# Characterization of a *Thermobifida fusca* β-1,3-Glucanase (Lam81A) with a Potential Role in Plant Biomass Degradation†

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ABSTRACT: *Thermobifida fusca* is a filamentous soil bacterium that plays a major role in the breakdown of plant biomass. In this paper, we report the cloning, expression, purification, and characterization of the *T. fusca* enzyme, Lam81A. The Carbohydrate Active Enzymes Database (http://afmb.cnrs-mrs.fr/CAZY/) indicates that Lam81A belongs to a relatively uncharacterized family of β-1,3-glucanases, family GH-81 [Coutinho, P. M., and Henrissat, B. (1999) in *Recent Advances in Carbohydrate Bioengineering* (Gilbert, H. J., Davies, G., Henrissat, B., and Svensson, B., Eds.) pp 3−12, The Royal Society of Chemistry, Cambridge, U.K.]. Microarray analysis suggests that Lam81A plays a role in biomass degradation, where its natural substrate may be the plant cell wall polysaccharide, callose, which is a polymer of β-1,3-linked glucose. Characterization of Lam81A has shown that the enzyme is specific for β-1,3-linked glucose polysaccharides, is endohydrolytic, and utilizes an inverting mechanism for substrate hydrolysis. In addition, the enzyme has a broad pH optimum from 5.5 to 10, a temperature optimum of 50 °C, and demonstrates substrate inhibition, as well as showing a high basal level of expression.

In recent years, extensive research has been carried out on microbial-mediated biomass hydrolysis, which is an important step in global carbon recycling (*2*, *3*). The breakdown of biomass into fermentable sugars to generate ethanol shows promise for industrial production of an alternative fuel (*3*, *4*). However, plant biomass is a complex cell wall-derived material containing many polymers and is resistant to enzyme degradation, such that efficient hydrolysis requires the concerted action of multiple glycanases [see Clarke (*5*) for review].

*Thermobifida fusca*, a thermophilic bacterium (*Actinomycetaceae*) that operates optimally at 55 °C, is a primary degrader of plant biomass in soil, compost heaps, and rotting hay and has been adopted as a model system for characterizing the complex interplay of glycanases involved in biomass hydrolysis. Extensive work in this organism has resulted in the identification and characterization of six extracellular cellulases (four endoglucanases and two exoglucanases), an intracellular β-glucosidase, two xylanases, a xyloglucanase, and the protein CelR, which regulates the induction of cellulases and related enzymes (*6*, *7*). Using the recently completed *T. fusca* genome sequence, determined by the Joint Genome Institute of the Department of Energy, our laboratory has begun to search for additional enzymes that may be involved in biomass hydrolysis.

In an effort to characterize the full complement of *T. fusca* enzymes that are necessary for efficient biomass degradation, microarray analysis was used to identify new *T. fusca* genes with potential roles in plant cell wall hydrolysis. *T. fusca*, grown on cellulose (Solka Floc), showed increased levels of mRNAs associated with gene 2130 (Genpet-AAZ56163.1), whose product is a hypothetical protein encoding a family-81 glycosyl hydrolase (*8*). This family, which currently includes about 40 members from bacteria, fungi, and plants, contains β-1,3-glucanase of which the catalytic residues and basic protein fold are unknown. β-1,3-Glucanase activity in this family was first attributed to Eng1p, which is involved in cell wall separation in *Saccharomyces cerevisiae* (*9*). Other members of the family include the *S. cerevisiae* protein, Eng2p/Acf2p, which is associated with cortical actin assembly, and several plant proteins, including β-glucan elicitor binding proteins, which are involved in host defense against pathogens (*9*−*11*). Several members of the family have no ascribed function, and none is reported to function in biomass degradation. However, the bacterium *Clostridium thermocellum* contains a gene that codes for a cellulosomal family GH-81 enzyme, which is consistent with a role in biomass degradation (*12*). The upregulation of Lam81A in conjunction with cellulases, in *T. fusca* grown on cellulose (Solka Floc), suggests a possible role for this enzyme in plant cell wall hydrolysis.

A potential natural substrate for Lam81A is callose, which is a polymer composed of β-1,3-linked glucose that is found in pollen tubes, the cell plate, and at sites of plant wounding or pathogen infection (*13*). The callose content of a typical living plant is low, but in decomposing tissues there is evidence for callose accumulation. For example, work by Gechev et al. has demonstrated that *Arabidopsis thaliana* mutants with a knockout in the tomato disease resistance gene homologue (Asc) are sensitive to programmed cell death

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(PCD)[1] in the presence of the fungal AAL toxin (*14*). Treatment with AAL toxin results in a plant hypersensitive response (HR) prior to PCD (*14*). Microarray analysis and cytochemical studies of these plants showed induction of callose synthase genes and subsequent callose accumulation, which is characteristic of the HR (*14*). In this work we characterize the substrate specificity, mode of action, and mechanism stereochemistry of Lam81A. These experiments further characterize a little studied family of glycosyl hydrolases and support a role for specific members in biomass degradation.

