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Characterization of monoclonal antibodies to wood-derived b-1,4-xylanase of *Postia placenta* and their application to detection of incipient decay *

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Summary. Murine monoclonal antibodies (mAbs) were produced to the extracellular β-1,4-xylanase fraction of the brown-rot fungus, *Postia placenta*. The immunizing antigen was derived from decayed southern yellow pine wood blocks extracted in phosphate-buffered saline and Triton X-100, and fractionated by column chromatography. Five IgG monoclonal antibodies were selected for characterization by enzyme-linked immunosorbent assay (ELISA) and western blot. Each mAb exhibited binding to two major protein bands on western blot at 32 kDa and 36 kDa. Characterization by chemical and enzymatic modification of the antigen determined whether mAbs were protein specific or specific for the carbohydrate portion of the glycoprotein. Affinity chromatography with the protein specific mAbs demonstrated specific binding to β-1,4-xylanase and cross-reactivity with β-1,3-glucanase, β-1,4-glucanase, and β-1,4-mannanase. These mAbs were also immunoreactive with polysaccharidases from liquid culture filtrates when tested by ELISA. The mAbs have been utilized to successfully detected *P. placenta* by ELBA in decayed southern yellow pine at a weight loss of 3.6%.

Introduction

Brown-rot decay fungi produce a variety of extracellular polysaccharidases that attack the wood cell wall. However, we know virtually nothing about the release of these enzymes from the fungus and their delivery to sites of attack within wood. Successful colonization and initial penetration of the wood may depend on the utilization of hemicelluloses, which are important cell wall polysaccharides. Hemicelluloses are removed preferentially, early in the decay process, by brown-rot decay fungi (Kirk & Highley, 1973; Highley, 1987). β-1,4-Xylanase, of the brown-rot fungus *Postia placenta* (Fr.) M. Lars. et Lomb. [= *Poria placenta* (Fr.) Cke.] was partially purified by our laboratory (Green et al., 1989a) and used as antigen to produce murine monoclonal antibodies (mAbs). This glycoprotein typically co-migrates with other hemicellulases and glucanases, and is comprised of fifty percent carbohydrate, necessitating characterization with carbohydrate modifying procedures, in addition to standard characterization methods which include enzyme-linked immunosorbent assay (ELISA), western blots, and affinity chromatography. This paper reports the

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first successful attempt to produce murine mAbs to xylanase of a brown-rot decay fungus isolated from wood, and their use in detection of incipient decay at low wood weight loss, i.e. less than 10%.

Methods

Antigen Preparation

Southern yellow pine *(Pinus* sp.) wood blocks inoculated with *P. placenta* (Mad 698), were incubated at 27 °C for 9 weeks. An extract was prepared by grinding the decayed wood blocks in phosphate buffered saline (PBS), 0.1% Triton X-100 (Sigma, St. Louis, Missouri), 0.85% NaCl, pH 7.2, and filtering the homogenate through cheesecloth. The resulting extract was dialyzed, ultracentrifuged at 128,000 × *g* for 2 h, and passed sequentially through Sepharose 6 B [1] (Pharmacia, Piscataway, New Jersey) and Fractogel TSK HW-55 (F) (EM Science, Gibbstown, New Jersey) columns, both equilibrated with PBS, pH 7.2. Chromatography fractions were eluted in PBS, and those eluants containing xylanase activity served as antigen as previously published (Green et al., 1989a).

Protein and enzyme assays

Total protein was determined by the Bradford microassay (Simpson & Sonne, 1982) using bovine serum albumin (BSA) (Sigma, St. Louis, Missouri) as standard. Activities of endo-β-1,4-xylanase, endo-β-1,4-glucanase (CMCase), endo-β-1,3-glucanase (laminarinase), and endo-β-1,4-mannanase were assayed by the reducing-sugar microassay (Green et al., 1989 b). One unit of enzyme activity was defined as the amount needed to liberate reducing power equivalent to 1 µg glucose per 24 h at 40°C using D-glucose (Fischer Scientific Co., Fair Lawn, New Jersey) as standard. β-D-Galactosidase, α-D-galactosidase, β-D-glucosidase, α-D-glucosidase, and β-D-xylosidase activities were assayed by determining the liberation of p-nitrophenol from respective p-nitrophenol substrates utilizing a microassay adaptation of the methods of Agrawal & Bahl, 1968 and Young et al., 1987. A unit of enzyme activity was defined as the amount that will liberate 1 µM of p-nitrophenol per hour.

