Appl Biochem Biotechnol (2008) 146:79–87
DOI 10.1007/s12010-007-8074-2

Heterologous Expression of Two Ferulic Acid Esterases from *Penicillium funiculosum*

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Received: 25 May 2007 / Accepted: 27 September 2007 /
Published online: 7 December 2007

Abstract Two recombinant ferulic acid esterases from *Penicillium funiculosum* produced in *Aspergillus awamori* were evaluated for their ability to improve the digestibility of pretreated corn stover. The genes, *faeA* and *faeB*, were cloned from *P. funiculosum* and expressed in *A. awamori* using their native signal sequences. Both enzymes contain a catalytic domain connected to a family 1 carbohydrate-binding module by a threonine-rich linker peptide. Interestingly, the carbohydrate binding-module is N-terminal in FaeA and C-terminal in FaeB. The enzymes were purified to homogeneity using column chromatography, and their thermal stability was characterized by differential scanning microcalorimetry. We evaluated both enzymes for their potential to enhance the cellulolytic activity of purified *Trichoderma reesei* Cel7A on pretreated corn stover.

Keywords Ferulic acid esterase · Hemicellulose · Cellulose · Biomass digestion · Heterologous expression

Introduction

The use of lignocellulosic materials from agricultural crops for the production of fuels and chemicals has received a considerable amount of interest recently and is a major part of the Advanced Energy Initiative outlined by the President of the United States in both the 2006 and 2007 State of the Union addresses [1]. Currently, ethanol derived from biomass is produced by the conversion of either sugar or starch containing crops. The production of

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ethanol from lignocellulosic agricultural residues has become increasingly important due to the volumetric limitations of using corn kernels and will directly address the United States goal of reducing gasoline usage by 20% in the next 10 years [1]. Examples of underutilized lignocellulosic materials that are produced in large quantities worldwide include maize stem, wheat, barley, rice, and rye straw.

Lignocellulosic materials, having a structure and composition that is recalcitrant to degradation, require pretreatment followed by enzymatic hydrolysis to obtain soluble sugars for fermentation. It is thus critical that pretreatment and enzymatic saccharification maximize the release of these soluble sugars. One barrier to efficient sugar release is the degree of accessibility of the crystalline cellulose to the saccharification enzymes. Plant cell walls are structurally complex with networks of cross-linked hemicellulose and lignin intercalated between the cellulose fibrils. The natural breakdown of plant cell wall material requires the synergistic action of several enzymes including those that break ester cross-links between lignin and xylan. Ferulic acid is a hydroxycinnamic acid that cross-links hemicelluloses and lignin in plant cell walls through ester linkages [2]. This lignin/carbohydrate complex may contribute to the natural recalcitrance of biomass to microbial degradation. Microbial feruloyl esterases (EC 3.1.1.73) are generally secreted, catalyze the hydrolysis of ester and ether bridges between hydroxycinnamic acids and polysaccharides and/or lignins in plant cell walls, and have been classified into four groups based on substrate utilization: A, B, C, D [3].

An important characteristic of enzymes having plant cell wall deconstruction activities is the presence of a carbohydrate binding module (CBM). These modules have been found on enzymes with a wide range of activities and are crucial to targeting the enzyme at the substrate level leading to increased activity on insoluble substrates [4]. Interestingly, the majority of known fungal ferulic acid esterases do not contain CBMs. Those without CBMs include FaeA and FaeB from *Aspergillus niger* [5, 6], FaeB and FaeD from *Neurospora crassa* [7, 8], and FaeC from *Talaromyces stipitatus* [9]. However, while the gene encoding FaeB from *T. stipitatus* has not been fully sequenced, it does show significant sequence homology to FaeB from *Penicillium funiculosum*, which does have a CBM [10]. This lack of a CBM in the majority of the ferulic acid esterases is somewhat surprising, as CBMs have been shown to be required for maximal utility for a variety of cellulolytic enzymes [4].

Here, we report the heterologous expression of two ferulic acid esterases from *P. funiculosum* and their ability to enhance the release of cellobiose by *Trichoderma reesei* Cel7A from pretreated corn stover.

