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The *faeA* Genes from *Aspergillus niger* and *Aspergillus tubingensis* Encode Ferulic Acid Esterases Involved in Degradation of Complex Cell Wall Polysaccharides

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We report the cloning and characterization of a gene encoding a ferulic acid esterase, *faeA*, from *Aspergillus niger* and *Aspergillus tubingensis*. The *A. niger* and *A. tubingensis* genes have a high degree of sequence identity and contain one conserved intron. The gene product, FAEA, was overexpressed in wild-type *A. tubingensis* and a protease-deficient *A. niger* mutant. Overexpression of both genes in wild-type *A. tubingensis* and an *A. niger* protease-deficient mutant showed that the *A. tubingensis* gene product is more sensitive to degradation than the equivalent gene product from *A. niger*. FAEA from *A. niger* was identical to *A. niger* FAE-III (C. B. Faulds and G. Williamson, Microbiology 140:779–787, 1994), as assessed by molecular mass, pH and temperature optima, pI, N-terminal sequence, and activity on methyl ferulate. The *faeA* gene was induced by growth on wheat arabinoxylan and sugar beet pectin, and its gene product (FAEA) released ferulic acid from wheat arabinoxylan. The rate of release was enhanced by the presence of a xylanase. FAEA also hydrolyzed smaller amounts of ferulic acid from sugar beet pectin, but the rate was hardly affected by addition of an endo-pectin lyase.

Cinnamic acids have been demonstrated to be covalently bound to polysaccharides (arabinoxylans and pectins) in cell walls of gramineous plants (16, 22, 28, 31). Here, they have a possible function in decreasing cell wall biodegradability (5) and in regulating cell growth (15) by covalently cross-linking cell wall polymers. To degrade these cell wall polymers, many bacteria and fungi produce a wide range of hemicellulases, as well as cinnamic acid esterases (8). Several cinnamic acid esterases have been isolated from *Aspergillus niger* (13, 14, 23, 33). Most of these esterases are active on methyl esters of ferulic and *p*-coumaric acids, but other hemicellulases increase the hydrolysis of these cinnamic acids from xylan. So far, only a cinnamoyl esterase (CinnAE) from *A. niger* has been shown to release ferulic acid from sugar beet pectin (24).

We isolated a ferulic acid esterase (FAEA) from a commercial pectinase preparation which is similar to FAE-III (14). We used the partial amino acid data generated to clone the FAEA-encoding genes from *A. niger* and *Aspergillus tubingensis* and subsequently overexpressed these genes. To our knowledge, this is the first paper that describes the isolation and characterization of a gene encoding a specific ferulic acid esterase.

MATERIALS AND METHODS

Strains, libraries, and plasmids. *A. niger* N402 (*cspA1*) and NW219 (*nicA1 leuA1 pyrA6*) were derived from strain N400 (= CBS 120.49). The *prtF* mutation present in *A. niger* NW154 (*pyrA6 prtF28*) has been described previously (34). The *A. tubingensis* strains used were NW756 and NW241 (*pyrA2 fwnA1*). *Escherichia coli* DH5αF′ (BRL, Life Technologies Inc., Gaithersburg, Md.) was used for routine plasmid propagation. *E. coli* LE392 was used as a host for phage λEMBL3. pBluescript (30) was used for subcloning. The genomic libraries of *A. niger* and *A. tubingensis* and the cDNA library of *A. niger* have been described previously (11, 17, 21).

Chemicals. Methyl ferulate was obtained from APIN Chemicals Ltd. Ferulic acid esterase was purified from a commercial product, Pectinase PII from Amano. Nitrophenyl acetate, phenylmethylsulfonyl fluoride, ferulic acid, oat spelt xylan, and endoproteinase lysC were obtained from Sigma. Sugar beet pectin was obtained from Danisco Ingredients. Lyophilized bovine plasma gamma globulin was obtained from Bio-Rad Laboratories. Endoproteinase V8 was obtained from Boehringer Mannheim. Xylanase 1 was purified from the commercial product Grindamyl H121 (Danisco Ingredients) and is equivalent to XLNA from *A. tubingensis* (11). Pectin lyase was purified from the commercial product Pektolase CA (Danisco Ingredients). Water-insoluble pentosan (WIP) from wheat arabinoxylan was isolated by the method of Gruppen et al. (19).