Production of mAbs

Six-week-old female BALB/c mice were immunized intraperitoneally (IP) with 10 µg protein per 0.5 ml in 50% Freund's complete adjuvant, and boosted IP at two week intervals with the same antigen in PBS. Mice were bled at 4 weeks and their serum was tested by ELISA (Stahli et al., 1983). Five days prior to the fusion, the mouse with

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[1] The use of trade of firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service

the highest titer was inoculated intravenously (IV) with 0.1 ml, and IP with 0.5 ml on two successive days prior to fusion. The spleen was surgically removed and collected splenocytes were fused with 10^7 NS-1 myeloma cells. Fused cells were suspended in Dulbecco's Modified Eagle's Minimal Essential Medium (DME) (Gibco, Grand Island, New York) supplemented with penicillin, streptomycin, 20% fetal calf serum (FCS) (Gibco), hypoxanthine, aminopterin, and thymidine and plated in sterile 96-well cell culture plates. Hybridomas were selected by ELISA and western blot. Dilution cloning of the hybridomas yielded five clones from different colonies. Monoclonal antibodies were isotyped using a commercially available kit utilizing an Ouchterlony assay (Binding Site, San Diego, California). Cell lines were frozen at 4×10^6 cells ml^{-1} in DME, 20% FCS, penicillin, streptomycin, and 10% dimethyl sulfoxide (DMSO) and maintained in liquid N_2.

ELISA Screening

Flat bottom, 96-well ELISA plates were coated with antigen (2 µg protein ml^{-1}) overnight at 25 °C. Supernatants from hybridoma cell cultures were added to the wells at 100 µl per well, incubated overnight at 25°C and blocked with 50 mM Tris, 150 mM NaCl, pH 7.4, 1:1 with calf serum and supplemented with 0.25% gelatin. Alkaline phosphatase conjugated goat anti-mouse IgG at a concentration of 125 ng ml^{-1} was added (100 µl per well) and incubated for 2 h at 25°C. Plates were washed and 200 µl of 4-methylumbelliferyl phosphate (MUP) disodium salt in 1 M 2-amino-2-methyl-1-propanol, pH 9.9 (Sigma) was added. Fluorescence was read with a Fluroscan redder (Flow Labs, McLean, Virginia) (Engvall & Perlmann, 1971, Yolken & Stopa, 1979).

SDS-PAGE and Western Blotting

Gel chromatography fractions described above were separated on sodium dodecyl sulfate (SDS) polyacrylamide gels consisting of a 4% stacking gel and a 12% resolving gel. Gels were cast using a Biorad Protean II apparatus (Rockville Centre, New York). Prestained molecular weight markers (Biorad) were run for molecular weight estimations on the immunoblots and duplicate gels were stained with Coomassie blue. Proteins were transferred to nitrocellulose paper (NCP) using a Biorad Transblot apparatus with 25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3 running buffer, precooled to - 20 °C. Transfers were conducted for 2 h at 100 V. Blotted NCP was blocked for 1 h in Tris buffered saline (TBS) (0.02 M Tris, 0.5 M NaCl, pH 7.5) with 3% gelatin, rinsed briefly with distilled water, and placed in a Miniblotter II (Immunetics, Cambridge, Massachusetts). Supernatants from single colony clones were added to each channel and allowed to bind for 2 h. Blots were washed with TTBS (TBS plus 0.05% Tween 20) before adding 1 µg ml^{-1} horseradish peroxidase conjugated goat anti-mouse IgG (H + L) in TBS with 1% gelatin. After a 2 h incubation at 25°C the blot was washed again in TTBS and developed with 60 mg 4-chloro-1-naphthol (Sigma) in 20 ml methanol and 60 µl hydrogen peroxide in 100 ml TBS (Towbin et al., 1979).

Ascites Generation and IgG Purification

Ten-week-old mice were primed with 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane) (Sigma) IP, 10 days before injecting 2×10^6 hybridoma cells per 0.5 ml IP for each mouse. Ascites fluid was collected 10 to 18 days later, centrifuged at 5,000 × *g* for 5 min, and frozen at -20°C. Total IgG was purified and concentrated from ascites using Beckman's r protein A antibody purification kit and purification protocol (Beckman, Fullerton, California).

