A NOVEL ENZYME THAT CLEAVES A PHENOLIC ETHER BOND BETWEEN C6 OF MANNOSE AND A MONOLIGNOL ANALOG

Nancy G. Kravit[1,2], Yang Yu[1,2] and Katherine A. Schmidt[1,2]

1. Tethys Research LLC, Bangor, ME 04401

2. Department of Chemical and Biological Engineering, University of Maine

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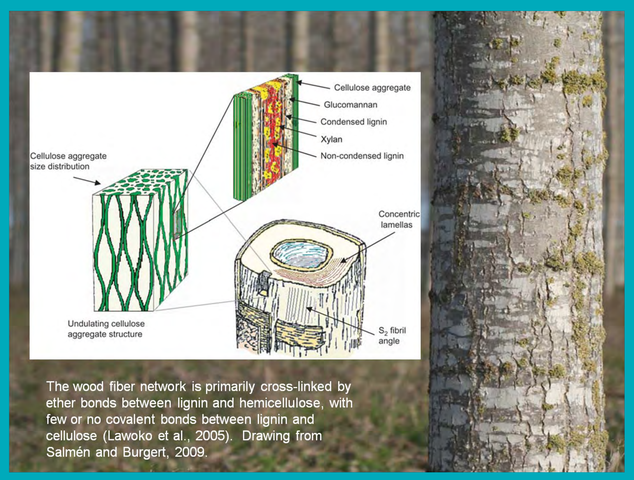
ABSTRACT

Hemicellulose from wood, a waste product in papermaking, can be utilized for production of biofuels. However, ether bonds between lignin and hemicellulose are a major impediment to their extraction and subsequent efficient fermentation. Because of the nature of the biosynthesis of the wood matrix, we hypothesize that these cross-links are non-glycosidic. Because naturally-occurring organisms can completely degrade wood, we conclude that naturally-occurring organisms express enzymes that can break non-glycosidic ether bonds between lignin and hemicellulose, and that these hemicellulose:lignin etherases (HLEs) can be exploited commercially to aid in fractionation of wood into cellulose, hemicellulose and lignin.

A novel polymeric fluorogenic model compound that mimics non-glycosidic phenolic ether bonds between lignin and galactomannan was developed. Commercial cellulases and hemicellulases, which are specific for glycosidic bonds, do not cleave the model substrate. The model substrate was used in bioprospecting experiments to isolate several organisms that appear to express an HLE activity. We have characterized in particular the activity expressed by Microbe B603, a small, aerobic autofluorescent organism that has not been previously described. We have demonstrated that the activity is due to an enzyme. The enzyme is heat tolerant, requires a mineral co-factor, and is inactive against the commercial substrates used to assay for several glycosidases. The enzyme activity has been shown to be expressed in a cyclical pattern with a periodicity of approximately 27 hours. The cDNA for the enzyme has been cloned from an expression library based on the activity of its fusion protein against the model substrate. Tests of the enzyme on wood pulp are currently underway.



The wood fiber network is primarily cross-linked by ether bonds between lignin and hemicellulose, with few or no covalent bonds between lignin and cellulose (Lawoko et al., 2005). Drawing from Salmén and Burgert, 2009.

Introduction

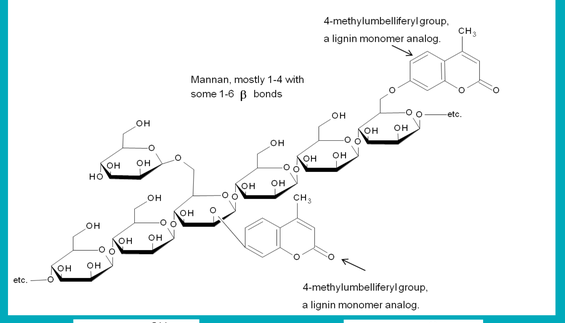
Wood is held together by a variety of different chemical bonds. Ether bonds between lignin and hemicellulose are particularly stubborn. These bonds are formed by free radical conjugations during the assembly of lignin after the hemicellulose is already polymerized via glycosidic bonds. This mechanism of cross-linking limits the linkages between lignin and hemicellulose to the hemicellulose carbons that are not involved in glycosidic bonds. Enzymes that break glycosidic bonds are well known, but enzymes that specifically break non-glycosidic ether bonds between a sugar and another organic group have not been previously described.

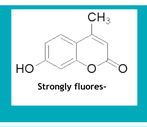
But, if a compound is found in nature, some microorganism will inevitably manage to break it apart and use it for energy or building blocks. We set out to bioprospect for microorganisms that could do just that—cleave the non-glycosidic ether bonds between mannans and the phenyl groups of lignin. To do so, we designed a model substrate based on mannan derivatized to a phenyl ring of 4-methylumbelliferone through an ether bond. 4-methylumbelliferone is highly fluorescent, but when derivatized through an ether bond, it is much less fluorescent (<1%). 4-methylumbelliferone was not released from the substrate by a variety of glycosidases in control experiments.