## MATERIALS AND METHODS

*Cloning of Lam81A.* A PCR product of the Lam81A gene (*T. fusca* gene 2130) was generated from *T. fusca* YX-ER1 strain genomic DNA. The PCR product was made using the forward primer, 5′-GTTGTTCTCCCCCTGATCATATGGG-CTGCGTCATGTCCCATGCCTCACG, which inserts an *Nde*I site (underlined) at the location of the start codon of the gene (italic), and the reverse primer, 5′-GGTGGC-GAACCCGACCACTAGTGGGAGTACTCGGGAGGAG, which adds an *Spe*I site (underlined) following the C-terminal codon. pD1396 was made by digesting a modified pET26b+ vector with the restriction enzymes *Nde*I and *Spe*I. The resulting 5.2 kb fragment was gel purified and ligated to the digested and gel-purified Lam81A PCR product. The ligation mixture was initially transformed into *Escherichia coli* DH5α cells for plasmid preparation and screening. The plasmid containing the Lam81A gene was sequenced and found to contain a point mutation resulting in the conversion of T291 to A; however, the mutation was not in a conserved residue, and so the recombinant enzyme was purified and characterized.

*Protein Expression and Purification.* pD1396 was transformed into *E. coli* BL-21 Codon Plus (DE3)-RIPL (Stratagene) for expression and purification. Frozen stocks containing the Lam81A insert were grown up overnight at 37 °C in LB broth with 0.5% glucose and 60 μg/mL kanamycin. The overnight culture was used to inoculate flasks of LB broth containing 60 μg/mL kanamycin (1:33 inoculation ratio), which was grown at 30 °C until an $OD_{600}$ of 0.8, induced with 0.5 mM isopropyl thio-β-D-galactoside (IPTG), and allowed to grow overnight at 30 °C. Lam81A is a secreted protein, so the cells were pelleted by centrifugation and the supernatant was saved. Phenylmethanesulfonyl fluoride (PMSF) and $(NH_4)_2SO_4$ were added to the supernatant to a final concentration of 0.1 mM and 1.2 M, respectively; it was microfiltered through a CUNO Beta Pure polyolefin 46368-02L cartridge and loaded onto a phenyl-Sepharose CL-4B column (10 mL/L of supernatant). The column was washed with 2 column volumes of 1.2 M $(NH_4)_2SO_4$ + 0.01 M NaCl + 5 mM $KP_i$ buffer (pH 6.8). Further washing was done with 3 column volumes of 0.5 M $(NH_4)_2SO_4$ + 0.005 M NaCl + 5 mM $KP_i$ buffer (pH 6.8) followed by elution in 3 column volumes of 5 mM $KP_i$ buffer (pH 6.8). The Lam81A fractions containing the fewest extraneous proteins, as determined by SDS−PAGE, were pooled and loaded onto a Q-Sepharose Fast Flow column (3 mg of protein/mL of packed resin). The column was washed with 2 column volumes of 10 mM Bis-Tris buffer (pH 6.0) + 10% glycerol and eluted with a gradient of 0−0.5 M NaCl + 10 mM Bis-Tris buffer (pH 6.0) + 10% glycerol (gradient 20× column volume). The purest Lam81A-containing fractions were pooled and concentrated using a PTGC 10000 MWCO ultrafiltration membrane (Millipore) and stored at −80 °C in 5 mM Na/$KP_i$ buffer (pH 6.8) + 10% glycerol (overall yield approximately 7 mg/L).

*Assays.* Unless otherwise specified, activity assays for Lam81A were conducted using 200 μL of (5 mg/mL) substrate + enzyme in 5 mM Na/$KP_i$ buffer (pH 6.8) in a final volume of 400 μL. Low ionic strength buffer was used because aliquots were removed from the assays and analyzed by thin-layer chromatography, which is more amenable to low salt concentrations. Assays were also run at higher ionic strength (0.5M buffer) with no change in activity (data not shown). Pachyman, CM-pachyman, laminarin, and barley β-glucan substrates were obtained commercially (Megazyme). All reactions were carried out at 50 °C in triplicate. Following the reaction, 1.0 mL of dinitrosalicylic acid (DNS) reagent was added, and the samples were boiled for 15 min according to the method of Miller (*15*). The amounts of reducing sugars were determined by measuring the absorbance at 600 nm using 0−300 μg of glucose to generate a standard curve. With insoluble substrates, samples were centrifuged at 13000 rpm for 5 min before absorbance values were read.