Carbohydrate-Modfying Procedures

Antigen was modified either chemically or enzymatically as described in the following sections (Wycoff et al., 1987; Tarentino et al., 1985). Since ConA binds to cc-linked mannose and glucose residues, it was used to determine completeness of each carbohydrate-modifying procedure (Wycoff et al., 1987; Beeley, 1985). In each procedure, ConA added to control wells at 100 µg ml^{-1} in TBS plus 1 mM $CaCl_2$ + 1 mM $MnCl_2$ was allowed to bind for 1 h. ConA also binds the goat anti-mouse IgG conjugate used in the ELISA to test treated antigen, so the same ELISA procedure was used for all wells.

a. Mannosidase digestion. Antigen coated plates were washed with 50 mM sodium acetate, pH 4.5, and blocked with PBS plus 0.5% BSA. Wells to be treated were filled with 100 µl α-mannosidase (Sigma), 2.7 units ml^{-1} in 100 mM sodium acetate buffer, pH 4.5 + 5 mM $ZnCl_2$ + 0.5% BSA. Control wells received buffer only. Plates were incubated 24 h at 37°C washed, and incubated with mAbs (as described for ELISA) or ConA as described above.

b. Endo-H digestion. Antigen was pretreated with 0.2 µg ml^{-1} endo-H (endoglycosidase H) (Sigma) in 200 mM sodium acetate buffer, pH 5.8, for 24 h at 37 °C before binding to the ELISA plates. The control mixture consisted of buffer and antigen only. Plates were probed with mAbs and ConA.

c. PGNase F treatment. Antigen was pretreated with 8 µg ml^{-1} glycopeptidase F (Boehringer-Mannheim Biochemicals, Indianapolis, Indiana) for 18 h at 37°C in 100 mM sodium acetate buffer, pH 5.0, before binding to the ELISA plates. Controls were treated with buffer only. Plates were probed with mAbs or ConA, as described.

Detection of Antigen in Decayed Wood and Liquid Culture

Southern yellow pine wood blocks were exposed to *P placenta* for 4 wk at 27°C, 80% relative humidity TRH). At four day intervals, blocks were removed, ground to 20 mesh, and extracted in PBS, 0.1% Triton X-100 (Sigma) for 18 h. Extracts were tested in ELISA against mAbs designated F1 and C9 and polyclonal antibodies (pAbs), and correlated to wood block weight loss.

Monoclonal antibodies F1 and C9 were also tested for their ability to bind to extracellular enzymes from liquid cultures of the test organism. *Postia placenta* was grown in 25 ml stationary flasks with 1% cellobiose in basal salts solution (Highley, 1973) and incubated at 27°C, 80% RH for 4 wk. Culture filtrate served as antigen for this ELISA.

Affinity Chromatography

Individual columns were prepared by coupling either F1 or C9 to CNBr-activated Sepharose 4 B (Pharmacia) according to Pharmacia Principles and Methods. Briefly, gel swelled in 1 mM HCl was washed and coupled to mAbs at 4°C for 18 h. Antigen from either decayed wood or liquid culture filtrate was applied to the column in 50 mM phosphate buffer, 0.15 M NaCl, and 0.1% Tween-20. After thoroughly washing unbound antigen from the column, bound antigen was eluted in 100 mM glycine-HCl, pH 2.6. 0.15 M NaCl, and 0.01% Tween-30, and neutralized with 1 M phosphate buffer, pH 7.5. The eluted protein peak was assayed for enzyme activities as previously described (Green et al., l989b).

Results

Monoclonal antibodies to the xylanase fraction of *P. placenta* isolated by gel chromatography were characterized by ELISA. Western blot, chemical and enzymatic modification of the antigen, and affinity chromatography. Five mAbs were isotyped as IgG1 or IgG2a. and they bound on a Western blot from SDS-PAGE to two major protein bands at 32 kDa and 36 kDa (Fig. 1).

Carbohydrate-modifying procedures were used to test the specificity of each mAb to the protein moiety of our antigen (Table 1). After 24-h treatment with α-mannosidase. the binding of ConA to the antigen was reduced by 26%; similar reductions occurred in C10, E4, and D4 (28. 23, and 30%, respectively). There was only a slight reduction (10%) in the binding ability of C9, and F1 was unaffected (Table 1). In contrast, endo-H treatment reduced the binding ability of ConA by 81%, and C9, C10, D4, and E4 by 30% to 75%, while F1 showed binding enhancement. PNGase F treatment for 18 h gave a 71% binding reduction of ConA. Antigen binding in C10, F1, E4, and D4 was reduced by 13 to 24%, and C9 binding was enhanced.

