MAD 698-liquid&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.95</td>
<td>21.54</td>
<td>3.77</td>
<td>3.55</td>
<td>1.40</td>
<td>307</td>
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<tr>
<td>Distilled water</td>
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<td></td>
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<tr>
<td>MAD 698-wood</td>
<td>13.86</td>
<td>0.50</td>
<td>0.16</td>
<td>0.20</td>
<td>1.33</td>
<td>615</td>
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<tr>
<td>ME-20-wood</td>
<td>1.12</td>
<td>0.16</td>
<td>0.37</td>
<td>0.48</td>
<td>0.51</td>
<td>292</td>
</tr>
</tbody>
</table>

<sup>a</sup>Micromoles of p-nitrophenol per milliliter. GAL is galactosidase
<sup>b</sup>CMCase is carboxymethylcellulase, LAMase is laminarinase, and MANase in mannanase.
<sup>c</sup>Mycelial mat
<sup>d</sup>Culture filtrate.
estimated molecular weight for laminarinase (55 kDa) was also not detected on western blot (i.e., antibodies did not bind to laminarinase). Affinity chromatography showed a total lack of recognition of ME-20 by the Mab. ME-20 xylanase has a molecular weight of 22 kDa as estimated by gel filtration chromatography.

Table 2. Carbohydrate Content of Extracellular Enzyme Extracts From ~.placenta Grown on Various Substrates

<table>
<thead>
<tr>
<th>Treatment and substrate</th>
<th>Glucose (mO/ml)</th>
<th>Galactose (mO/ml)</th>
<th>Arabinose (mO/ml)</th>
<th>Xylose (mO/ml)</th>
<th>Mannose (mO/ml)</th>
<th>Total (mO/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 % Triton X-100</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MAD 698-wood</td>
<td>2.45</td>
<td>3.17</td>
<td>0.12</td>
<td>1.00</td>
<td>2.66</td>
<td>9.40</td>
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<tr>
<td>MAD 698-agar</td>
<td>2.45</td>
<td>10.65</td>
<td>0.28</td>
<td>0.94</td>
<td>0.06</td>
<td>14.90</td>
</tr>
<tr>
<td>MAD 698-mat</td>
<td>2.12</td>
<td>5.78</td>
<td>0.10</td>
<td>0.99</td>
<td>3.48</td>
<td>12.47</td>
</tr>
<tr>
<td>MAD 698-liquid</td>
<td>5.87</td>
<td>6.80</td>
<td>0.17</td>
<td>2.20</td>
<td>6.80</td>
<td>21.54</td>
</tr>
<tr>
<td>Distilled water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAD 698-wood</td>
<td>0.23</td>
<td>0.04</td>
<td>0.03</td>
<td>0.06</td>
<td>0.13</td>
<td>0.50</td>
</tr>
<tr>
<td>ME-20 – wood</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.02</td>
<td>0.02</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Figure 1 shows polysaccharidases activities of fractions eluted from liquid chromatography and compares them to ELISA results. The highest ELISA readings correspond to the peak polysaccharidases activity for xylanase, with a drop to background readings around 305-ml elution volume.

Treatment of the MAD 698 liquid culture filtrate with the deglycosylating enzyme, PGNase F, eliminated the slight binding of the antigen in affinity chromatography. Similar results were demonstrated in ELISA. Untreated antigen had an OD_{490} of 0.742, and treated antigen decreased antibody recognition to an OD_{490} of 0.019.
DISCUSSION

Extracellular enzymes of the brown-rot fungus *P. placenta* MAD 698 and ME-20 grown on artificial or wood substrates were analyzed for differences in enzyme activities, protein and carbohydrate content, and antigen recognition by a monoclonal antibody (Mab). Artificial medium enhanced enzyme activity and availability but also increased the glucan production twofold compared to that of the wood substrate. Despite increased enzyme activities, a Mab to b-1,4-xylanase preferentially bound to wood-derived xylanase and CMCase, suggesting that xylanase-CMCase from liquid culture is either conformationally different from xylanase-CMCase in wood or is disguised by the excess glucan produced in liquid culture. Because the carbohydrate component of the liquid culture enzyme complex was composed of the same sugars as the enzyme complex from wood, it seems unlikely that the Mab would be recognizing a carbohydrate from wood not present in liquid culture.