Assays to determine the pH optimum of Lam81A were run as above with the buffers reported in Irwin et al. (*16*). The temperature stability of the enzyme was determined on 400 μL samples containing 200 μL of pachyman substrate (5 mg/mL) + 0.08 μM enzyme in Na/$KP_i$ buffer (pH 6.8), which were incubated for 18 h at temperatures ranging from 0 to 75 °C, followed by a Lam81A activity assay run at 50 °C for 15 min. This assay was conducted within the linear range for product formation over time.

Thin-layer chromatography (TLC) was performed on reaction mixtures containing 1.5 mM substrate with 0.4 μM enzyme in a volume of 400 μL at 50 °C. Samples were prepared for TLC by removing 15 μL aliquots of the reaction mixture and quenching the reaction with 5 μL of 2 M $NH_4$-OH. Samples were spotted onto TLC plates, run, stained (100 mL of acetic acid, 1 mL of *p*-anisaldehyde + 2 mL of concentrated $H_2SO_4$), and developed by heating the plate at 95 °C for 1 h (*16*−*18*). The solvent system was ethyl acetate, water, and methanol (40:15:20). Oligosaccharide substrates and standards were obtained from Seikagaku or Megazyme.

For NMR analysis, the Lam81A enzyme was concentrated and brought up in $D_2O$. The enzyme was added to a final concentration of 11.2 μM to 560 μL of laminarin (5 mg/mL) in $D_2O$ + 50 mM sodium acetate (pH 6.8). The reaction was run at 50 °C and monitored in an INOVA 600 MHz NMR in the Department of Chemistry and Chemical Biology NMR facility at Cornell University.

Kinetic constants were calculated for reactions containing 3.35 pmol of Lam81A and laminarin substrate concentrations of 7−148 μM in 0.05M Na/$KP_i$ buffer (pH 6.8), final volume 400 μL. All reactions were run at 50 °C in triplicate. Reactions were quenched at specific time points (30 s−60

[1] Abbreviations: PCD, programmed cell death; HR, hypersensitive response; IPTG, isopropyl thio-β-D-galactoside; PMSF, phenylmethanesulfonyl fluoride; CM-pachyman, carboxymethyl-pachyman; DNS, dinitrosalicylic acid; TLC, thin-layer chromatography; TBS buffer, Tris-buffered saline; DMSO, dimethyl sulfoxide.

min depending on substrate concentration) with 1.6 mL of 2,2′-bicinchoninate reagent and heated at 80 °C for 30 min, and reducing sugars were quantified by measuring the absorbance at 560 nm, according to the method of Doner et al. (*19*). Plots were made showing the reducing sugars produced by each assay versus time. The plots indicated that the majority of time points were in the linear range for product formation over time. The $K_m$ and $V_{max}$ values were determined by fitting the Michaelis−Menten equation to a plot of the initial rates for each reaction against substrate concentration.

*Supernatant Activity Assays.* A starter culture of *T. fusca* was grown for 48 h with 5 mg/mL glucose as the carbon source in Hagerdahl media (*20*). The starter culture (100 $\mu$L) was used to inoculate 3 mL cultures of fresh Hagerdahl media containing various carbon sources (5 mg/mL). Cultures were grown for 72 h and spun down at 13000 rpm for 10 min, and the supernatant was collected. A Bradford assay was used to determine the protein concentration of the supernatant. Various amounts of supernatant were added to 400 $\mu$L reactions containing 200 $\mu$L of laminarin (5 mg/mL) and Na/$KP_i$ buffer, pH 6.8, to the final volume. Reactions were run for 3 h at 50 °C and stopped by the addition of 1 mL of DNS; reducing sugars were determined by reading the samples at 600 nm.

*Western Blots. T. fusca* supernatants grown on various carbon sources were run on SDS−PAGE to separate the proteins and then transferred to an Immobilon-P poly(vinylidene difluoride) membrane (Millipore). The primary antibody was rabbit antiserum raised against Lam81A, and the secondary antibody was a goat anti-rabbit Ig alkaline phosphatase conjugate (Bio-Rad). Western blots were developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad protocol).

*Immunoprecipitation. T. fusca* supernatant (250 $\mu$L) was added to 125 $\mu$L of Lam81A antiserum and 625 $\mu$L of 1× TBS buffer (0.02 M Tris, pH 7.5, + 0.5 M NaCl). Samples were left at 37 °C for 1 h and then at 4 °C for 12 h. Samples were spun down at 7000 rpm for 15 min, and the supernatant was used to perform reducing sugar activity assays as reported above.