Media and culture conditions. Minimal medium (MM) contained (per liter) 6.0 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of KCl, and 0.5 g of $MgSO_4$, as well as trace elements (39) and 1 to 3% glucose as a carbon source unless otherwise indicated. For complete medium MM was supplemented with 2% (wt/vol) tryptone, 1% (wt/vol) yeast extract, 1% (wt/vol) Casamino Acids, and 0.5% (wt/vol) RNAs. Liquid cultures were inoculated with 10^6 spores/ml and incubated at 30°C in a Gallenkamp orbital shaker at 250 rpm. Agar was added at a concentration of 1.5% for solid medium. For the growth of strains with auxotrophic mutations, the corresponding supplements were added to the medium.

For characterization, transformants were grown for 6 days on MM containing a crude wheat arabinoxylan fraction at a concentration of 1% (wt/vol). Supernatant samples were analyzed by Western analysis and activity measurement. For production of recombinant FAEA, transformant NW154::pIM3207.7 was grown at 30°C in three batches of 300 ml of MM containing 0.5 g of ferulic acid per liter and 10 g of oat spelt xylan per liter.

Purification of ferulic acid esterases. FAEA was purified from 10 g of Pectinase PII which was dissolved in 300 ml of 50 mM sodium phosphate buffer–1 mM EDTA (pH 7.0), stirred for 30 min, centrifuged (10,000 × *g* for 30 min), and filtered (pore size, 0.8 μm). FAE-III was purified from culture supernatants (10 liters) of *A. niger* CBS 120.49 grown on oat spelt xylan as described previously (14). Recombinant FAEA was purified from 600 ml of culture filtrate from a 3-day-old culture of NW154::pIM3207.7 grown on MM containing 0.5 g of ferulic acid per liter and 10 g of oat spelt xylan per liter. For all three sources of enzyme, the ferulic acid esterase was purified by ammonium sulfate precipitation, hydrophobic interaction chromatography, and anion-exchange chromatography as described previously for the purification of FAE-III (14). The purity of isolated ferulic acid esterases was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Characterization of ferulic acid esterases. Molecular masses were determined by SDS-PAGE (Novex 4 to 12% Tris-glycine gel with the electrophoresis conditions given by the manufacturer) by using a Pharmacia LMW electrophoresis

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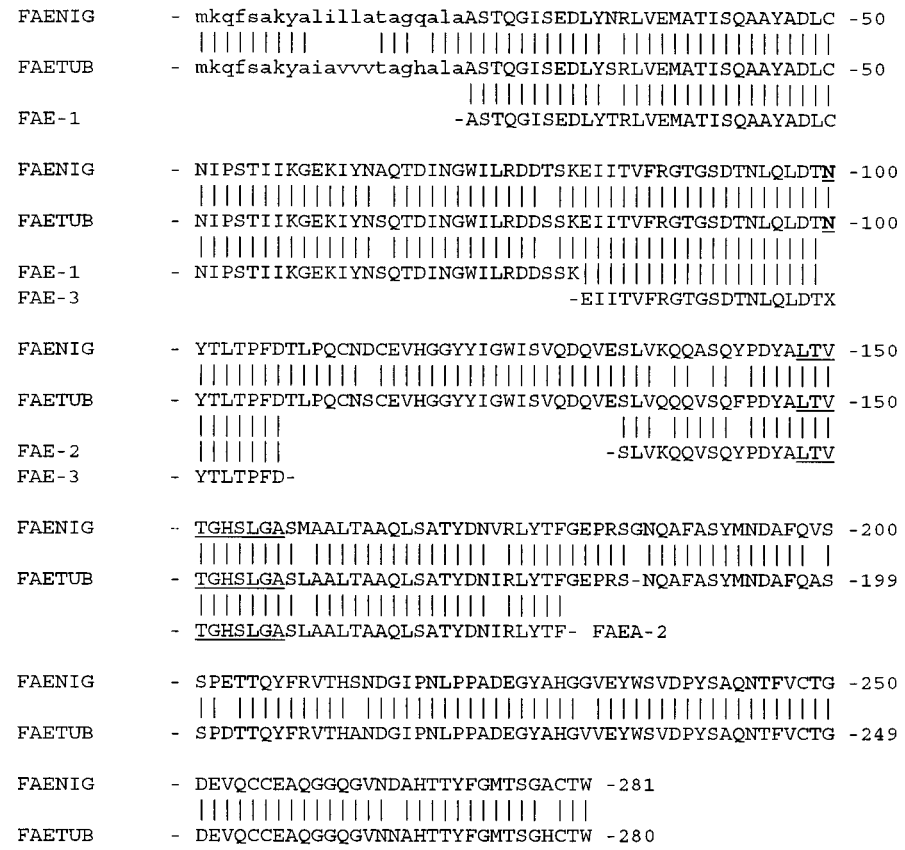

