Novel Family of Carbohydrate Esterases, Based on Identification of the *Hypocrea jecorina* Acetyl Esterase Gene[†]

Xin-Liang Li,^{1*} Christopher D. Skory,² Michael A. Cotta,¹ Vladimir Puchart,³ and Peter Biely³

Fermentation Biotechnology Research Unit, National Center for Agricultural Utilization Research, United States Department of Agriculture-Agricultural Research Service, 1815 N. University Street, Peoria, Illinois 61604¹; Bioproducts and Biocatalysis Research Unit, National Center for Agricultural Utilization Research, United States Department of Agriculture-Agricultural Research Service, 1815 N. University Street, Peoria, Illinois 61604²; and Institute of Chemistry, Slovak Academy of Sciences, 845 38 Bratislava, Slovakia³

Received 8 April 2008/Accepted 6 October 2008

Plant cell walls have been shown to contain acetyl groups in hemicelluloses and pectin. The gene *aes1*, encoding the acetyl esterase (Aes1) of *Hypocrea jecorina*, was identified by amino-terminal sequencing, peptide mass spectrometry, and genomic sequence analyses. The coded polypeptide had 348 amino acid residues with the first 19 serving as a secretion signal peptide. The calculated molecular mass and isoelectric point of the secreted enzyme were 37,088 Da and pH 5.89, respectively. No significant homology was found between the predicated Aes1 and carbohydrate esterases of known families, but putative *aes1* orthologs were found in genomes of many fungi and bacteria that produce cell wall-degrading enzymes. The *aes1* transcript levels were high when the fungal cells were induced with sophorose, cellulose, oat spelt xylan, lactose, and arabinose. The recombinant Aes1 produced by *H. jecorina* transformed with *aes1* under the cellobiohydrolase I promoter displayed properties similar to those reported for the native enzyme. The enzyme hydrolyzed acetate ester bond specifically. Using 4-nitrophenyl acetate as substrate, the activity of the recombinant enzyme was enhanced by D-xylose, D-glucose, cellobiose, D-galactose, and xylooligosaccharides but not by arabinose, mannose, or lactose. With the use of 4-nitrophenyl-β-D-xylopyranoside monoacetate as substrate in a β-xylosidase-coupled assay, Aes1 hydrolyzed positions 3 and 4 with the same efficiency while the *H. jecorina* acetylxyylan esterase 1 exclusively deacetylated the position 2 acetyl group. Aes1 was capable of transacetylating methylxyloside in aqueous solution. The data presented demonstrate that Aes1 and other homologous microbial proteins may represent a new family of esterases for lignocellulose biodegradation.

Acetylxyylan esterases are common components of hemicellulolytic and cellulolytic enzyme systems of microorganisms proliferating on plant biomass residues. Their function is to deesterify partially acetylated hardwood 4-O-methyl-D-glucuronoxylan and xylans of annual plants (3, 6, 7). These esterases are part of hemicellulolytic enzyme systems and found in 8 of 15 carbohydrate esterase (CE) families (cazy.org/fam/acc_CE.html). Most of the CEs are serine-type esterases, also attacking low-molecular-mass substrates such as 4-nitrophenyl (4-NP) or 4-methylumbelliferyl acetate (4, 36). An exception in this regard is the acetylxyylan esterases of CE family 4, which also contain chitin deacetylases. These enzymes do not operate on the two above-mentioned aryl acetates and also do not possess the Ser-His-Asp catalytic triad. They are aspartate deacetylases, activated with bivalent metal cations coordinated with two histidines (27, 35).

The fungus *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is capable of secreting many hydrolytic enzymes for lignocellulose biodegradation (10, 22). Among the hemicellulases are two acetylxyylan esterases (10) and one glucuronoyl esterase

(GE1) (19) encoded by *cip2* (10). The two acetylxyylan esterases, Axe1 and Axe2, both belong to CE family 5, but only Axe1 possesses a carbohydrate-binding module, while GE1 has been recently assigned to CE family 15. The three-dimensional structure of the Axe1 catalytic domain was determined (11). Both *axe1* and *axe2* are upregulated on cellulose or during induction with sophorose (10); however, the product of *axe2* has not yet been described. Besides Axe1, Axe2, and GE1, *H. jecorina* also produces an acetyl esterase which alone does not deacetylate polymeric substrates such as acetylxyylan but is capable of deacetylating acetylated xylooligosaccharides generated from acetylxyylan by the action of endoxylanases (24, 25, 26). The enzyme is found to be efficient in catalyzing transacetylation from vinyl acetate (ViAc) to a variety of carbohydrates even in aqueous medium (13, 14). Despite its role in hemicellulose biodegradation and potential application in carbohydrate transacetylation, the gene coding for the enzyme and its regulation are not known.

In this work we report the identification of the gene (*aes1*) coding for the *H. jecorina* acetyl esterase (Aes1) previously described (25), based on amino-terminal sequencing, tryptic peptide tandem mass spectrometry (MS/MS) analysis, overexpression, and characterization of the recombinant enzyme. The expression/regulation patterns of *aes1* together with the enzymatic properties demonstrate that Aes1 is a new hemicellulase and that Aes1 and other enzymes encoded by the orthologs of *aes1* should be assigned a new CE family.

* Corresponding author. Present address: Youtell Biotechnology, Inc., Suite 100, North Creek Corporate Center, 19310 North Creek Parkway, Bothell, WA 98011. Phone: (425) 485-8218. Fax: (425) 485-8830. E-mail: shinli88@hotmail.com.

[†] Supplemental material for this article may be found at <http://aem.asm.org/>.

[‡] Published ahead of print on 31 October 2008.