*Callose Induction and Extraction.* The *Nicotiana tabacum* suspension cell line 1 (NT1) was a gift from the laboratory of Maureen Hanson at Cornell University. The cells were cultured on autoclaved KCMS medium (pH 5.5) prepared according to the method of Brants et al. (Murashige-Skoog, MS medium with 0.1 mg/L kinetin, 0.2 mg/L 2,4-dichlorophenoxyacetic acid, and 0.2 g/L $KH_2PO_4$) (*21*). Cells were subcultured every 7 days by transferring 1 mL of culture into 50 mL of fresh medium in a 250 mL Erlenmeyer flask. To induce callose synthesis, cells were harvested at 6 days by centrifugation and washed twice with calcium medium [3 mM $CaCl_2$ + 3% (w/v) sucrose, pH 5.0] according to the method of Ikegawa et al. (*22*). After washing, cells were resuspended in calcium medium at a concentration of 5 g fresh weight/500 mL of medium. Cells were treated with $AlCl_3 \cdot 6H_2O$ and $FeSO_4$ (final concentrations of 50 and 5 $\mu$M, respectively) and left on a rotary shaker for 24 h (*22*). To extract callose, cells were filtered through a nylon mesh membrane, resuspended in 100% ethanol, and left on an agitator for 3 days. The ethanol was changed frequently during the 3 day period. Cells were filtered through nylon mesh to remove the ethanol and ground with a glass homogenizer. After homogenizing, the cells were allowed to dry before being resuspended in dimethyl sulfoxide (DMSO); the mixture was placed in a sealed container and boiled for 30 min according to the method of Kohler et al. (*23*). Samples were centrifuged to remove the solids, and DMSO was removed using a speed vac, leaving a colorless gel.

Table 1: Specific Activity of Lam81A on Various Glucose Polysaccharides[a]

| substrate (linkage type) | specific activity [$\mu$mol of disaccharide produced $min^{-1}$ ($\mu$mol of Lam81A)$^{-1}$] |
|---|---|
| pachyman (1,3) | 168 |
| laminarin (1,3; some 1,6) | 1431 |
| CM-pachyman (1,3) | 349 |
| $\beta$-glucan (1,3;1,4) | 0.8 |
| lichenan (1,3;1,4) | 0.6 |
| CM-cellulose (1,4) | − |
| swollen cellulose (1,4) | − |
| pustulan (1,6) | − |

[a] All activities were measured at 5% digestion of substrate. No activity detected (−).

## RESULTS

*Expression and Purification.* The mature sequence of Lam81A was compared to those of 15 family-81 glycosyl hydrolases, including *C. thermocellum*. Lam81A shares 11−43% identity with other members of this family, with the highest similarity to the *C. thermocellum* enzyme and the most divergence from the yeast enzymes. The mature enzyme appears to lack a carbohydrate-binding module with only a catalytic domain. Lam81A was expressed as a recombinant protein in BL-21 Codon Plus (DE3)-RIPL cells, giving approximately 7 mg/L after purification with a purity of greater than 90% based on SDS−PAGE (data not shown). SDS−PAGE also indicated that the majority of the enzyme was secreted into the supernatant and not retained in the shock fluid (data not shown). Lam81A was expressed with its native signal sequence, with predicted cleavage between residues 35 and 36 to give a mature peptide with a calculated mass of 78773 Da (www.cbs.dtu.dk/services/SignalP/). Analysis of the purified Lam81A by mass spectrometry revealed a single major peak corresponding to 78824 Da, confirming the cleavage site and suggesting that no posttranslational modifications were present.

*Characterization.* Lam81A was assayed for its ability to hydrolyze a variety of substrates (Table 1). Callose is not commercially available, so other substrates with a similar structure were assayed including laminarin, pachyman, and carboxymethyl-pachyman (CM-pachyman). Lam81A had the highest activity on laminarin, a soluble polysaccharide composed primarily of $\beta$-1,3-linked glucose with occasional intrastrand 1,6-linked glucose linkages or branch points. Lam81A also showed substantial activity on the 1,3-linked pachyman and CM-pachyman substrates but less than on laminarin due to the relative insolubility of these substrates. The enzyme displayed much lower activity on the 1,3;1,4-linked substrates, mixed-linkage barley $\beta$-glucan and lichenan. Negligible activity was observed with the exclusively 1,4-linked substrates, (carboxymethyl)cellulose (CMC) and swollen cellulose, or the 1,6-linked pustulan.

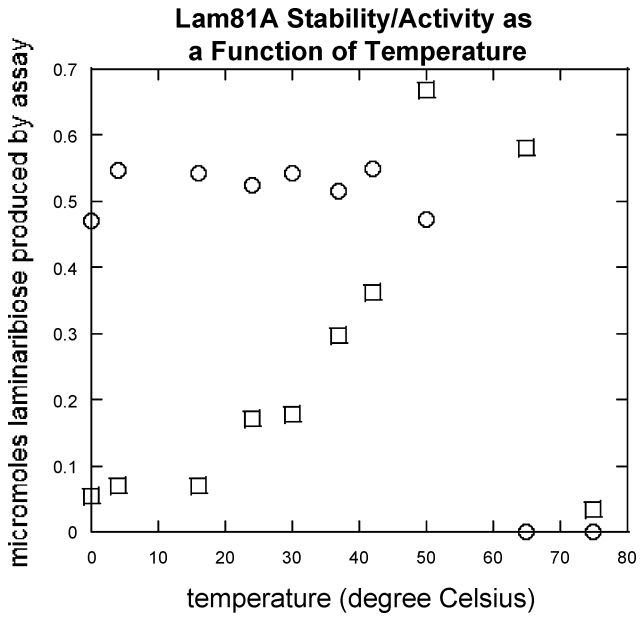


FIGURE 1: Plot of Lam81A activity (squares) and stability (circles) as a function of temperature.