FIG. 1. Alignment of the deduced amino acid sequences for FAEA from *A. niger* and *A. tubingensis*. The sequence identity is 92.5%. The determined amino acid sequences of the pectinase ferulic acid esterase (FAE-1, FAE-2, and FAE-3) are also aligned with the protein sequences. The putative N-glycosylation site is in boldface type and underlined. The signal sequence is given in lowercase letters. The region homologous to the putative lipase serine active site is underlined. FAENIG, *A. niger* FAEA; FAETUB, *A. tubingensis* FAEA.

calibration kit and by mass spectrometry performed with a MALDI-TOF mass spectrometer (Voyager; Perceptive) as described previously (38). The protein concentrations of purified pectinase ferulic acid esterase, recombinant FAEA, and FAE-III were determined by measuring the A_{280} and using the extinction coefficient for FAEA at 280 nm (43,660 M^{-1} cm^{-1}), which was estimated from the amino acid sequence as previously described (18), and the molecular weight obtained by mass spectrometry. pI values were determined on a Novex isoelectric focusing (IEF) gel (pH 3 to 7) by using an IEF calibration kit from Pharmacia. The temperature optimum was determined by measuring activity over a temperature range from 35 to 70°C. The pH optimum was determined by measuring activity at 37°C in 100 mM sodium acetate buffer with pH values ranging from 4.2 to 5.8 and in 100 mM morpholineethanesulfonic acid (MES) buffer with pH values ranging from 5.5 to 7.0. Kinetic measurements for methyl ferulate were performed in 100 mM morpholinepropanesulfonic acid (MOPS) (pH 6.0) at 37°C with substrate concentrations ranging from 0.1 to 2.0 mM. The assay mixtures had a total volume 0.5 ml and contained 20 ng of protein. At this protein concentration, reactions were linear up to 60 min; reactions were terminated after 15 min by the addition of 0.5 ml of 11.3% acetic acid in methanol. Hydrolyzed samples were assayed by high-performance liquid chromatography (HPLC) as described below. Raw data were analyzed by the method of Wilkinson (41).

Western analysis and protein determination. Western analysis of supernatant samples from *Aspergillus* cultures was performed by using polyclonal antibodies raised in mice against purified FAEA. Protein concentrations in supernatants and throughout purification procedures were estimated with the Bio-Rad protein assay reagent by using lyophilized bovine serum albumin as a standard (4a). The method is based on protein dye binding (6).

Enzyme assays. Ferulic acid esterase activities in supernatant samples and throughout enzyme isolation were measured by using methyl ferulate as a substrate. Samples (0.02 ml) were incubated in 100 mM sodium acetate buffer (0.48 ml, pH 4.8) containing methyl ferulate (2 mg/ml). After 15 min, the incubation mixture (0.1 ml) was transferred to an Eppendorf tube containing methanol (0.5 ml) and MilliQ water (0.4 ml). This effectively stopped the enzyme reaction. An aliquot (0.05 ml) of this mixture was analyzed by HPLC with a type RP-8 reverse-phase column (Spectra-Physics, Santa Clara, Calif.). Separation of substrate (methyl ferulate) and product (ferulic acid) was achieved by using a linear gradient starting with 32% (vol/vol) methanol–0.3% (vol/vol) acetic acid–67.7% (vol/vol) water and ending with 64% (vol/vol) methanol–0.3% (vol/vol) acetic acid–35.7% (vol/vol) with detection at 325 nm. *endo*-Xylanase, β-xylosidase, and pectin lyase activities were determined as described previously (2, 27, 36).

Analysis of hydrolysis products from wheat arabinoxylan and sugar beet pectin. Hydrolysis by FAEA of sugar beet pectin and arabinoxylan was measured in 0.1 M sodium acetate buffer (pH 4.8) containing 1% (wt/vol) WIP (isolated from wheat flour) and 1% (wt/vol) sugar beet pectin, respectively, as the substrate. To 10 ml of the solutions enzymes were added in a total volume of 1 ml. Samples were incubated for 1 and 24 h, respectively, at 50°C, after which a 5-ml sample was transferred to a boiling water bath and incubated for 15 min to stop the reaction. The samples were filtered (pore size, 45 μm) and subsequently analyzed by HPLC.