MATERIALS AND METHODS

Organisms. The fungal strain used throughout this project was *H. jecorina* (*Trichoderma reesei*) Rut-C30 (ATCC 56765), a hypercellulolytic mutant of the wild-type *T. reesei* QM 6a. Over the course of mutagenesis using UV light and nitrosoguanidine for cellulase production improvement, various mutations occurred, and the major ones identified so far have been summarized by Seidl et al. (23, 30). *Escherichia coli* DH5 α was used for routine cloning and propagation of plasmids.

Homologous protein overexpression. Forward primer (5'-TCCCGCGGACT GCGCATCATGCGGTCCATTCTGGTGATTC-3') and reverse primer (5'-A ACTGCGACTACCACTGCTCCCAATAAGTGC-3') were synthesized and used to amplify the genomic region coding for Aes1. To replace a 0.3-kb fragment between SacII (underlined) and PstI (double underlined) in the expression vector pT3C (17) with the *aes1* PCR product, SacII and PstI restriction sites were added to the forward and reverse primers, respectively. PCR was carried out on a Thermocycler (Bio-Rad) in a tube containing 2.5 ng genomic DNA of *H. jecorina* Rut-C30 (17, 23) and a high-fidelity DNA polymerase provided in the iProof kit (Bio-Rad). The PCR product and pT3C were digested with the two restriction enzymes, purified, and ligated. Plasmid with the correct insert was identified by restriction digestion and nucleotide sequencing. This allowed the fusion of *aes1* between the *H. jecorina cel7A* (encoding cellobiohydrolase I [CBHI]) promoter and terminator, followed by a hygromycin B resistance gene (8, 17). The plasmid, named pTSP-TAE, was linearized with EcoRI and used to transform *H. jecorina* Rut-C30 according to the protocol of Hazell et al. (12). After purification of the transformants through two cycles of conidium isolation, the cultures were grown with shaking (250 rpm) in 250-ml shake flasks each containing 40 ml lactose medium (17) at 28°C for 6 days. Aes1 overexpression was detected by analyzing supernatant samples on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue R-250 staining and by measuring the increase of activity in culture supernatants hydrolyzing 4-NP acetate. Production of CBHI in culture supernatants was detected by Western analysis using the CBHI-specific antibodies (16). A transformant that produced the highest acetyl esterase activity and displayed a heavy band at approximately 42 kDa was chosen to prepare the crude recombinant Aes1 in 5-liter bench-top fermenters according to the method of Li et al. (17).

Purification and characterization of the *H. jecorina* acetyl esterase. Upon completion of fermentation, culture broth was chilled on ice, and then supernatant and solid were separated by centrifugation (6,000 $\times$ g, 30 min) at 4°C using a Sorvall RC5C Plus centrifuge (Kendro Laboratory Products, Newtown, CT). Proteins in the clarified supernatant were concentrated 5 $\times$ and equilibrated in 10 mM sodium citrate buffer, pH 4.2, by using a Pellicon system with a membrane cassette (10,000-Da-cutoff pore size; Millipore Corp., Billerica, MA). The liquid column chromatography scheme to purify Aes1 was virtually the same as that described by Poutanen et al. (26). The activity of the fractions against 4-NP acetate as described below was used to monitor the presence of Aes1, and SDS-polyacrylamide gels were used to assess the enrichment and purity of Aes1. The concentration of proteins was determined with the DC protein assay kit (Bio-Rad) for crude samples using bovine serum albumin as a standard and using UV₂₈₀ for purified Aes1 based on an extinction coefficient of 87,290.

Protein and bioinformatic analyses. Purified native and recombinant acetyl esterase samples (~2.0 μ g) were subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes using the Criterion system (Bio-Rad). Protein bands were visualized by amido black (Sigma) staining and excised from the gel with a razor blade. Ten amino acid residues at the N-terminal ends of the proteins were identified at the Wistar Institute Proteomics Facility (Philadelphia, PA) on an Applied Biosystems model 477A gas-phase sequencer equipped with an automatic online phenylthiohydantoin analyzer. The partial amino acid sequence was used to search for homologous sequences in the NCBI BLAST protein database and translated draft sequences of the *T. reesei* genome on 4 May 2006. Nucleotide sequences (1.0 kb) upstream and downstream of the fragment coding for the short amino acid sequence were used to search for homologous sequences in the nucleotide collection (nr/nt) and expressed sequence tag (EST) databases of NCBI using the BLAST program. Sequence analysis, multiple sequence alignment, and phylogenetic analysis were done using DNASTar software (Madison, WI). To obtain mass signals of tryptic peptides, protein bands after SDS-PAGE were visualized by Coomassie brilliant blue staining and were excised from the gel. The gel pieces were smashed and subjected to trypsin digestion. The extracted peptides were analyzed at the Wistar Institute Proteomics Facility using microcapillary reverse-phase high-pressure liquid chromatography nanospray MS/MS on a ThermoFinnigan LTQ quadrupole ion trap mass spectrometer. Mass signals were used to search against the NCBI nr databases using the MASCOT program.

Northern blot analysis. *H. jecorina* Rut-C30 conidia (1.0×10^8) were cultivated at 28°C by being shaken (250 rpm) in a 250-ml shake flask containing 50 ml basal medium (17) with 1.0% (vol/vol) glycerol as carbon source. After 24 h, the culture was left on ice for 30 min and then centrifuged (5,000 $\times$ g, 4°C) for 15 min. Supernatant was discarded, and the mycelia were resuspended in 50 ml ice-cold sterile 50 mM sodium phosphate buffer, pH 5.5. The mycelial suspension (2.0 ml each) was inoculated into 38 ml basal medium plus 1.0% (wt/vol) D-glucose, D-xylose, Solka-Floc 100 cellulose, oat spelt xylan, lactose, L-arabinose, 0.5 mM sophorose, 4.0 mM sodium D-glucuronate, or sodium acetate. The sophorose, D-glucuronate, and acetate cultures also had 1.0% (wt/vol) glycerol. The cultivations in 250-ml shake flasks were carried out by shaking at 28°C and 250 rpm for 24 h and then stopped by passing the cultures through filter paper. Collected mycelia were immediately frozen in liquid nitrogen and disrupted using the Beat Mill MM 301 (Retsch, Newtown, PA). Total RNA was extracted from the disrupted mycelia using the Qiagen RNeasy plant kit. An *aes1* probe was generated by PCR as described above except that 1.0 μ l digoxigenin-dUTP (1 mM; Roche) per 100 μ l of PCR solution was added. DNA hybridization probe for *axe1* (21) was also prepared by PCR using the two oligonucleotide primers AXEF1 (5'-AGCCAGTAGATGGAGAGACCG-3') and AXER1 (5'-TTGA GAACCGCTGAGGAGAGC-3'). The amplicon covered nucleotides 105 to 773 of Z69256 (21). RNA gel electrophoresis, transfer to nylon membranes, and Northern blot analysis were done as described before (18).