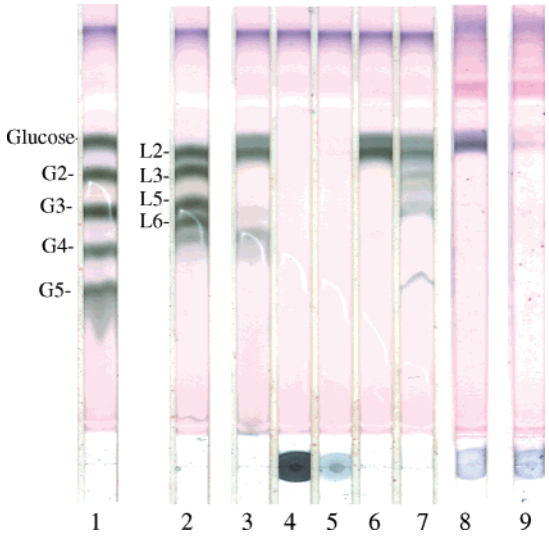


FIGURE 2: Thin-layer chromatography (TLC) of Lam81A hydrolysis products on various substrates. Hydrolysis reactions were conducted at 50 °C for 36 h. Lanes: 1, $\beta$-1,4-linked standards (glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose); 2, $\beta$-1,3-linked standards (laminaribiose, laminaritriose, laminaripentaose, and laminarihexaose); 3, laminarin; 4, barley $\beta$-glucan; 5, lichenan; 6, pachyman; 7, carboxymethyl- (CM-) pachyman; 8, Al/Fe-treated *N. tabacum* extract (callose induction); 9, no Al/Fe treatment of *N. tabacum* extract (minimum callose).

The optimum pH and temperature for Lam81A activity were determined, and the enzyme retained greater than 80% of its activity on pachyman across a broad pH range, from 5.5 to 10, with a maximum activity at pH 7.0 (data not shown). The enzyme was active over a wide range of temperatures, with the highest activity at 50 °C, and was stable for 18 h at temperatures ranging from 0 to 50 °C, although activity was rapidly lost at higher temperatures (Figure 1).

The products of Lam81A hydrolysis of several substrates were analyzed using TLC (Figure 2). After 36 h, the laminarin substrate was completely hydrolyzed to yield three major products: glucose, laminaribiose, and lesser amounts

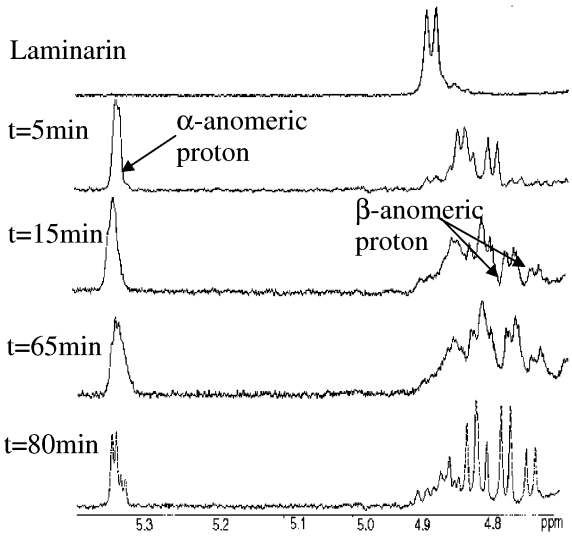


FIGURE 3: $^1$H NMR of laminarin hydrolysis by Lam81A after 5, 15, 65, and 80 min. The α-anomeric proton peak appears after initial hydrolysis, followed more slowly by the $\beta$-anomeric proton peak due to mutorotation.

of a larger unidentified oligosaccharide. Similar hydrolysis of the pachyman substrate yielded laminaribiose and glucose as the major products, while the products of CM-pachyman hydrolysis were laminaribiose and glucose with some larger oligosaccharides present in smaller amounts. Hydrolysis of mixed-linkage barley $\beta$-glucan and lichenan showed minimal product formation.