Determination of N-terminal and internal peptide sequences of pectinase ferulic acid esterase. Amino acid sequences were determined by traditional procedures (1, 32). Purified ferulic acid esterase was digested with endoproteinases lysC and V8 and with CNBr. The digests were applied to reverse-phase columns (lysC and V8 digests were applied to C18 columns [Vydac] and the CNBr digest was applied to C2 columns [Brownlee]). The columns were equilibrated with 0.1% (vol/vol) trifluoro acetic acid (TFA) in water, and peptides were separated by elution with 0.1% (vol/vol) TFA in acetonitrile in a 10 to 40% (vol/vol) gradient. Individual peptides were applied to micro-TFA filters and analyzed with an Applied Biosystems amino acid sequencer (model 476A).

PCR cloning of a specific fragment of the *faeA* genes. Two degenerate oligonucleotides (5′-CARACIGAYATHAAYGGNTGGAT-3′ and 5′-GCRTART CNGGRTAYTG-3′, with Y, R, N, H, and I representing C/T, A/G, A/C/G/T, A/C/T, and ionisine, respectively) were designed and synthesized with an Applied Biosystems model 392 DNA synthesizer. PCR were performed with a Sensa model 949E DNA processor by using these oligonucleotides at 55°C and chromosomal DNA from *A. niger* N402 and *A. tubingensis* NW756. The resulting fragments were cloned into the pGEM-T vector system (Promega), and a sequence analysis was performed as described below.

Isolation, cloning, and characterization of the *faeA* genes. Plaque hybridization by using nylon replicas was performed as described by Benton and Davis (4).

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- ATGAAGCAATTCTCTGCAAAATACGCCCTCATTCTTTTGGCGACTGCAGG -50
- ACAAGCCCTCGCGGCCTCCACGCAAGGCATCTCCGAAGACCTCTACAATC -100
- GCTTGGTAGAGATGGCCACTATCTCCCAAGCCGCCTACGCCGACCTATGC -150
- AATATTCCATCGACTATTATCAAAGGAGAGAAAATTTACAACGCTCAAAC -200
- TGATATCAACGGATGGATCCTCCGCGACGACACCAGCAAAGAAATTATCA -250
- CCGTCTTCCGTGGCACTGGCAGTGACACAAACCTACAGCTCGATACTAAC -300
- TACACGCTCACGCCATTCGACACTCTACCTCAATGCAACGATTGCGAGGT -350
- ACACGGTGGATACTATATTGGATGGATCTCAGTCCAAGACCAAGTCGAGT -400
- CGCTTGTCAAACAACAGGCTAGCCAGTATCCGGACTATGCGCTTACCGTG -450
- ACAGGCCATAggtatgcgtgcttaccgtcttacaatttgttgtatgatat -500
- atactaactttatgataGTCTGGGAGCGTCGATGGCAGCACTCACTGCCG -550
- CCCAGCTGTCCGCGACATACGACAACGTCCGTCTGTACACATTCGGCGAA -600
- CCGCGCAGCGGCAACCAGGCCTTCGCGTCGTACATGAACGATGCGTTCCA -650
- GGTCTCGAGCCCGGAGACGACCCAGTACTTCCGGGTCACTCATTCCAACG -700
- ACGGCATCCCAAACTTGCCCCCGGCGGACGAGGGTTACGCCCATGGTGGT -750
- GTAGAGTACTGGAGCGTTGATCCTTACAGCGCCCAGAACACGTTTGTCTG -800
- TACTGGGGATGAAGTACAGTGCTGTGAGGCACAGGGCGGACAGGGGGTGA -850
- ATGATGCGCATACTACTTATTTTGGGATGACGAGCGGAGCTTGTACTTGG -900
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FIG. 2. Nucleotide sequence of the *faeA* structural gene from *A. niger*. The intervening region (positions 461 to 517) is given in lowercase letters.