Materials and Methods

Media, Strains, Plasmid Construction, and Genetic Techniques

Genomic DNA from *P. funiculosum* ATCC 62998 grown in CM-glucose media [11] was isolated using the DNeasy Plant Maxi Kit (Qiagen, cat. no. 68161) as per the manufacturer's directions. The genes *faeA* (AJ312296) and *faeB* (AJ291496) were amplified by the PCR and the appropriate primers (Table 1) for sub-cloning into the fungal expression vector, pFE2 [11]. The following cycle parameters and the PfuTurbo polymerase (Stratagene, cat. no. 600250) were used; 99 °C 10 min, 30 cycles of 95 °C for 30 s, 58 °C for 1 min, 72 °C for 2:30 min, followed by a final extension at 72 °C for 5 min. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen, cat. no. K4510–20) for recovery then sub-cloned into the

Table 1 PCR primers.

Primer name	Sequence
FaeAfor	**CCTCAGC**AATGGTGAAATCGTACATTATCGGGGCAT
FaeArev	**TCTAGA**TTAGTGGAATAGAGAGAAGAAACTCCAGAT
FaeBfor	**CCTCAGC**ATGGCGATTCCCTTGGTCCT
FaeBrev	GA**TCTAGA**TCACAGGCACTGGGAATAATAATCGT

Start and stop codons are underlined. Restriction sites used for sub-cloning into pFE2 are in bold (*Bbv*CI and *Xba*I).

pFE2 vector for fungal expression. Plasmid DNA of the expression vector–gene construction from a large-scale isolation (Qiafilter Plasmid Maxi Kit, Qiagen, cat. no. 12262) was sequenced using the Applied Biosystems Automated 3730 DNA Analyzer and Big Dye Terminator chemistry with AmpliTaq-FS DNA Polymerase at the Cornell Biotechnology Resource Center. *Aspergillus awamori* ATCC 22342 was transformed as described [11]. Spores from potential transformants were frozen for later use.

Protein Expression and Purification

Frozen *A. awamori* spores were thawed and inoculated into 50 ml CM-maltose medium and grown at 32 °C, 225 rpm in 250 ml baffled flasks. After 2–3 days, the cultures were transferred to 1.0 l CM-maltose fermentation medium in 2,800 ml Fernbach flasks and grown in the above conditions. The flasks were harvested by filtration through Miracloth (Calbiochem, cat. no. 475855) after 4–6 days of growth and frozen at −20 °C. The broth was thawed and clarified through a glass fiber filter (Pall Life Sciences, cat. no. 66084). Protein was allowed to precipitate out in 90% ammonium sulfate for 2 days at 4 °C, then centrifuged at 9,000×*g* for 20 min at 4 °C for removal from the supernatant. Precipitated protein was dissolved in 20 mM Bis–Tris at pH 6.8 and desalted on a Pharmacia Hi-Prep Desalting (GE Healthcare Bio-Sciences, Uppsala, Sweden) column into 20 mM Bis–Tris pH 6.8. The desalted proteins were separated by anion exchange chromatography using a FineLine Pilot 35 column (GE Healthcare Bio-Sciences, Uppsala, Sweden) packed with 96 ml of Resource SourceQ (GE Healthcare Bio-Sciences, Uppsala, Sweden) in 20 mM Bis–Tris pH 6.8 as the running buffer over a 0- to 1-M NaCl gradient. Both proteins eluted in a sharp peak within the initial 5% of the salt gradient. Purified FaeA was then buffer-exchanged into 20 mM sodium acetate, 100 mM NaCl at pH 5.0 (SEC buffer) using a 26/60 SuperDex200 size exclusion chromatography (SEC) column (GE Healthcare Bio-Sciences, Uppsala, Sweden) aliquoted, and stored at −80 °C. Pooled FaeB fractions were further purified on a Resource ISO (1 ml) HIC column (GE Healthcare Bio-Sciences, Uppsala, Sweden) running 20 mM sodium acetate buffer at pH 5.0 over a 1.5- to 0-M ammonium sulfate gradient. Purified FaeB was then buffer-exchanged, aliquoted, and stored as above. Purity of the proteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and each was quantified by absorbance at 280 nm. Purified proteins were sequenced at the Cornell Proteomics and Mass Spectrometry core facility as described [12]. *T. reesei* Cel7A was purified chromatographically from Spezyme CP (Genencor International Inc., Palo Alto, CA) via two anion exchange steps followed by an affinity step. Spezyme CP was diafiltered into 20 mM Bis–Tris pH 6.2 using an Amicon CH-2 hollow fiber concentrator (Amicon, Beverly, MA.) equipped with PM10 cartridges and