The ability of mAbs F1 and C9 to recognize *P. placenta* derived from infected wood and liquid culture was tested by ELISA (Fig. 2). Liquid culture filtrates gave optical density (O. D.) readings at 490 nm of 1.10 and 2.00 when tested against F1 and

Table 1. ELISA binding ability of mAbs after carbohydrate-modifying procedures

mAb	Isotype	Relative binding ability[a]		
		α-Mannosidase	Endo-H	PGNase F
F1	IgG2a	1.05	1.28	0.81
C9	IgG2a	0.90	0.70	1.30
Cl0	IgG2a	0.72	0.25[b]	0.87
D4	IgG1	0.70	0.33b	0.76
E4	IgG1	0.77	0.46[b]	0.79
ConA		0.74	0.19b	0.29[b]

[a] Values are ratios of treated sample to untreated control. ELISA results are the mean of three replicates.
[b] Binding ability reduced > 50%

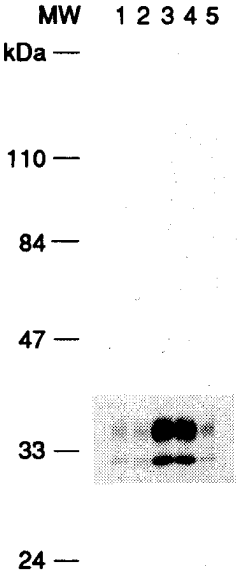

Fig. 1. Western blot analysis after SDS-PAGE of xylanase indicating binding patterns of the monoclonal antibodies: lane 1: C10, lane 2: D4, lane 3: F1, lane 4: C9, lane 5: E4. Molecular weight standards are indicated

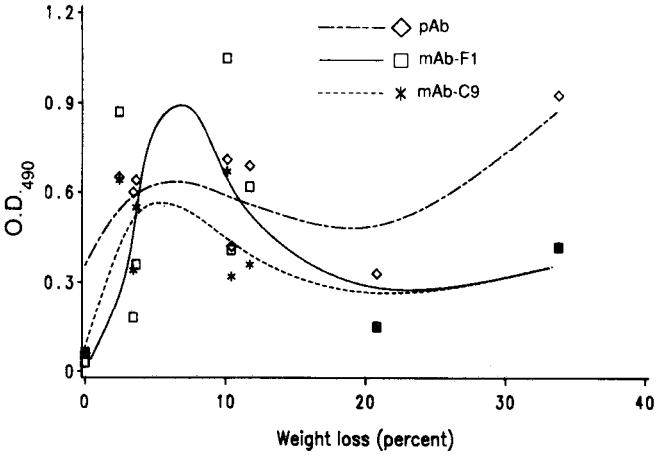

Fig. 2. Detection of *P. placenta* in southern yellow pine comparing percent of wood block weight loss to binding ability ($O.D._{490}$) of monoclonal and polyclonal antibody in ELISA. Data represent the mean of three replicates. $O.D._{490}$ of undecayed SYP = 0.07

C9, respectively. Extracts of *P. placenta* antigen from decayed wood blocks had O. D. readings of 0.4 to 0.6 at 3.6% weight loss while an undecayed southern yellow pine control had an O. D. reading of 0.07.

Affinity chromatography using F1 or C9 coupled to CNBr-activated Sepharose 4B demonstrated that both mAbs have specific affinity for xylanase (Table 2). A single

Table 2. Enzyme activity after afftnity chromatography of *P. placenta* wood extracts and culture filtrate on Sepharose 4B-CNBr coupled to monoclonal antibody F1 or C9

Enzyme	Enzyme activity (µg glucose ml^{-1} (% total activity))			
	Wood extract		Culture filtrate	
	F1	C9	F1	C9
Xylanase	576 (39)	653 (31)	54 (6.8)	298 (19.3)
CMCase	493 (33.4)	404 (19)	234 (29.4)	213 (13.8)
Mannanase	83 (5.6)	168 (8)	239 (30)	253 (16.4)
Laminarinase	324 (22)	884 (42)	269 (33.8)	777 (50.4)

protein peak was eluted. Cross reactivity of both F1 and C9 was measurable for CMCase, laminarinase, and mannanase. Glycosidase activity was uniformly negative.