Hybridizations were performed overnight at 65°C by using the PCR fragment as a probe. Filters were washed with 0.2× SSC–0.5% (wt/vol) SDS (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.6). Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and were purified by rescreening at low plaque density. Standard methods were used for other DNA manipulations, such as Southern analysis, subcloning, DNA digestion, and λ phage and plasmid DNA isolations (29). In vivo excision of positive cDNA clones was performed as recommended by the supplier (Stratagene). Chromosomal DNA was isolated as previously described (10). Sequence analysis was performed with both strands of DNA by using a Sequenase DNA sequencing kit (United States Biochemical Corp.) and a T7 sequencing kit (Pharmacia LKB) with additional oligonucleotides. Nucleotide and amino acid sequences were analyzed by using the computer programs of Devereux et al. (12). *Aspergillus* transformations were performed as described by Kusters-van Someren et al. (25) by using 1 μg of a construct carrying the *pyrA* gene for selection of transformants and 20 μg of the plasmid with the gene of interest. Contour-clamped homogeneous electric field–Southern analysis of *A. niger faeA* was performed as described by Verdoes et al. (37) by using modifications described by van den Hombergh et al. (35).

Nucleotide sequence accession numbers. The EMBL accession numbers for the nucleotide sequences of *faeA* from *A. niger* and *A. tubingensis* are Y09330 and Y09331, respectively.

RESULTS

Purification and amino acid sequence analysis of FAEA from Pectinase PII. FAEA was purified approximately 100-fold to electrophoretic homogeneity with a recovery of 34% from Pectinase PII, a commercial *A. niger* product. Peptides were prepared by endopeptidase lysC and V8 and CNBr digestion of the purified sample. Sequencing of the N-terminal part of the protein and several internal peptides resulted in the sequences shown in Fig. 1. FAE-1 is a combination of the N-terminal sequence and a LysC peptide. FAE-2 is a combination of LysC, V8, and CNBR peptides. FAE-3 is a LysC peptide. A total of 132 amino acids were sequenced.

Cloning and sequence analysis of the ferulic acid esterase (*faeA*) gene from *A. niger* and *A. tubingensis*. By using two degenerate oligonucleotides based on peptides FAE-1 and FAE-2, a 256-bp fragment was generated and subcloned for both *A. niger* and *A. tubingensis*. Translation of the DNA sequence of the two PCR fragments identified all three amino acid sequences (FAEA 1, 2, and 3), as determined by amino acid sequencing. Screening of the genomic libraries of *A. niger* and *A. tubingensis* by using the PCR fragments as probes resulted in four and three positive λ clones, respectively. Restriction analysis of these clones was performed, and a 1.5-kb *Pst*I-*Hin*dIII fragment and a 4-kb *Kpn*I-*Bam*HI fragment from an *A. niger* clone and a 2-kb *Pst*I-*Bam*HI fragment and a 3-kb *Pst*I-*Xho*I fragment from an *A. tubingensis* clone were subcloned into pBluescript SK+, resulting in plasmids pIM3202, pIM3204, pIM3205, and pIM3206, respectively. Sequence analysis demonstrated that the *A. niger* and *A. tubingensis* structural genes contain 900 bp (Fig. 2) and 897 bp, respectively. Sequencing of the cDNA clones confirmed the presence in both genes of an intron of 57 bp starting at position 461 of the structural gene. Alignment of the DNA sequences of the two genes showed an identity of 87.1%. Translation of the DNA sequences from *A. niger* and *A. tubingensis* resulted in amino acid sequences containing 281 and 280 amino acids, respectively (Fig. 1). Comparison of the deduced amino acid sequences to the determined amino acid sequences of the mature *A. niger* protein indicated that there was a signal peptide of 21 amino acids. This peptide has all of the characteristics of a typical signal peptide in fungi (40). The enzymes from *A. niger* and *A. tubingensis* contain a single putative N-glycosylation site, and alignment of their amino acid sequences showed an identity of 92.5%. The calculated molecular mass is 28 kDa for both proteins. The *A. niger* and *A. tubingensis* sequences differ at five and three positions, respectively, from the determined amino acid sequences of pectinase FAEA. The asparagine residue of the putative N-glycosylation site is likely to be glycosylated as this residue could not be determined experimentally. Analysis revealed high homology of a region of the enzymes to a putative lipase serine active site (Fig. 3). This region is aligned with the putative serine active sites of *Rhizopus delemar* (20), *Mucor miehei* (7), *Humicola lanuginosa*, and *Penicillium camembertii* (42). A search of gene and protein libraries did not produce any other genes or proteins with significant homology to these esterases. Contour-clamped homogeneous electric field–Southern analysis demonstrated that the *A. niger faeA* gene is located on chromosome I.