Enzyme assays and other analyses. Esterase activity against 4-NP formate, acetate, butyrate, and canoate (Sigma) was assayed with a microplate method on a temperature-controlled Benchmark-Plus System (Bio-Rad). Routine assays were set up with 150 μ l of 1.0 mM substrate in 100 mM sodium phosphate buffer, pH 5.8, followed by preincubation at 30°C for 5 min. Reactions were initiated by the addition of 5.0 μ l of enzyme preparations, and optical density at 410 nm was recorded every 30 s. 4-NP was used as standard for the quantification of products released. For determination of kinetic parameters, a stock solution of 20 mM 4-NP acetate was first made in dimethyl sulfoxide and then diluted to a concentration range of 0.05 to 5.0 mM with 100 mM sodium phosphate buffer. Michaelis-Menten kinetic parameters were obtained by fitting the velocity data into the enzyme kinetic module of Sigmaplot 8.0 (Systat Software, Inc., San Jose, CA). Positional specificity of the enzyme was determined on 4.0 mM 2-, 3-, and 4-O-acetyl 4-NP β -D-xylopyranoside substrates in the β -xylosidase-coupled assays according to the method of Biely et al. (5).

The ability of the recombinant Aes1 to catalyze transacetylation was examined at 40°C in water and in 100 mM sodium phosphate buffer, pH 6.0, using methyl β -D-xylopyranoside (25 mM) as the acetyl group acceptor and ViAc as the acetyl group donor. The volume of ViAc was half of the volume of the aqueous phase. Aliquots of the aqueous phase were analyzed for reaction products by thin-layer chromatography on Silicagel (Merck Chemicals, Ltd., Gibbstown, NJ) in ethyl-acetate-benzene-1-propanol (2:1:0.2, vol/vol/vol). After diffusion, sugar derivative identification was achieved as described by Kremniký and Biely (13).

Nucleotide sequence accession number. The sequence described below was submitted to GenBank with accession number DQ866149 (see Fig. S1 in the supplemental material).

RESULTS

Identification of the gene coding for the *H. jecorina* acetyl esterase. A small sample (~30 μ g) of the *Hypocrea jecorina* acetyl esterase was purified as described previously (25, 26). After being subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and sequenced by Edman analysis, the enzyme gave an N-terminal amino acid sequence of FPKPHDDFKY. No identical or homologous hits were identified when the sequence was used to BLAST search the protein database of NCBI. The sequence, however, matched translated hypothetical amino acid residues encoded by nucleotides 507545 to 507574 of scaffold 5 of the first draft version of the *T. reesei* genome sequence database. When used to search the EST databases of GenBank, EMBL, and DDBJ, the region matched three cDNA sequences (CF883740.1, CF873987.1, and CB903531.1) from the fungus grown on cellulose (10). Nucleotide sequence comparison between scaffold 5 and the three cDNAs revealed that there were two introns,

53 and 52 bp in the coding region. GT and GC served as 5' ends of the first and second introns, respectively, while both introns had an AG 3' end, matching the two most common types of introns found in fungi (15). The protein, designated Aes1, had 348 amino acid residues with the first 19 as a secretion signal peptide. It matched perfectly a region located on scaffold 7 of the *T. reesei* genome sequence database v2.0 (genome.jgi-psf.org/Trire2/Trire2.home.html) that codes for a 348-amino-acid protein (protein identification no. 121418) annotated as a lipolytic enzyme based on the presence of the GDSY motif (amino acid residues 34 to 37 [see Fig. S1 in the supplemental material]), highly similar to the GDSL signature motif found in many esterases/lipases (1). The four invariant important catalytic residues Ser, Gly, Asn, and His in blocks I, II, III, and V, respectively, in the GDSL enzymes (1) were also present in Aes1 (see Fig. S1 in the supplemental material) and other homologous but functionally unknown proteins (see Fig. S1 in the supplemental material). The nucleotide sequence of DQ866149 was identical to that in the genome database. However, the protein sequence posted in the genome database had Thr instead of Ile at position 323 for DQ866149 due to an error that occurred during our analysis. Search for Aes1 homologs in the genome database of *T. reesei* using *aes1* revealed another protein (protein identification no. 103825; fgenes5_pg.C_scaffold_2000413) located in scaffold 2 that was also annotated as a lipolytic enzyme. The protein, designated Aes2, had 342 amino acid residues, about 40% identity with Aes1, and the GDSL signature motif. However, no sequence corresponding to the gene in the EST database was found, indicating that the gene might not be expressed when the fungus grew on cellulose (10).