Fig. 1 Alignments of our sequence (*top*) and the sequences deposited in GenBank (*bottom*). Amino acids are *numbered on either end* and differences in AA sequences are *underlined*. Peptides isolated by sequencing are in *bold*. *Symbols* are located under the corresponding sequences: *closed triangles*; Putative start of the family 1 CBM. *Hatch filled boxes* delineate linker regions. The GXSXG sequence of the conserved serine protease motif is *enclosed in a box*

loaded onto a 53-ml DEAE anion exchange column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Bound proteins were eluted with a linear 0 to 1.0 M NaCl gradient in the same buffer. Fractions containing Cel7A were pooled, desalted, and loaded onto a 200-ml SourceQ anion exchange column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Cel7A was eluted with a 0.0- to 1.0-M NaCl gradient, and active fractions were pooled and bound to SigmaCell 101 (SigmaAldrich, St. Louis, MO) with cellulose loadings of 0.1 g/ml. After binding overnight at 4 °C, the cellulose was washed twice with SEC buffer via centrifugation (4,000×*g*) to remove unbound protein. Bound Cel7A was eluted with 100% ethylene glycol and concentrated with a 10-kDa PES MWCO membrane in a stirred pressure cell (Amicon, Beverly, MA.) before buffer exchange on a 26/60 SuperDex200 SEC column into SEC buffer.

Protein Stability Measurements

The thermal stability of the proteins was measured by differential scanning microcalorimetry (DSC) using a Microcal model VP-DSC calorimeter (Microcal, Inc., Northampton, MA), with data analysis by Origin for DSC software (Microcal). Thermograms were collected for samples containing 50 μg/ml protein at pH 5.0 in SEC buffer. Calorimeter scan rate was 60 °C/h.

Ferulic Acid Esterase Activity Analysis

Activity of the purified enzymes was assessed on methyl ferulate (methyl 4-hydroxy 3-methoxy cinnamate, MF). Assays were run for 30 min at 50 °C in 50 mM citrate buffer at pH 4.8 with initial MF concentrations of 50 to 750 μM and an enzyme concentration of 50 nM. The reactions were terminated after 30 min by boiling for 10 min and analyzed for MF and ferulic acid content via C18 high performance liquid chromatography (HPLC) over a 0 to 100% acetonitrile gradient with 0.1% formic acid in all solutions. Preliminary isothermal titration calorimetry (ITC) on both ferulic acid esterase proteins was conducted in SEC buffer on a Microcal VP-ITC system. All reactions were carried out at 37 °C using MF as a substrate.

Corn Stover Digestions

The corn stover used for this study was harvested in 2003 at the Kramer Farm in Wray, Colorado. The stover was pretreated in a flow-through hot water pretreatment reactor at Dartmouth College under subcontract with the National Renewable Energy Laboratory (NREL). The pretreatment was conducted at 200 °C for 16 min with a 2.5% (*w*/*v*) solid loading. Carbohydrate and lignin composition of the pretreated material was determined by a two-stage sulfuric acid hydrolysis treatment using NREL Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin in Biomass" [13]. Briefly, 300 mg of biomass is hydrolyzed by 3.0 ml 72% (*w*/*v*) H_2SO_4 for 1 h at 30 °C with stirring every 5–10 min. After dilution to 4% H_2SO_4 (*w*/*v*), the sample was autoclaved at 121 °C for 1 h. Aliquots were analyzed by UV absorbance for acid soluble lignin, gravimetrically for acid-insoluble lignin, and by HPLC for structural sugars. The glucan, xylan, and lignin content of the pretreated material was 0.59, 0.14, and 0.19 g/g, respectively. Digestions were run with 2.5 mg FaeA or FaeB /g cellulose in addition to 10 mg Cel7A/g cellulose for 24 h at 50 °C in 50 mM citrate buffer, pH 4.8, with the biomass loaded to achieve a 1.0% (*w*/*v*) glucan concentration in solution. FaeA and FaeB were assessed separately and in combination with each other.