Rhizopus delemar lipase	-VIVTGHSLGG...59...D...53...H-
Mucor miehei lipase	-VAVTGHSLGG...59...D...53...H-
Humicola lanuginosa lipase	-VVFTGHSLGG...55...D...57...H-
Penicillium camembertii lipase	-LVVVGHSLGA...55...D...60...H-
Aspergillus niger FAEA	-LTVTGHSLGA...61...D...53...H-
consensus	-..VT**GHSLG**.....**D**.....**H**-

FIG. 3. Alignment of the putative serine active sites of a number of lipases with a region of the *A. niger* FAEA. Only the regions containing the putative active site are shown. In the consensus sequence, amino acids which are conserved in at least four of the five enzymes are shown. Amino acids which are conserved in all five enzymes are indicated by boldface type.

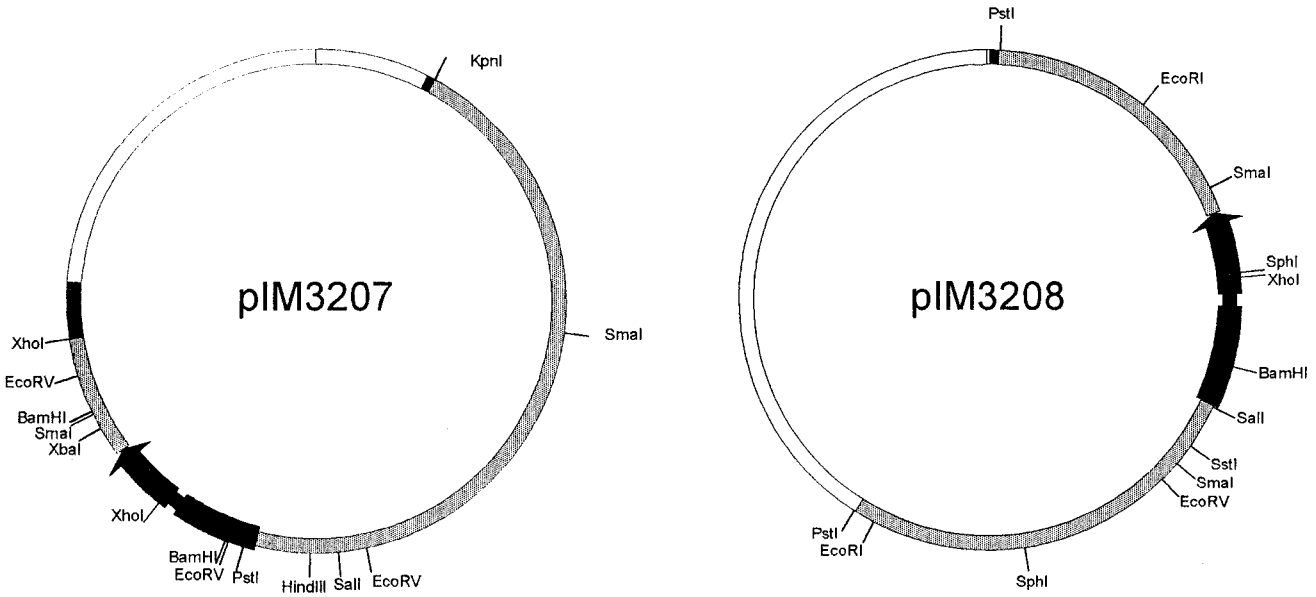

FIG. 4. Expression constructs containing the *faeA* gene from *A. niger*(pIM3207) and *A. tubingensis*(pIM3208). The genes are depicted as black arrows. The intervening regions are indicated by the narrow regions in the arrows. Chromosomal regions flanking the genes are stippled. The polylinker regions of the plasmid are depicted as black boxes. pIM3207 is a combination of the 1.5-kb *Hin*dIII-*Pst*I fragment from pIM3202 and the 3.9-kb *Kpn*I-*Pst*I fragment from pIM3204. pIM3208 is a combination of the 2-kb *Bam*HI-*Pst*I fragment of pIM3205 and the 2.2-kb *Bam*HI-*Pst*I fragment of pIM3206.