To confirm the identity and annotation of the gene coding for Aes1, we subjected purified Aes1 to tryptic digestion followed by liquid chromatography-MS/MS analysis. A MASCOT search revealed that peptide mass spectrometry signals matched the calculated mass spectra for peptides covering more than half of the mature protein (Table 1). The coverage was much higher than that set for the protein identification with MS/MS analysis (28). Furthermore, oxidized Met- and alkylated Cys-containing peptides were also detected (Table 1). Therefore, the mass spectrometry signal data plus the perfect match of the deduced and detected N-terminal sequences unambiguously showed that the acetyl esterase is encoded by *H. jecorina aes1*. The presence of other mass signals at significant levels plus the lack of signal matching other regions of the protein suggests that these regions could be posttranslationally modified.

Homology analysis. The deduced amino acid sequence of Aes1 displayed significant (>50% identity) homology to that of many fungal proteins, and all of them were obtained through random genome sequencing (Fig. 1). Some of these proteins were annotated as lipases/fatty acyltransferases. Most fungi which possessed such genes, like *H. jecorina*, *Chaetomium globosum*, *Magnaporthe grisea*, *Aspergillus clavatus*, and *Aspergillus fumigatus*, could secrete many hemicellulases while those of *A. clavatus* and *A. fumigatus* had a putative family 1 carbohydrate-binding module. Hypothetical bacterial proteins also had homology with Aes1. The homologous regions were shorter and less apparent (e.g., <25% [data not shown]). Therefore, the functions of the bacterial proteins are less certain. No signifi-

TABLE 1. Peptide fingerprinting of the *H. jecorina* acetyl esterase using matrix-assisted laser desorption/ionization-time of flight mass spectrometry after in-gel tryptic digestion

m/z observed	m/z calculated	Peptide ^a	Position
758.2900	757.78	PHDDFK	23–28
1,621.6500	1,620.71	YLITFGDSYTDNGR	29–42
2,415.0600	2,414.74	WAAFMNANYPSIITDEI	103–123
		PSFK	
2,431.0600	2,430.73	WAAFM*NANYPSIITDEI	103–123
		PSFK	
3,173.7931	3,173.50	GVTSANTVYALWIGTNDLS	133–162
		YTGILSDSQVK	
1,849.0100	1,848.15	FVILNNNALQLTGLYR	188–203
2,659.0756	2,659.09	MLEYTTSSNTMIDYGVFPF	231–253
		LLVK	
2,675.7841	2,675.08	M*LEYTTSSNTMIDYGVFPF	231–253
		HLLVK	
2,674.3486	2,675.08	MLEYTTSSNTM*IDYGVFPF	231–253
		HLLVK	
2,692.4746	2,691.08	M*LEYTTSSNTM*IDYGVFPF	231–253
		HLLVK	
2,233.9700	2,233.55	VAVYDIHSLIMDIYNQPSR	261–279
2,250.0400	2,249.54	VAVYDIHSLIM*DIYNQPSR	261–279
1,578.7300	1,577.77	YLEPPHNVVGYGYYK	280–292
1,720.6800	1,719.73	HC**DVNGTNC**LYGPGR	293–307
1,020.4300	1,020.14	EFLNVVSGR	330–338
1,141.4300	1,140.19	YGTWEHW	341–348

^a M* refers to an oxidized Met residue with the mass increase of 15.9949 Da, and C** refers to a cysteine residue reduced and alkylated with a mass increase of ~57 Da.

cant homology (<30% identity) was found between Aes1 and any of the CEs listed in the known CE families (cazy.org/fam/acc_CE.html). Phylogenetic analysis suggested that Aes1, Aes2, and their tightly clustered orthologs were distantly branched for other well-established CE family esterases (Fig. 1).

Gene analysis and regulation by carbon sources. To assess regulation of *aes1* by carbon sources, the fungus was grown on various carbon sources and RNA samples isolated from the cultures were used in Northern blot analyses using *aes1*-specific and *axe1*-specific (21) DNA probes. Growth conditions were similar in all the cultures. Harvested mycelia were frozen, and total RNA was extracted. High levels of *aes1*-specific RNA were detected in cultures on sophorose, Solka Floc cellulose, oat spelt xylan, and lactose, followed by significant levels when grown on arabinose (Fig. 2). Possible transcriptional elements involved in the regulation of *aes1* (see Fig. S1 in the supplemental material) including transcriptional initiation sequence TATA, the carbon catabolic repressor (CreI)-binding sequence (GCGGAG) (33), and the ACEII-binding sequence (GGCTAAA) (2) were found in the upstream region while the possible ACEI recognition site (AGGCA/TGCCT) (29) and the HAP 2/3/5 protein complex binding site (ATTGG/CCAAT) (37) were also found upstream in the antisense orientation. No typical terminator site (AATAAA) downstream was found within the region examined. It is interesting that a low but detectable level of *aes1* RNA was produced when the fungus was grown on acetic acid plus glycerol (lane 6, Fig. 2). In the case of *axe1*, high levels of RNA were detected in sophorose-, Solka Floc cellulose-, and lactose-grown cultures but not in cultures grown on the other carbon sources tested (Fig. 2). In contrast to *aes1*, no binding sequence within 1.0 kb

FIG. 1. An unrooted neighbor-joining tree with 1,000 bootstrap replicates was generated based on the multiple sequence alignment by the Clustal W method of esterases related to hemicellulose biodegradation. Putative protein sequences obtained through genome sequencing plus the two *H. jecorina* acetyl xylan esterases (Axe1 and Axe2 [10]) were chosen for the analysis. Acetyl xylan esterase sequences already assigned to various CE families were randomly picked from the CAZy database (cazy.org/fam/acc_CE.html), and their CE family numbers are given in parentheses. Protein accession numbers are used, and for sequences significantly homologous to Aes1, the fungal species names are also given.