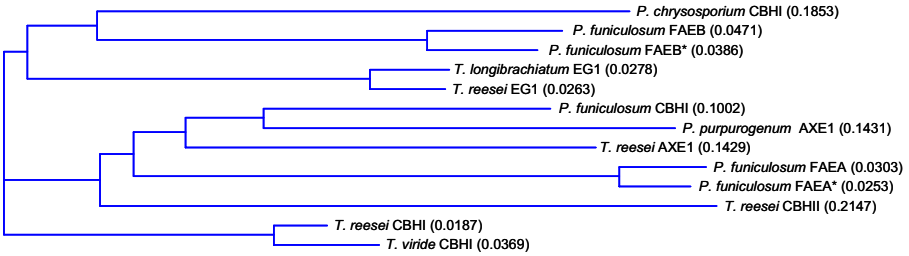

Fig. 2 Relatedness of FaeA and FaeB Family One CBMs. *asterisks*: denotes our sequence for FaeA or FaeB

Results and Discussion

Cloning and Heterologous Expression

The genes *faeA* and *faeB* were amplified from genomic DNA of *P. funiculosum* using the PCR and cloned into the pCR2.1-TOPO vector. The genes were then sub-cloned into the fungal expression vector pFE2 [11] at the *Bbv*CI and *Xba*I sites to allow for fungal expression driven by the glucoamylase promoter with secretion based on the gene's native signal sequence. The sequence of the cloned DNA revealed that one intron was present in each gene. These introns were left intact and were properly processed by *A. awamori* as shown by the expression of a functional protein.

Several amino acid differences between our cloned sequences and the sequences reported in GenBank for *faeA* (AJ312296; no specific strain designation was given) and *faeB* (AJ291496; *P. funiculosum* IMI-134756), respectively, were present (Fig. 1). It is not surprising and quite common that minor AA sequence differences are present in the same proteins from different strains. Peptide sequences derived from chymotrypsin digests followed by nano-liquid chromatography/mass-spectrometry/mass spectrometry (LC-MS/MS) analysis were used for positive identification of the purified enzymes. Four peptides were recovered for each protein representing 13 and 14% coverage of FaeA and FaeB, respectively (Fig. 1).

Both enzymes have a family 1 CBM; however, they differ in that the carbohydrate binding module is N-terminal in FaeA and C-terminal in FaeB (Fig 1). This situation mirrors that of Cel6A (N-terminal CBM) and Cel7A (C-terminal CBM) of *T. reesei*. The

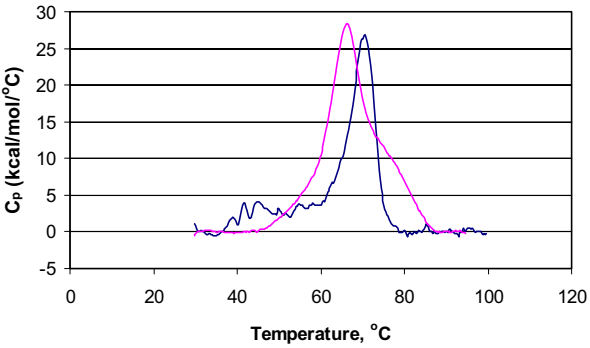

Fig. 3 Thermal stability of FaeA and FaeB. DSC thermograms were acquired as described in the Materials and Methods section

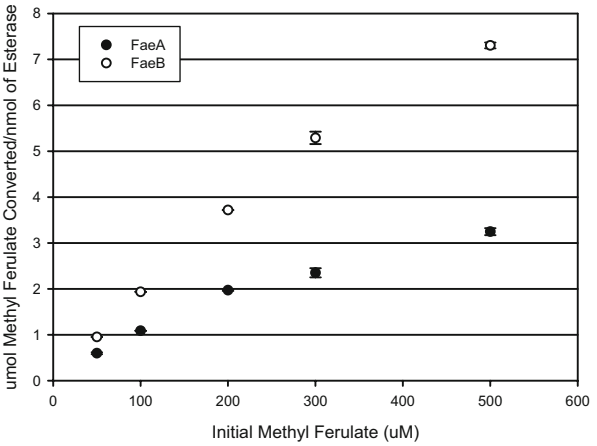

Fig. 4 Activity of the two esterases on methyl ferulate. *filled circles*: FaeA; *empty circles*: FaeB

CBM's of our sequences have two and three AA differences for FaeA and FaeB, respectively, compared to the sequences listed in GenBank. As a result of these AA differences, the sequences we cloned are slightly closer to their respective branch points than for the sequences currently deposited in GenBank as estimated by the neighbor-joining method [14] (Fig. 2). We did not characterize the CBM–cellulose interaction of FaeA and FaeB. Both enzymes bound effectively to Sigmacell 101 as a step in our initial purification, but due to concerns about the effects of eluting with ethylene glycol, we did not use this step in our final purification scheme (data not shown). It is curious that enzymes that do not act on crystalline cellulose have a CBM that effectively binds crystalline cellulose. One hypothesis is that the CBM allows the enzyme to maintain a higher effective concentration near its preferred substrate; thus, by binding to cellulose, the ferulic acid esterases can more effectively target their activity. In this regard, the enzymes from *P. funiculosum* are unique in possessing CBMs, and as more ferulic acid esterases are sequenced and characterized, it will be interesting to see whether the majority of these enzymes contain or lack CBMs.