Overexpression of *faeA* from *A. niger* and *A. tubingensis*. Combination of fragments from plasmids pIM3202 and pIM3204 and plasmids pIM3205 and pIM3206 resulted in functional constructs for *faeA* from *A. niger* and *A. tubingensis*, respectively (plasmids pIM3207 and pIM3208) (Fig. 4). These constructs were transformed to *A. niger* NW219 and *A. tubingensis* NW241 and to an *A. niger prtF* mutant (NW154).

Western analysis performed with a specific antibody against the pectinase FAEA was applied to select transformants with a high level of FAEA production. Six high-producing transformants were chosen, and the copy number and FAEA activity after 6 days of growth on MM containing a crude wheat arabinoxylan preparation at a concentration of 1% (wt/vol) were determined (Table 1). The activity in the *prtF* strain was higher than the activity in the wild-type *A. niger* strain for the *A. niger* gene product, while the copy numbers were comparable. For the *A. tubingensis* gene product the same difference was observed, with a lower number of copies in the *prtF* strain. Similarly, the *A. tubingensis* strain also exhibited higher activities than the wild-type *A. niger* strain for both gene products.

Transformant NW154::pIM3207.7 was used to compare the levels of induction on wheat arabinoxylan and sugar beet pectin. The highest levels of FAEA activity in supernatant samples were observed when this transformant was grown on a crude wheat arabinoxylan preparation (Table 2). FAEA activity was also found in supernatant samples from media containing WIP and sugar beet pectin, demonstrating that the *faeA* gene is not specifically induced on only xylan but also on pectin.

FAEA from *A. tubingensis* is more sensitive to degradation than FAEA from *A. niger*. Four of the transformants described above were grown for 6 days on MM containing 1% (wt/vol) oat spelt xylan. Supernatant samples were isolated and concentrated five times, and aliquots (0.02 ml) of the concentrated samples were subjected to Western analysis. Several low-molecular-weight bands were detected (Fig. 5). N-terminal amino acid sequencing showed identical sequences for the mature protein and the second-largest protein, presumably produced by truncation, indicating that this peptide originated by C-terminal degradation. In both backgrounds (*A. niger prtF* and *A. tubingensis*) the amount of degraded protein compared to the amount of mature protein was much higher in the transformant containing the *A. tubingensis* construct than in the transformant containing the *A. niger* construct, indicating that the *A. niger* gene product is less sensitive to degradation.

Recombinant *A. niger* FAEA is identical to *A. niger* FAE-III. Recombinant FAEA (from transformant NW154::pIM3207.7), FAE-III, and the pectinase FAEA were purified to electrophoretic homogeneity (see above). None of the purified preparations contained detectable *endo*-xylanase, β-xylosidase, or endo-pectin lyase activity. FAE-III and recombinant FAEA

TABLE 1. FAEA activities and copy numbers of six transformants

Strain[a]	FAEA activity[b]	Copy no.[c]	Strain description
NW219	0.06	1	
NW154	0.13	1	
NW241	0.00	1	
NW219::pIM3207.28	1.60	20	*A. niger faeA* in *A. niger* NW219
NW154::pIM3207.7	7.71	25	*A. niger faeA* in *A. niger* NW154 (*prtF* mutant)
NW241::pIM3207.15	3.16	10	*A. niger faeA* in *A. tubingensis* NW241
NW219::pIM3208.10	0.00	6	*A. tubingensis faeA* in *A. niger* NW219
NW154::pIM3208.5	0.97	8	*A. tubingensis faeA* in *A. niger* NW154 (*prtF* mutant)
NW241::pIM3208.6	0.80	7	*A. tubingensis faeA* in *A. tubingensis* NW241

[a] Transformant designations are given as follows: strain::plasmid.transformant number.

[b] FAEA activity is expressed as micromoles of ferulic acid produced per minute per milliliter of supernatant.

[c] Copy numbers were determined by hybridization with the *faeA* gene, after which intensities were compared.

TABLE 2. Ferulic acid esterase activities in culture supernatants of transformant NW154::pIM3207.7 after 6 days of growth

Substrate[a]	Activity[b]
Crude wheat arabinoxylan	3.08
WIP	1.39
Sugar beet pectin	0.19

[a] Substrates were added at a concentration of 0.5% (wt/vol).
[b] Ferulic acid esterase activity is expressed as micromoles of ferulic acid produced per milliliter of supernatant per minute.

were identical in all respects (Table 3). All three enzymes were identical with respect to apparent M_r as determined by SDS-PAGE, pH optimum, and temperature optimum. However, the pectinase FAEA had a slightly higher pI than FAEA and FAE-III and a lower V_{max} for methyl ferulate.