A $^1$H NMR spectrum was taken during the hydrolysis of laminarin by Lam81A to determine the stereochemistry (inverting or retaining) of the reaction (Figure 3). An α-anomeric proton peak appeared after initial hydrolysis, followed more slowly by a $\beta$-anomeric proton peak due to mutorotation. The initial appearance of the α-anomeric proton peak indicates Lam81A utilizes an inverting hydrolytic mechanism.

The products of Lam81A-mediated hydrolysis of laminarihexaose were examined by TLC to determine whether the enzyme is an endo- or exoglucanase. If Lam81A is an exoglucanase, the initial hydrolysis products would be laminaribiose and laminaritetraose in a 1:1 ratio, and the laminaritetraose would then be completely hydrolyzed to laminaribiose. However, if Lam81A is an endoglucanase and the substrate binds randomly within the active site, the expected hydrolysis products would be laminaribiose, laminaritriose, and laminaritetraose in a 1:2:1 ratio. Subsequent cleavage would then yield laminaribiose and glucose as the final products. TLC analysis showed that the initial products of the reaction correspond to those expected for an endoglucanase with band intensities approximating a 1:2:1 ratio of laminaribiose:laminaritriose:laminaritetraose (Figure 4).

Lam81A was tested for its ability to hydrolyze callose extracted from Al/Fe-induced cultures of *N. tabacum*. TLC analysis of the products resulting from co-incubation of callose with Lam81A showed laminaribiose and glucose to be the primary hydrolytic products (Figure 2). Similar incubation of Lam81A with callose extracted from uninduced cultures showed minimal product formation.

The apparent $K_m$ and $V_{max}$ of Lam81A are 13.7 $\mu$M and 0.023 $\mu$mol of glucose produced/min, respectively. A plot of initial rates versus substrate concentration shows strong

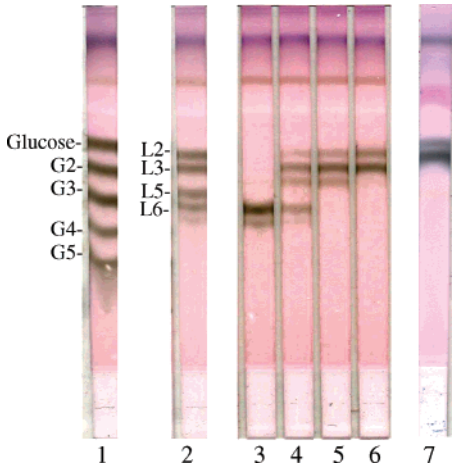


FIGURE 4: Thin-layer chromatography (TLC) of laminarihexaose + Lam81A hydrolysis products over time. The formation of three products, laminaribiose, laminaritriose, and laminaritetraose, in a 1:2:1 ratio suggests that Lam81A is an endoglucanase. Lanes: 1, $\beta$-1,4-linked standards (glucose, cellobiose, cellotriose, cellotetraose, and cellopentaose); 2, $\beta$-1,3-linked standards (laminaribiose, laminaritriose, laminaripentaose, and laminarihexaose); 3−6, Lam81A + laminarihexaose at 0, 5, 10, and 20 min; 7, Lam81A + laminarihexaose final products after 18 h.

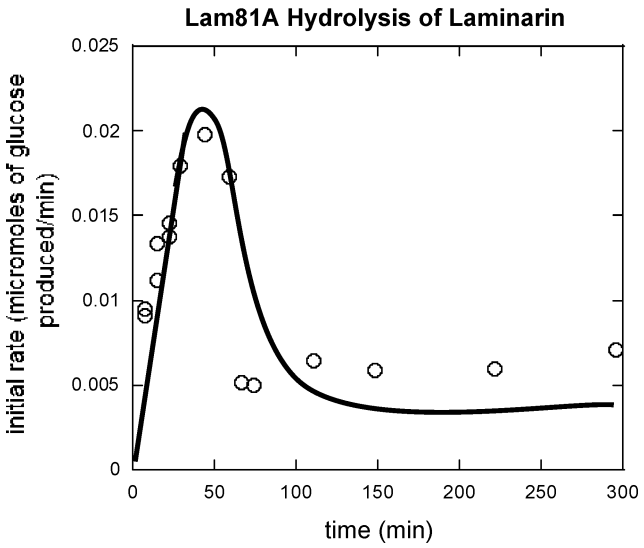


FIGURE 5: Plot of initial rates versus substrate concentration for the reaction of Lam81A with laminarin. The concentration of laminarin was determined by assuming an average length of polymerization of 25 glucose units as determined by Read et al. (*26*). The curve is drawn to help with visualization and is not intended to be a fit.

substrate inhibition at concentrations of laminarin exceeding 50 $\mu$M (Figure 5).