Discussion

Five monoclonal antibodies to β-1,4-xylanase of *P. placenta* were selected for characterization. Each mAb reacted prominently with two major protein bands at 32 and 36 kDa on a western blot from SDS-PAGE. Previous estimations of the molecular weight of xylanase appear to be reduced by more complete solubilization with Triton X-100 and reduction in carbohydrate residues as reported by other authors (Gallagher & Evans, 1990; Magnuson et al., 1991). Xylanases characterized from other organisms are most commonly observed in the molecular weight range of 12 - 45 kDa (Mishra et al., 1990). This raises the possibility that complete solubilization would yield individual enzymes of smaller molecular weight.

The double banding pattern on the Western blot may represent binding to two different enzymes or isotypes of the same enzyme carrying the same epitope (Micales et al., 1991). It is not uncommon for a mAb to recognize a similar but non-identical epitope with a lower affinity (Lane & Koprowski, 1982). Based on column chromatography, we postulate that the 36 kDa band represents laminarinase and the 32 kDa band represents xylanase, mannanase, and CMCase. The latter three enzymes typically co-migrate. Multiple activities have been attributed to a single enzyme in *Neurospora crassa* and *Irpex lacteus* by Deshpande et al., 1986 and Kanda et al., 1985, respectively, and our data indicate the same is true for *P. placenta* (Highley et al. 1981, Green et al., 1989). Electrophoretic comigration of xylanase and endoglucanase (CMCase) activity was observed by Ishihara et al., 1978, and Ramachandra et al., 1987. Unlike the co-migration of xylanase and CMCase, it is possible to separate laminarinase from other activities chromatographically. Conformationally, laminarin (β-1,3) is similar to other wood polysaccharides (β-1,4) which may account for the cross-reactivity observed in affinity chromatography.

Monoclonal antibodies C9 and F1 appeared to be protein specific; chemical or enzymatic removal of carbohydrate from the xylanase molecule did not reduce the binding affinity of C9 or F1 in ELISA. These two mAbs were of particular interest because of their protein specificity, and emphasis was placed on further analyzing

their characteristics. Affinity chromatography columns prepared with C9 or F1 confirmed specific binding to xylanase. The ability of F1 and C9 to bind to extracellular enzymes derived from liquid culture confirms experimentally that polysaccharidases from wood are antigenically similar to those derived from liquid culture in vitro, however. a different distribution of enzyme activities between liquid culture and wood was observed. Often, extracellular enzymes are isolated from liquid culture, but the affinity chromatography data suggests that mAbs specific for xylanase more readily recognize xylanase from wood than from culture filtrate (5.7 times more for F1 and 1.6 times more for C9).

Three monoclonal antibodies, D4, E4, and C10 were carbohydrate specific. Binding reductions similar to the ConA control were noted following treatment with x-mannosidase and endo-H suggesting specificity to the carbohydrate moiety of the enzyme. Due to the bond (or bonds) affected by α-mannosidase, treatment of xylanase with the enzyme is likely to eliminate more terminal α-linked mannose residues causing a decreased binding of ConA as we observed. Endoglycosidase H, a member of the endo-β-N-acetylglucosaminidase enzyme family. splits the linkage between the two N-acetylglucosamine residues with the core region of N-glycosidic units (Sharon & Lis, 1982). This enzyme acts on oligosaccharides of the high-mannose or hybrid type (Beeley, 1985). Only F1 showed a binding enhancement after treatment with α-mannosidase or endo-H. Removing the carbohydrate from the protein may account for increases in binding of treated antigen. After peptide : N-glycosidase F (PNGase F) treatment, binding reductions were slight compared to the ConA control. Only one mAb, C9, showed enhanced binding after treatment with PNGase F enzyme. PNGase F cleaves between asparagine and the carbohydrate chain of all classes of N-linked oligosaccharides (Tarentino et al., 1985).

MAbs F1 and C9 were able to detect antigen extracted from *P. placenta*-decayed wood blocks at very low weight losses (3.6%), i.e. low levels of hyphal colonization. The sensitivity and specificity demonstrated by C9 and F1 in ELlSA are being utilized to detect incipient decay by brown-rot fungi before strength loss of the wood occurs. This technology is currently expanding to incorporate other immunodiagnostic procedures, as well as immunolabeling of wood blocks to study the mechanism of decay.

In conclusion, monoclonal antibodies were produced to xylanase extracted from wood blocks decayed by the brown-rot fungus *Postia placenta in vitro.* Use of these antibodies for immuno-detection of wood decay enzymes *in situ* and *in vitro* will aid in developing field tests for incipient decay, and will give us a better understanding of the mechanism of decay of brown-rot fungi.

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