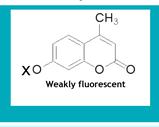
We sampled sites of softwood decay in Maine forest and set up mixed cultures using the substrate as the sole carbon source. Cell-free culture supernatants of cultures that developed fluorescence were tested for their ability to release 4-methylumbelliferone from fresh substrate. Cultures whose cell-free supernatants contained an activity that cleaved the fluorogenic substrate were plated onto agar plates containing the substrate as sole carbon source and colony-purified. Pure cultures were screened on a non-fluorogenic medium to eliminate autofluorescent false positives. As a control, a fluorogenic mimic of an ether bond within lignin was synthesized. Cultures that were positive on the fluorogenic lignin-hemicellulose model substrate but not on the fluorogenic lignin model substrate were presumed to have a specific enzyme rather than a system that generates free radicals, such as lignin-peroxidase. We have called the activity hemicellulose:lignin etherase (HLE)

We report here on the activity from an organism designated B603.

Indicating (Fluorogenic) Substrate







Free 4-methyl-umbelliferone is strongly fluorescent.

Ethers of 4-methyl-umbelliferone are minimally fluores-

Microorganisms that can cleave phenyl ether bonds between 4-methylumbelliferone and C6 of residues of mannan will generate fluorescence.

Glycosidases Do Not Cleave 4-Methylumbelliferone from Indicating Substrate

Glycosidases Tested:

α-galactosidase, β-galactosidase, mixed commercial hemicellulase (xylanases, mannanases, xylosidases, mannosidases, etc.), α-mannosidase, β-mannosidase, amylase, isoamylase, pullanase

Examples:

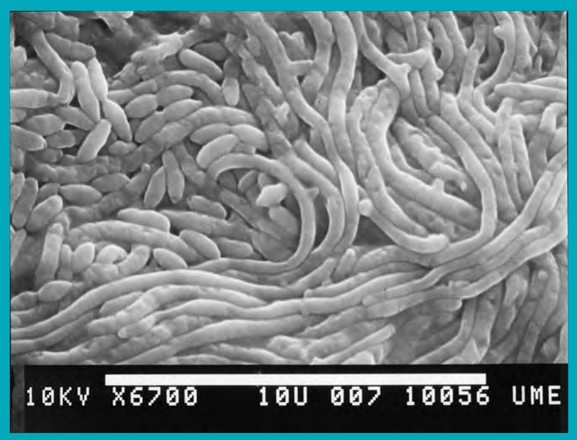
Enzyme	Negative Control	Cognate 4MU-pyranoside	4MU-LBG	4MU-LBG + cognate 4MU-pyranoside (internal control)
α-galactosidase	94	50,971	94	49,471
β-galactosidase	92	127,112	100	116,781
α-mannosidase	114	115,450	94	97,181
β-mannosidase	127	120,332	114	113,240

Bioprospecting

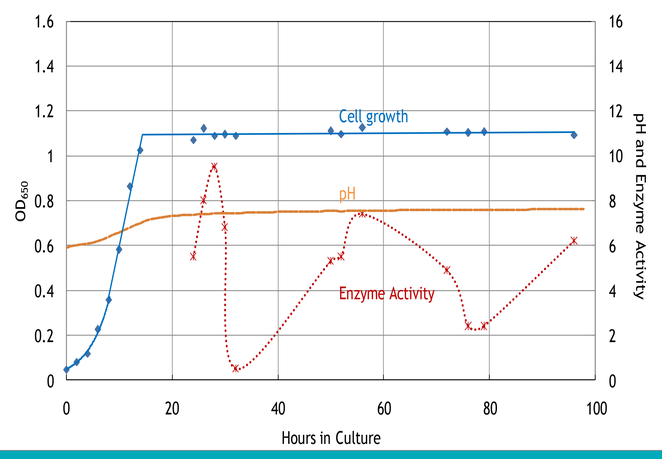
Environmental samples were taken from Maine forests at sites of softwood decay. Samples of soil, decaying wood, etc. were cultured in the dark on indicating substrate as sole carbon source. Cultures that developed fluorescence were checked to see if the fluorescence was due to autofluorescence. A sample of culture medium was filtered through 0.2μ filters to remove cells and incubated with fresh indicating substrate. An increase in fluorescence could not be due to new synthesis of fluorescent molecules and was taken as evidence of an enzyme activity. Positive mixed cultures were plated onto agar medium containing indicating substrate as the sole carbon source and colony purified. Culture collections were are screened.

Three candidates were identified. B603 is the subject of this report.

Scanning Electron Micrograph of B603



Enzyme Activity is Produced Cyclically

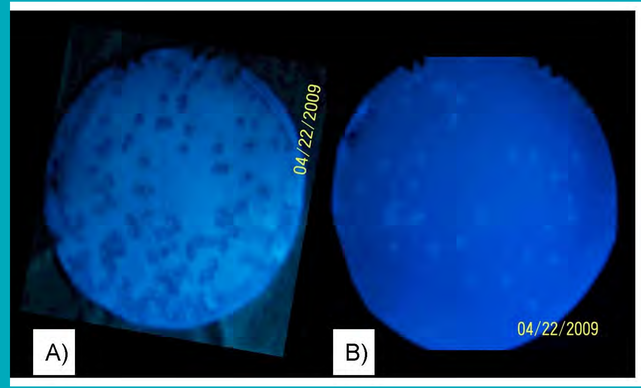


Characteristics of HLE Activity:

- ◊ Digestion with proteases destroys activity
- ◊ pH optimum at usual pH of culture supernatant, pH 5 ±0.5
- ◊ Optimal NaCl concentration is 0.25-0.5M NaCl.
- ◊ Desalting destroys activity. Adding back lyophilized column wash restores activity.
- ◊ Very temperature stable. Treatment for 10 min to 65°C has no measurable effect.
- ◊ More than 80% of the enzyme activity is located in the supernatant of a culture during peak expression.