*Induction. T. fusca* was grown on several different carbon sources for 72 h; the supernatants were then isolated and assayed for $\beta$-1,3-glucanase activity on laminarin (Table 2). All supernatants tested show detectable $\beta$-1,3-glucanase activity, suggesting a high basal level of expression. The highest levels of activity were observed in the supernatants derived from *T. fusca* grown on pachyman, Solka Floc cellulose, and laminaribiose, demonstrating their ability to induce Lam81A. These results were confirmed by Western blot analysis using polyclonal antibodies raised against Lam81A. The blot showed that Lam81A was present in all supernatants assayed (Figure 6).

Table 2: Specific Activities for the Hydrolysis of Laminarin by *T. fusca* Supernatant Grown on a Variety of Carbon Sources[a]

| substrate | specific activity [$\mu$mol of disaccharide produced min$^{-1}$ ($\mu$mol of protein)$^{-1}$] |
|---|---|
| glucose | 0.161 |
| cellobiose | 0.643 |
| Solka Floc | 1.340 |
| corn fiber | 0.247 |
| alfalfa | 0.263 |
| pachyman | 1.265 |
| laminaribiose | 1.211 |

[a] Supernatant was isolated from cells grown for 72 h at 50 °C. Assays were run for 3 h at 50 °C. The extent of hydrolysis was quantified by the dinitrosalicylic acid (DNS) colorimetric assay for reducing sugars.

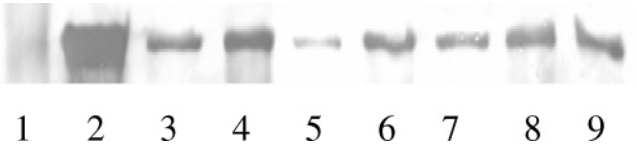


FIGURE 6: Western blot analysis of *T. fusca* supernatants using Lam81A antibody (10 $\mu$L of supernatant loaded in each lane). Lanes: 1, MW marker, 85 kDa; 2, 0.05 $\mu$g of pure Lam81A (Lam81A, 78.8 kDa); 3, cellobiose; 4, laminaribiose; 5, glucose; 6, Solka Floc; 7, corn fiber; 8, pachyman; 9, alfalfa.

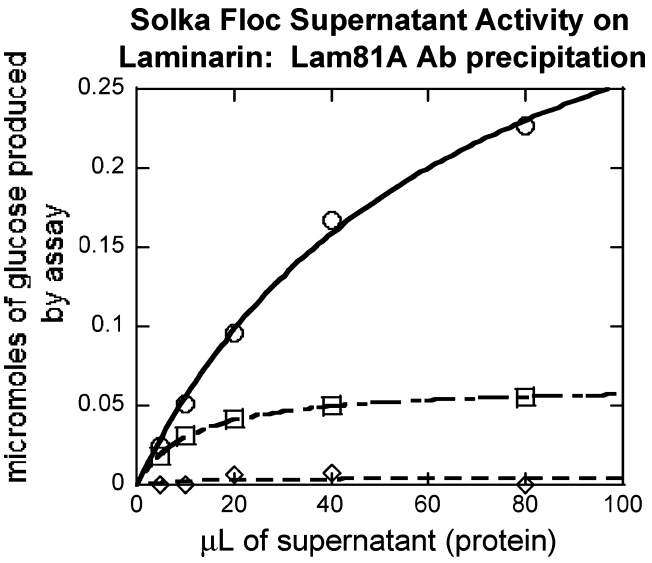


FIGURE 7: Hydrolysis of laminarin by *T. fusca* supernatant grown on Solka Floc for 72 h with and without Lam81A immunoprecipitation. Key: supernatant unprecipitated (circles), supernatant precipitated with 1 part antiserum to 2 parts supernatant by volume (squares), and supernatant precipitated by 1 part antiserum to 1 part supernatant by volume (diamonds).

The above activity assays assume that all $\beta$-1,3-glucanase activity is due to Lam81A, and a BLAST search of the *T. fusca* genome shows no additional known $\beta$-1,3-glucanases. To verify this, Lam81A antiserum was used to immunoprecipitate Lam81A from *T. fusca* supernatants, and then the activity assays were repeated. Upon antibody precipitation of Lam81A, all $\beta$-1,3-glucanase activity was lost from the supernatant, demonstrating that Lam81A was solely responsible for the observed activity (Figure 7).