FIG. 2. Northern blot analysis using *aes1*- and *axe1*-specific probes. *H. jecorina* Rut-C30 was grown in glycerol medium, and total RNA was extracted after the fungus was cultivated further in medium containing D-glucose (lane 1), D-xylose (lane 2), sophorose (lane 3), Solka-Floc 100 cellulose (lane 4), oat spelt xylan (lane 5), sodium acetate/glycerol (lane 6), lactose (lane 7), sodium D-glucuronate/glycerol (lane 8), and L-arabinose (lane 9). Gel1 and Gel2 after ethidium bromide staining and photography were used for transfer of RNA to nylon membranes that were subjected to Northern analysis using the *aes1* and *axe1* probes, respectively.

upstream of *aes2* was found for CreI, ACEII, or HAP 2/3/5 proteins, further suggesting that *aes2* might not be regulated in the same fashion as are typical biomass-degrading enzyme genes.

Aes1 overexpression and purification. Transformation with plasmid pTSP-TAE yielded 24 transformants that displayed robust growth in the presence of hygromycin B. Ten of the 24 purified transformants when cultivated in lactose medium displayed elevated (5- to 20-fold) activity against 4-NP acetate assayed at 30°C, pH 5.5. For those with higher esterase activity, a heavy protein band with a mass of about 45 kDa was detected (Fig. 3, lane 1) and the size of the band matched that previously reported (25). Untransformed culture lacked the 45-kDa band (19). Western blot analysis using the CBHI-specific antibodies revealed that all the transformants still produced the CBHI protein (data not shown). Cultivation of the transformant (no. 22) which produced the highest esterase activity was scaled up to 3.5 liters in a 5.0-liter bench-top bioreactor, and the total protein level in the culture supernatant was 6.7 mg/ml after 5 days. One liter of the supernatant containing 5,640 mg protein with a specific activity of 0.21 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ after concentration was used for the purification of the recombinant enzyme. Purification and properties of the recombinant Aes1 were virtually the same as those reported elsewhere (25, 26), indicating that the recombinant enzyme had very similar structural and physiochemical properties. Over 300 mg Aes1

FIG. 3. SDS-PAGE analysis of protein samples using an 8 to 16% gradient gel. Lane M, 8.0 μ l prestained Precision plus protein standards (Bio-Rad); lane 1, 20 μ g supernatant proteins of transformant 22; lane 2, 5.0 μ g protein after CM-Sepharose FF column; lane 3, 2.0 μ g protein after the DEAE FF column.

was purified from 1 liter culture supernatant in this manner, substantially more than that obtained by Poutanen and Sundberg (25), reflecting a much higher level of the esterase in the crude preparation (Table 2; Fig. 3, lane 1). The purity of the purified Aes1 was assessed with SDS-PAGE (Fig. 3, lane 3), and its N-terminal sequence perfectly matched that of the native enzyme.

Properties of the recombinant enzyme. The molecular mass of the purified recombinant Aes1, 45 kDa, matched that of the purified native acetyl esterase (25) but was larger than the calculated mass (37,088 Da) from amino acid sequence of the mature polypeptide. The discrepancy could be due to the presence of glycosylation. Treatment of the purified Aes1 with endoglycosidase H produced lower-mass polypeptides, while treatment with *O*-glycosidase did not cause the size shift of the esterase, indicating that N glycosylation might be the predominant form of posttranslational modifications (Fig. 4). This is in agreement with analysis of Aes1 sequence with NetNlyc 1.0,

FIG. 4. Analysis of the recombinant *H. jecorina* Aes1 without deglycosylating enzymes (lane 1) and after treatment with endoglycosidase H (lane 2), *O*-glycosidase (lane 3), and both endoglycosidase H and *O*-glycosidase (lane 4). Lane M was loaded with 10.0 μ l prestained Precision plus protein standards (Bio-Rad), but only the 50-, 37-, and 25-kDa bands are shown.

where residues Asn45, Asn146, and Asn198 were highly likely and Asn204 and Asn 278 were also possibly N glycosylated. The lack of O glycosylation might be due to the fact that Aes1 lacked a linker sequence, commonly found in many multidomain cellulases and hemicellulases (10). The reason for the presence of a heavy band with a mass lower than the calculated mass after endoglycosidase H treatment (Fig. 4) was not clear. The pI value of the purified recombinant Aes1 was determined to be around pH 6.0 (data not shown), close to the calculated pI of 5.89 and those of the purified native forms (6.0 and 6.8) (25). The posttranslational modifications such as N glycosylation shown above could be responsible for the forms with slightly different pI values.

The enzyme hydrolyzed 4-NP acetate at least 4 orders of magnitude faster than it hydrolyzed 4-NP formate, butyrate, or canoate (specific activities were $1.21, 2.13 \times 10^{-4}, 1.09 \times 10^{-4}$, and $4.19 \times 10^{-5} \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, respectively), demonstrating that the esterase is a highly specific acetyl esterase and that therefore the annotation of the enzyme as a lipolytic enzyme should be corrected. Using 4-NP acetate as substrate at 30°C and pH 5.8, the V_{max} and K_m values were determined as $20.5 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and 0.23 mM, respectively. No kinetic parameter calculations were attempted for the activities against 4-NP formate, butyrate, or canoate due to the extremely low activities.

Rates of hydrolysis of 4-NP acetate by the purified Aes1 were inhibited by low concentrations (1.0 to 5.0 mM, Fig. 5A) but significantly enhanced by high concentrations (10.0 to 500 mM, Fig. 5B) of D-xylose. Sodium acetate at concentrations below 100 mM did not inhibit the hydrolysis. Higher concentrations inhibited the reactions (Fig. 5). Xylooligosaccharides (50 mM) were as effective as or more effective than D-xylose in the enhancement (Fig. 6). Monosaccharides tested such as D-glucose, D-galactose, and cellobiose also enhanced the hydrolysis. In contrast, arabinose, its α -1,5-linked oligosaccharides, mannose, or lactose did not significantly influence the hydrolysis rate (Fig. 6).