Thermal Stability Determination

The enzyme FaeB has previously been described [10], but thermal stability was not measured. In addition, the sequence for *FaeA* has been deposited in GenBank; however,

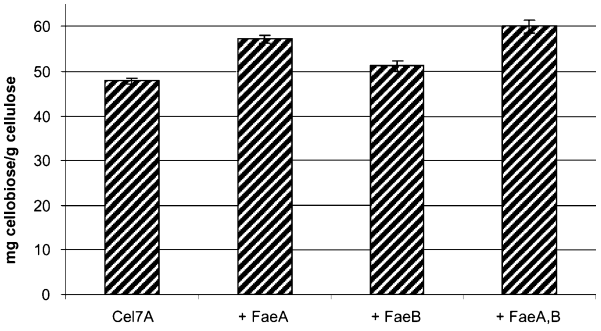

Fig. 5 Digestion of hot water pretreated corn stover with various enzyme combinations. Digestions were carried out for 24 h as described in the Materials and Methods section

there are no reports concerning that enzyme. Thermograms acquired by DSC showed that FaeB is slightly more thermally stable (T_{max} = 70.4 °C) than FaeA (T_{max} = 66.3 °C) when heterologously expressed (Fig. 3). Due to possible differences in the extent of glycosylation, their thermal stabilities when expressed in a recombinant host may differ from those when the proteins are expressed in their native host [15].

Activity Assays and Corn Stover Digestions

Activity assays on MF show that both esterases have activity when heterologously expressed in *A. awamori* (Fig. 4). FaeB was more active than FaeA, hydrolyzing the bulk of the available substrate during the 30-min incubation period. The identical loading of FaeA, in most cases, only consumed about half of the initial MF in each assay. Both enzymes are known to be active on MF with group A enzymes preferring methoxy substitutions on the phenolic ring, while group B enzymes prefer the hydroxyl substitutions [3]. In addition, preliminary ITC experiments with MF were run on both ferulic acid esterase proteins. The reactions of both proteins appears to be mildly endothermic, with apparent heats of reaction estimated as ~1,400 and ~2,000 cal/mol of MF reacted for FaeA and FaeB, respectively. The relatively small heats of reaction have made detailed kinetic analysis of the proteins on this substrate difficult to accurately discern in the presence of baseline drift in the calorimetry runs.

Previously, we have reported the presence of FaeA to be beneficial during the hydrolysis of hot water pretreated corn stover by the cellobiohydrolase Cel7A [16]. In this study, FaeA had a significant impact on Cel7A performance, releasing an additional 19% cellobiose, whereas the benefits of FaeB addition were smaller, releasing only an additional 7% cellobiose. The combination of the two esterase enzymes, however, had an additive gain releasing an additional 25% cellobiose (Fig. 5). Clearly, adding additional accessory enzyme activities can be beneficial in maximizing the enzymatic saccharification of recalcitrant lignocellulosic agricultural residues such as corn stover.

Conclusions

The ferulic acid esterase genes, *faeA* and *faeB*, from *P. funiculosum* were expressed in *A. awamori*, and after purification, the proteins were identified as FaeA and FaeB, respectively, by nano-LC/MS/MS peptide sequencing analysis. The heterologously expressed enzymes were determined to be active as judged by their ability to hydrolyze MF to ferulic acid and by their ability to increase the cellobiose release in digestions of pretreated corn stover with Cel7A. Additional studies involving the digestion of biomass with various enzymatic components will be critical to gain a more comprehensive understanding of the synergistic effects of individual enzymes on biomass deconstruction and will help in deconvoluting the myriad enzymatic pathways fungi have evolved to degrade complex recalcitrant materials present in their native habitats. With a better understanding of these synergisms, the development of an enzymatic suite capable of this same degradation in a timely fashion in the modern biorefinery will be greatly facilitated.

Acknowledgements We acknowledge Dartmouth College for the pretreatment of the corn stover and the MS-based protein identification service work provided by the Cornell Proteomics and Mass Spectrometry core facility. This work was funded by the Department of Energy Office of the Biomass Program.

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