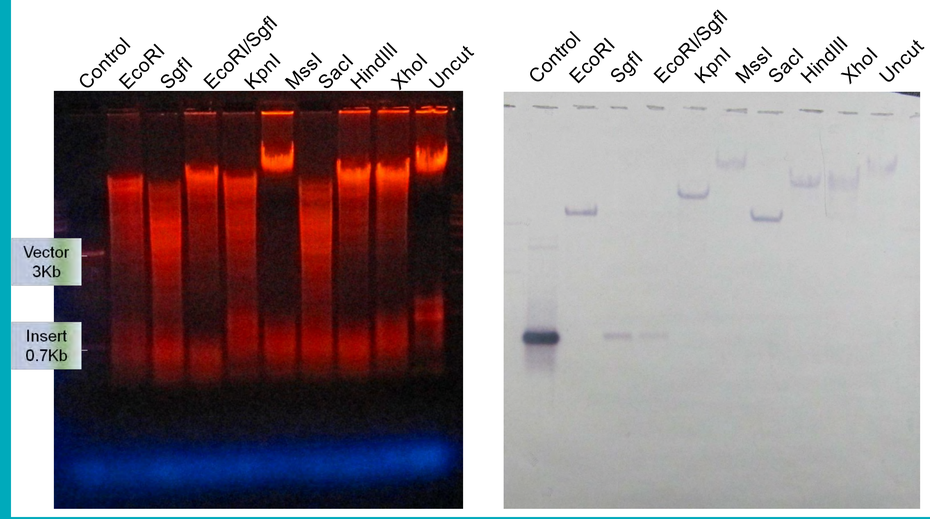
Cloning Strategy

mRNA was prepared from B603 cells expressing HLE activity. The mRNA was back-translated into cDNA and ligated into a Stratagene Lambda Zap expression vector to create a library. The library could be screened by hybridization, by antibody or by enzyme activity. The advantage to using a phage library in this case was that the substrate for activity screening was too large to enter cells. Use of λ ensured lysis of the host cells and good contact between the expressed fusion protein and the fluorogenic substrate.

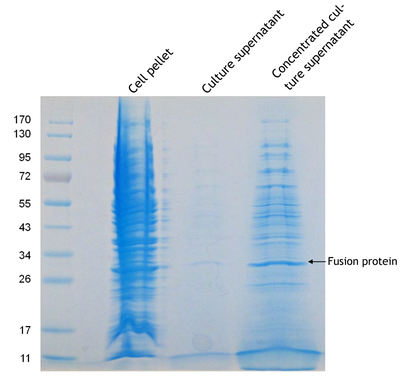


Short wave and long wave UV photograph of a plaque lift of an intermediate purification step of a cloned HLE cDNA. XL1-blue cells (Stratagene) were infected with a B603 λZAP library and plated with IPTG and 4MU-LBG. All of the plaques are visible under long wave UV light (A), but under short wave UV (b), only the plaques where HLE is expressed are visible. Fluorescent plaques were picked and plaque purified. In this intermediate plaque purification step, approximately half of the plaques are fluorescing under UV light, indicating that *hle* is being expressed.

Genomic Southern blots indicate that *hle* is a single copy gene

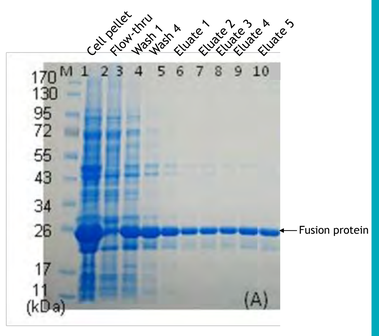


Expression of HLE Fused to α-Peptide of β-Galactosidase



A. HLE peptide fused to α peptide of β-galactosidase is active, but not very pure.

Purification of HLE Fused to HisLink™ on Nickel Affinity Resin



B. HLE peptide fused to HisLink™ forms inclusion bodies and can be easily isolated but is not active.

Experiments on the effect of recombinant HLE on softwood pulp are underway

A. Untreated softwood pulp

B. Softwood pulp treated with unconcentrated culture supernatant containing recombinant HLE peptide fused to α peptide of β-galactosidase. Note color change to lighter, more orange brown.

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Inquiries to:
Dr. Nancy Kravit, Chief Executive Scientist
Tethys Research LLC
53 Downing Rd.
Bangor, ME 04401
(207) 942-9044 (PH & FAX)
ngkravit@tethysresearch.com

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