Using 2-, 3-, and 4-O-acetyl 4-NP β -D-xylopyranosides as substrates in the β -xylosidase-coupled assay (3), Aes1 hydro-

TABLE 2. Purification of the recombinant Aes1 from *Hypocrea jecorina* Rut-C30 culture supernatant

Purification step	Total protein (mg)	Activity ($\mu\text{mol} \cdot \text{min}^{-1}$)	Sp act ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)
Culture supernatant	5,640	1,160	0.21
CM Sepharose FF	557	473	0.85
DEAE FF	325	364	1.12

FIG. 5. Effect of D-xylose (closed cycles) and sodium acetate (open cycles) on the hydrolysis of 4-NP acetate by Aes1. Activity levels were plotted against the low-concentration range (0 to 20 mM) (A) and the full range (0 to 500 mM) (B) of D-xylose and sodium acetate in the reaction mixtures.

lyzed the three substrates (4 mM) with the initial rate ratio of 1:19:17.7. In contrast, Axe1 had a ratio of 1:0.01:0. These data clearly suggest that Aes1 preferred positions 3 and 4 while Axe1 preferred the position 2 acetyl groups. Two of the three crude *H. jecorina* enzyme preparations had much higher activity deesterifying the position 2 acetyl group (5), strongly indicating that Axe1 could be more abundant than Aes1 in the preparations. However, due to the low activity of Axe1 against the position 3 acetyl group, Aes1 and Axe1 display complementary and possibly synergistic effects for the complete removal of acetyl groups during hemicellulose biodegradation.

The recombinant Aes1 displayed the same transacetylation ability as did the native acetyl esterase from *H. jecorina* (13, 14). Figure 7 shows the conversion of methyl β -D-xylopyranoside to its monoacetate in the presence of ViAc by Aes1. The reaction rate in the absence of the enzyme is negligible. These results further demonstrated that the purified native acetyl esterase (13, 14) and the Aes1 reported here were indistinguishable as far as their examined properties are concerned.

FIG. 6. Effect of monosaccharides and oligosaccharides (50 mM) on hydrolysis of 4-NP acetate by Aes1.

DISCUSSION

Here we report the identification of the gene designated *aes1* that encodes the acetyl esterase (25) of *H. jecorina*. Thanks to the databases made available by the genome and EST projects on the fungus, genomic, cDNA, and intron sequences for the gene were obtained based on a 10-amino-acid N-terminal sequence of the purified enzyme. The N-terminal sequencing also allowed us to identify the cleavage site for the removal of the secretion signal peptide. The definitive identification of the gene was also supported by the MS/MS analysis

FIG. 7. Acetylation of methyl β -D-xylopyranoside (MX; 25 mM in 100 mM sodium phosphate buffer, pH 6.0) with ViAc catalyzed by the recombinant Aes1 (40 $\mu\text{g}/\text{ml}$ in reaction mixture) for the production of methyl β -D-xylopyranoside-monoacetate (MXMA) followed by thin-layer chromatography on Silicagel in ethylacetate-benzene-1-propanol (2:1:0.2, vol/vol/vol). Controls (MX plus ViAc without enzyme addition) were tested (ViAc only).

of the tryptic digests of both the purified native and recombinant acetyl esterase samples. Other evidence that supports the identification includes the similarities between the reported native enzyme (14, 25, 34) and the recombinant enzyme on molecular masses, isoelectric points, their properties during column chromatography, and abilities to transacetylate saccharides.

According to the Northern analyses, *aes1* was upregulated when the fungus was induced with cellulose, xylan, lactose, and sophorose and to a lesser extent on arabinose and acetate. Expression was not observed in glucose, glycerol, or glucuronate cultures. These induction patterns strongly resemble those of other hemicellulase genes (20). Many sites that are involved in the binding of regulatory proteins are also present along the promoter region of *aes1*. All these further support the notion that *aes1* is a new hemicellulase gene. *axe1* under the same culturing condition was upregulated by cellulose, sophorose, and lactose. The expression was not detected when the fungus was grown on other carbon sources tested. *axe1* in *H. jecorina* QM9414 was found to be induced by cellulose, xylan, sophorose, and arabinose (20). The differences might reflect the use of different strains and sampling times during cultivation. The low level of *aes1* transcript detected when the fungus was grown on glycerol and acetate is interesting. This might indicate a novel induction mechanism, the glucose-repressed nature of *H. jecorina* Rut-C30, or just depletion of the repressor glucose. Further investigation is needed to differentiate these possibilities.

aes1 orthologs are widespread among fungi capable of secreting plant cell-degrading enzymes (Fig. 1), although no function for these sequences has been described. Some were annotated as lipolytic enzymes based on the presence of GDSL residues (1). The fact that Aes1 of *H. jecorina* is devoid of activity against typical lipase substrates suggests that the annotation should be corrected. The enzyme appears to specifically recognize acetate on the acid side of the active site. Shorter carboxyl acids such as formate did not fit into the site. We do not know at present what carbohydrates fit on the carbohydrate side best, but D-xylose, D-glucose, and D-galactose all influenced the activity and the enzyme deesterifies position 3 and 4 in the 4-NP D-xylopyranoside monoacetates. These data suggest that the carbohydrate side binding site offers more flexibility. The enzyme was also found to remove and transfer acetyl groups to xylo- (Fig. 7) and gluco- and mannooligosaccharides (13), indicating that the enzyme might participate in biodegradation of other hemicelluloses such as galactomannan and glucomannan where acetyl substitutions are also common (31). If this turns out to be the case, it might be more appropriate to designate the enzyme as acetyl esterase instead of acetyl xylan esterase.