## DISCUSSION AND CONCLUSIONS

The activity of Lam81A on various substrates indicates that the enzyme is specific for $\beta$-1,3-linked glucose polymers, which is consistent with a role for Lam81A in the hydrolysis of the plant polymer, callose. TLC showed that the major hydrolysis end products of Lam81A on all $\beta$-1,3-linked substrates are glucose and laminaribiose. Analysis of the hydrolysis products for Lam81A on laminarin also showed a larger hydrolysis product, which probably contains one or more $\beta$-1,6 linkages. These results, together with the inactivity of Lam81A on pustulan (a $\beta$-1,6-glucose polysaccharide), suggest that it cannot cleave $\beta$-1,6-linked glucose residues. The enzyme also has very low activity on mixed-linkage ($\beta$-1,3;1,4) glucan, indicating that Lam81A can only hydrolyze glycosyl bonds between adjacent $\beta$-1,3-linked glucose units, which are in low abundance in the mixed-linkage polysaccharides. Sequence analysis and Lam81A binding assays (data not shown) indicate that Lam81A has no known carbohydrate-binding module, and the lower activity on pachyman and CM-pachyman may reflect the insolubility of both substrates. The significant activity observed with CM-pachyman as a substrate confirms that Lam81A has typical endoglucanase activity because endoglucanases usually have open active site clefts that are able to accommodate the larger carboxymethylated sugars (*24*).

The $^1$H NMR experiment demonstrates that Lam81A hydrolyzes substrates with an inversion of configuration. This result is in agreement with recent results published by Fliegmann et al. on the family-81 $\beta$-glucan-binding protein (GBP) from soybean (*11*). With the exception of an inverting family GH-64 enzyme, reported by Nishimura et al., the other known $\beta$-1,3-glucanases, found in glycosyl hydrolase families-5, -16, and -17, all use retaining mechanisms (*25*). To date no mechanism has been reported for the family GH-55 $\beta$-1,3-glucanases. TLC data support the classification of Lam81A as an endoglucanase and are consistent with the endoglucanase activity that has been observed in other characterized family-81 glycosyl hydrolases, including Eng1p and Acf2p/Eng2p from *S. cerevisiae* (*9*).

The apparent $K_m$ for Lam81A of 13.7 $\mu$M is similar to the value reported by Fliegmann et al. for the soybean GBP, but they did not observe substrate inhibition (*11*). The observed substrate inhibition of Lam81A at concentrations of laminarin exceeding 50 $\mu$M could be explained by the existence of a secondary substrate-binding site, or by nonproductive binding of a substrate molecule into the multisubsite active site. Preliminary work, using stopped flow to visualize tryptophan fluorescence during the course of the mixing of Lam81A with laminarin, showed two distinct phases in the interaction, suggesting the presence of a lower affinity-binding site (data not shown). Additional work will be necessary to determine the exact mechanism of substrate inhibition.

Activity assays of *T. fusca* supernatants grown on various carbon sources show that Lam81A is induced by laminaribiose, $\beta$-1,3-linked polysaccharides, and cellulose. The increased activity of Lam81A in the cellulose substrate-related supernatant is consistent with microarray studies that showed elevated Lam81A transcript levels under similar growth conditions (*8*). In addition, the activity assays show a moderate induction of Lam81A when grown on cellobiose,

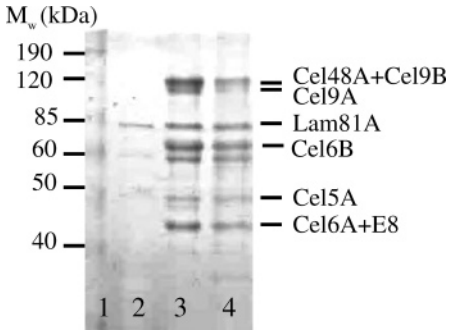


FIGURE 8: SDS gel of supernatants from *T. fusca* grown on glucose (lane 1), cellobiose (lane 2), and laminaribiose (lane 3). All supernatants were harvested from *T. fusca* cells grown for 72 h at 50 °C.

which was also suggested by microarray analysis (*8*). The assays also indicate that Lam81A is present under all conditions tested, suggesting that it has high basal expression (*24*). Western analysis of *T. fusca* supernatants grown on glucose demonstrates the presence of Lam81A and confirms that the enzyme synthesis is not subject to the strong catabolite repression that is typically seen with cellulases.

Using previous microarray studies to analyze other genes upregulated by cellulose (Solka Floc) and cellobiose, several interesting conclusions can be drawn regarding the regulation of cellulases versus Lam81A. Microarray analysis has shown that, in the presence of cellobiose, many genes containing the CelR regulatory sequence are upregulated, including all six cellulase genes, the regulatory protein CelR, and several carbohydrate-binding proteins (*8*).

Preliminary studies using SDS gels demonstrate that *T. fusca* grown on laminaribiose produces about the same level of all six cellulases as seen with cellobiose (Figure 8). These results are surprising because analysis of the *Lam81A* gene has failed to reveal the characteristic 14 bp repeat that serves as the binding site for CelR. Taken together, these results have several implications: (1) Lam81A may have a role in biomass degradation because it has an induction pattern that coincides with that of cellulases, (2) laminaribiose may be capable of binding CelR to relieve transcriptional repression in a comparable manner to cellobiose, and (3) there is an additional form of regulation for biomass hydrolyzing genes other than that involving CelR.

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