To determine if glucan interference was causing problems in antibody recognition, liquid culture filtrate was treated with PGNase F to enzymatically remove mannose and glucose residues from the enzyme. We successfully used PGNase F in enzymatic digestion studies for characterizing the Mab (Clausen et al., 1992). In the previous study, antigen treated with PGNase F was analyzed.
by ELISA to determine if the MAb would recognize the protein portion of the glycoprotein once the mannose and glucose residues were removed. The ability of the MAb to bind to treated antigen was not altered, and Con A, which binds to α-linked mannose and glucose residues and is used to assess the completeness of the digestion, showed a 77-percent reduction in binding for the control. In this study, however, untreated culture filtrate bound weakly to the affinity column whereas culture filtrate treated with PGNase F demonstrated no binding. Removing sugar residues from the antigen did not increase recognition by the MAb in affinity chromatography, again strongly suggesting the MAb is not specific for the carbohydrate moiety of the glycoprotein. In ELISA, treatment with PGNase F also reduced binding by the MAb to an undetectable amount. This suggests that although the MAb is protein specific for α,1,4-xylanase, there seems to be a second smaller protein that is recognized by the MAb or a repeating epitope in extracellular enzymes from liquid culture that is altered by enzymatic digestion.

Figure 1. Polysaccharidase activity of \textit{P. placenta} MAD 698 extract in gel chromatography fractions compared to ELISA readings.
Enzyme activities of the ME-20 and MAD 698 extracts were equivalent, but the MAb did not recognize ME-20 xylanase in ELISA, western blot, or affinity chromatography eluant. The estimated molecular weight of ME-20 xylanase was 10 kDa less than the estimated molecular weight of MAD 698 xylanase. The lack of glucan production by ME-20 may account for the differences in molecular weight and antibody recognition of this enzyme. The comparative information between these two isolates of *P. placenta* is valuable for our continuing efforts to discern the inability of ME-20 to degrade wood.

We conclude that antigenic characteristics of extracellular polysaccharide-degrading enzymes of *P. placenta* are substrate dependent. The differences between enzymes from liquid culture and those from wood may not be readily apparent in the absence of immunological comparisons. This is an important consideration when developing immunodiagnostic methodologies. We feel that it is necessary to develop detection methods compatible with naturally occurring substrate conditions. Such methods are equally important for *in situ* immunolabeling experiments to optimize antibody specificity (Green et al., 1992b).

**SUMMARY**

Most studies on the degradative enzymes of wood-decay fungi utilize enzymes produced in liquid culture and assume that the enzymes have the same properties as those produced by the fungus in wood. To determine if the culture medium affects enzyme characteristics, we compared the antigenic properties of extracellular enzymes from the brown-rot fungus *Postia placenta* grown in liquid culture, malt agar, or wood. Availability of low molecular weight sugars in artificial media resulted in excess production of glucan. Enzyme activities were higher in artificial media, and enzyme distribution and antigenic affinity were altered, as determined by affinity chromatography using monoclonal antibodies specific for xylanase derived from wood. This study demonstrates that enzyme characteristics of *P. placenta* are substrate dependent and that degradative enzymes produced *in vitro* differ from those produced in wood. These differences are critical considerations in the design of immunological methods for early detection of brown-rot fungi.
REFERENCES


Clausen, C. A., Green, F. III., and Highley, T.L. (1992), Characterization of monoclonal antibodies to wood derived b-1,4-xylanase *Postia placenta* and their application to detection of incipient decay. *Wood science and Technology*. (In press.)


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