H. jecorina is one of the most extensively studied fungi with respect to plant cell wall degradation. Although no enzymes for lignin biodegradation have been reported, a number of cellulase and hemicellulase genes have been described (10, 22). For hemicellulose hydrolysis, these include four xylanase, one β -xylosidase, two acetyl xylan esterase, two α -arabinofuranosidase, one β -mannanase, three α -galactosidase, and one α -glucuronidase gene (10). The regulation by carbon sources for most of the hemicellulase genes (20) has been reported. However, genes coding for feruloyl esterases that hydrolyze the

ester linkage between the arabinose side group and ferulic acid in arabinoxylan have not been reported. This is in agreement with the fact that no feruloyl esterase activity was detected in culture supernatants of the fungus growing on hemicellulose substrates (9). More recently, the gene product of *cip2* (10) was found to code for a glucuronoyl esterase (19) that specifically hydrolyzes methyl-glucuronic acid esters (32). Many lignocellulose-degrading fungi and bacteria have the glucuronoyl esterase orthologs which have been recently classified as a new CE family (CE family 15, cazy.org/fam/acc_CE.html). Finally, Aes1, Aes2, and their orthologs reported here may represent another novel CE family due to their phylogenetic tight clustering and distant branching from the existing family CEs.

ACKNOWLEDGMENTS

The work at USDA-ARS was supported by CRIS 3620-41000-118. We thank Jennifer Teresi, Kristina Glenzinski, and Timmy Ho for excellent technical assistance.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

REFERENCES

1. Akoh, C. C., G.-C. Lee, Y.-C. Liaw, T.-H. Huang, and J.-F. Shaw. 2004. GDSL family of serine esterases/lipases. *Prog. Lipid Res.* **43**:534–552.
2. Aro, N., A. Saloheimo, M. Ilmén, and M. Penttilä. 2001. ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *J. Biol. Chem.* **276**:24309–24314.
3. Biely, P., and G. L. Côté. 2005. Microbial hemicellulolytic carbohydrate esterases, p. 21–24. In C. T. Hou (ed.), *Handbook of industrial biocatalysis*. Taylor and Francis Group, Boca Raton, FL.
4. Biely, P., G. L. Côté, L. Kremnický, and R. V. Greene. 1999. Differences in catalytic properties of acetyl xylan esterases and non-hemicellulolytic esterases, p. 73–81. In H. J. Gilbert, G. J. Davies, B. Henrissat, and B. Svensson (ed.), *Recent advances in carbohydrate bioengineering*. Royal Society of Chemistry, Cambridge, United Kingdom.
5. Biely, P., M. Mastihubová, D. C. la Grange, W. H. van Zyl, and B. A. Prior. 2004. Enzyme-coupled assay of acetyl xylan esterases on monoacetylated 4-nitrophenyl β -D-xylopyranosides. *Anal. Biochem.* **332**:109–115.
6. Biely, P., C. R. MacKenzie, J. Puls, and H. Schneider. 1986. Cooperativity of esterases and xylanases in the enzymic degradation of acetyl xylan. *Bio/Technology* **4**:731–733.
7. Biely, P., J. Puls, and H. Schneider. 1985. Acetyl xylan esterases in fungal cellulolytic systems. *FEBS Lett.* **186**:80–84.
8. Cullen, D., S. A. Leong, L. J. Wilson, and D. J. Henner. 1987. Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. *Gene* **57**: 21–26.
9. Dien, B. S., X.-L. Li, L. B. Iten, D. B. Jordan, N. N. Nichols, P. J. O'Bryan, and M. A. Cotta. 2006. Enzymatic saccharification of hot-water pretreated corn fiber for production of monosaccharides. *Enzyme Microb. Technol.* **39**:1137–1144.
10. Foreman, P. K., D. Brown, L. Dankmeyer, R. Dean, S. Diener, N. S. Dunn-Coleman, F. Goedegebuur, T. D. Houfek, G. J. England, A. S. Kelley, H. J. Meerman, T. Mitchell, C. Mitchinson, H. A. Olivares, P. J. M. Teunissen, J. Yao, and M. Ward. 2003. Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*. *J. Biol. Chem.* **278**: 31988–31997.
11. Hakulinen, N., M. Tenkanen, and J. Rouvinen. 2000. Three-dimensional structure of the catalytic core of acetyl xylan esterase from *Trichoderma reesei*: insight into the deacetylation mechanism. *J. Struct. Biol.* **132**:180–190.
12. Hazell, B. W., V. S. J. Te'o, J. R. Bradner, P. L. Berquist, and K. M. H. Nevalainen. 2000. Rapid transformation of high secreting mutant strains of *Trichoderma reesei* by microprojectile bombardment. *Lett. Appl. Microbiol.* **30**:282–286.
13. Kremnický, L., and P. Biely. 2005. Unique mode of acetylation of oligosaccharides in aqueous two-phase system by *Trichoderma reesei* acetyl esterase. *J. Mol. Catal. B Enzym.* **37**:72–78.
14. Kremnický, L., V. Mastihubová, and G. L. Côté. 2004. *Trichoderma reesei* acetyl esterase catalyzes transesterification in water. *J. Mol. Catal. B Enzym.* **30**: 229–239.
15. Kupfer, D. M., S. D. Drabenston, K. L. Buchanan, H. Lai, H. Zhu, D. W. Dyer, B. A. Roe, and J. W. Murphy. 2004. Intron and splicing elements of five diverse fungi. *Eukaryot. Cell* **3**:1088–1100.
16. Li, X.-L., B. S. Dien, M. A. Cotta, Y. V. Wu, and B. C. Saha. 2005. Profile of enzyme production by *Trichoderma reesei* grown on corn fiber fractions. *Appl. Biochem. Biotechnol.* **121–124**:321–334.

17. Li, X.-L., C. D. Skory, D. B. Jordan, B. S. Dien, S. R. Hughes, E. A. Ximenes, and M. A. Cotta. 2007. Expression of an AT-rich xylanase gene from the anaerobic fungus *Orpinomyces* sp. strain PC-2 in and secretion of the heterologous enzyme by *Trichoderma reesei* (*Hypocrea jecorina*). *Appl. Microbiol. Biotechnol.* **74**:1264–1275.
18. Li, X.-L., H. Chen, and L. G. Ljungdahl. 1997. Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. *Appl. Environ. Microbiol.* **63**:628–635.
19. Li, X.-L., S. Špáníková, R. P. de Vries, and P. Biely. 2007. Identification of genes encoding microbial glucuronoyl esterases. *FEBS Lett.* **581**:4029–4035.
20. Margolles-Clark, E., M. Ihnen, and M. Penttilä. 1997. Expression patterns of ten hemicellulase genes of the filamentous fungus *Trichoderma reesei* on various carbon sources. *J. Biotechnol.* **57**:167–179.
21. Margolles-Clark, E., M. Tenkanen, H. Soderlund, and M. Penttilä. 1996. Acetyl xylan esterase from *Trichoderma reesei* contains an active-site serine residue and a cellulose-binding domain. *Eur. J. Biochem.* **237**:553–560.
22. Martinez, D., R. M. Berka, B. Henrissat, M. Saloheimo, M. Arvas, S. E. Baker, J. Chapman, O. Chertkov, P. M. Coutinho, D. Cullen, E. G. Danchin, I. V. Grigoriev, P. Harris, M. Jackson, C. P. Kubicek, C. S. Han, I. Ho, L. F. Larrondo, A. L. de Leon, J. K. Magnuson, S. Merino, M. Misra, B. Nelson, N. Putnam, B. Robertse, A. A. Salamov, M. Schmoll, A. Terry, N. Thayer, A. Westerholm-Parvinen, C. L. Schoch, J. Yao, R. Barbote, M. A. Nelson, C. Detter, D. Bruce, C. R. Kuske, G. Xie, P. Richardson, D. S. Rokhsar, S. M. Lucas, E. M. Rubin, N. Dunn-Coleman, M. Ward, and T. S. Brettin. 2008. Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nat. Biotechnol.* **26**:553–560.
23. Montenecourt, B. S., and D. E. Eveleigh. 1977. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol.* **34**:777–782.
24. Poutanen, K., and J. Puls. 1988. Characterization of *Trichoderma reesei* β -xylosidase and its use in the hydrolysis of solubilized xylans. *Appl. Microbiol. Biotechnol.* **28**:425–432.
25. Poutanen, K., and M. Sundberg. 1988. An acetyl esterase of *Trichoderma reesei* and its role in the hydrolysis of acetyl xylans. *Appl. Microbiol. Biotechnol.* **28**:419–424.
26. Poutanen, K., M. Sundberg, H. Korte, and J. Puls. 1990. Deacetylation of xylans by acetyl esterases of *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.* **33**:506–510.
27. Puchart, V., M. C. Gariépy, F. Shareck, and C. Dupont. 2006. Identification of catalytically important amino acid residues of *Streptomyces lividans* acetylxyloxy esterase A from carbohydrate esterase family 4. *Biochim. Biophys. Acta* **1276**:263–274.
28. Rappsilber, J., M. Moniatte, M. L. Nielsen, A. V. Podtelejnikov, and M. Mann. 2003. Experiences and perspectives of MALDI MS and MS/MS in proteomic research. *Int. J. Mass Spectrom.* **226**:223–237.
29. Saloheimo, A., N. Aro, M. Ilmén, and Penttilä. 2000. Isolation of the *ace1* gene encoding a Cys2-His2 transcription factor involved in regulation of activity of cellulase promoter cbh1 of *Trichoderma reesei*. *J. Biol. Chem.* **275**:5817–5825.
30. Seidl, V., G. Gamauf, I. S. Druzhinina, B. Seiboth, L. Hartl, and C. P. Kubicek. 2008. The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks an 85 kb (29 gene-encoding) region of the wild-type genome. *BMC Genomics* **9**:327–341.
31. Sims, I. M., D. J. Craik, and A. Bacic. 1997. Structural characterization of galactoglucomannan secreted by suspension-cultured cells of *Nicotiana glauca*. *Carbohydr. Res.* **303**:79–92.
32. Špáníková, S., and P. Biely. 2006. Glucuronoyl esterase—novel carbohydrate esterase produced by *Schizophyllum commune*. *FEBS Lett.* **580**:4597–4601.
33. Strauss, J., R. L. Mach, S. Zeilinger, G. Hartler, G. Stöffler, M. Wolschek, and C. P. Kubicek. 1995. Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Lett.* **376**:103–107.
34. Sundberg, M., and K. Poutanen. 1991. Purification and properties of two acetylxyloxy esterases of *Trichoderma reesei*. *Biotechnol. Appl. Biochem.* **13**:1–11.
35. Taylor, E. J., J. P. Turkenburg, T. M. Gloster, F. Vincent, A. M. Brzozowski, C. Dupont, F. Shareck, M. S. J. Centeno, J. A. M. Prates, L. M. A. Ferreira, C. M. G. A. Fontes, V. Puchart, P. Biely, and G. Davies. 2006. Structure and activity of two metal-ion dependent acetyl xylan esterases involved in plant cell wall degradation reveals a close similarity to peptidoglycan deacetylases. *J. Biol. Chem.* **281**:10968–10975.
36. Tenkanen, M., J. Eyzaguirre, R. Isoniemi, C. B. Faulds, and P. Biely. 2003. Comparison of catalytic properties of acetyl xylan esterases from three carbohydrate esterase families. *ACS Symp. Ser.* **855**:211–229.
37. Zeilinger, S., A. Ebner, T. Marosits, R. Mach, and C. P. Kubicek. 2001. The *Hypocrea jecorina* HAP 2/3/5 protein complex binds to the inverted CCAAT-box (ATTGG) within the cbh2 (cellobiohydrolase II-gene) activating element. *Mol. Genet. Genomics* **266**:56–63.