FAEA from *A. niger* is able to release ferulic acid from wheat arabinoxylan and sugar beet pectin. WIP (1%, wt/vol) from wheat arabinoxylan was incubated with recombinant FAEA and xylanase 1 for 1 and 24 h at 50°C. HPLC analysis of the samples showed that FAEA was able to release ferulic acid from arabinoxylan in the absence of other hemicellulases (Table 4). Addition of xylanase resulted in a strong increase in the amount of ferulic acid released. Depending on the conditions, a two- to sevenfold increase could be obtained. The alkali-extractable ferulic acid content of the WIP was estimated to be 0.86% (wt/wt). In the presence of xylanase 1, FAEA was able to release approximately 4.9% of the esterified ferulic acid in the WIP sample.

In a similar experiment 1% (wt/vol) sugar beet pectin was incubated with recombinant FAEA and a pectin lyase. FAEA was able to release ferulic acid from sugar beet pectin in the absence of other enzymes, but at a much lower level than it released ferulic acid from xylan (Table 4). Addition of pectin lyase had only a minor effect on the liberation of ferulic acid. The alkali-extractable ferulic acid content of the sugar beet pectin was estimated to be 1.30% (wt/wt). FAEA was able to release approximately 0.16% of the esterified ferulic acid in the sugar beet pectin sample.

DISCUSSION

Faulds and Williamson (14) purified from *A. niger* a ferulic acid esterase (FAE-III) with high activity against the methyl esters of several cinnamic acids. We purified an enzyme from a commercial enzyme preparation which was similar to FAE-III. Using reverse genetics, we cloned the corresponding gene (*faeA*) from *A. niger* and *A. tubingensis* and overexpressed both gene products.

The characteristics of the product from the *A. niger faeA* gene are identical to those of FAE-III (14), indicating that these enzymes are in fact the same enzyme. The differences observed in molecular mass, K_m, and V_{max} are well within the expected experimental variations. This conclusion was strengthened by

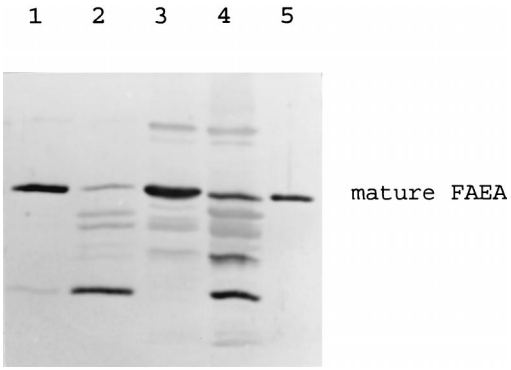

FIG. 5. Degradation patterns for FAEA from *A. niger* and *A. tubingensis* in the *A. niger prtF* mutant and in *A. tubingensis*. Lane 1, transformant NW154::pIM3207.7 (*A. niger faeA* in *A. niger*); lane 2, NW154::pIM3208.5 (*A. tubingensis faeA* in *A. niger*); lane 3, NW241::pIM3207.15 (*A. niger faeA* in *A. tubingensis*); lane 4, NW241::pIM3208.6 (*A. tubingensis faeA* in *A. tubingensis*); lane 5, purified mature FAEA from *A. niger*.

the identical N-terminal amino acid sequences of the two enzymes. The characteristics of FAEA isolated from the commercial pectinase preparation are very similar but not identical to those of the other two enzymes. This is probably due to strain differences as the origin of the preparation is unknown. The differences in the determined amino acid sequence of the enzyme from the commercial preparation and the amino acid sequence of the recombinant FAEA also indicate that the *A. niger* strain used for the production of the pectinase preparation was not *A. niger* N400 (=CBS 120.49). Further evidence for the identity of FAEA and FAE-III was obtained by heterologous Southern hybridization of *A. niger* N400 chromosomal DNA, which showed only one band for all individual restriction digests tested when *faeA* was used as a probe, thus demonstrating the presence of a single *faeA* gene (data not shown).

The differences in molecular mass observed when different methods of determination (SDS-PAGE and mass spectrometry) were used could be the result of a combination of two effects. FAEA is a very acidic protein (pI = 3.3), which may lead to reduced binding of SDS and reduced mobility on SDS-PAGE gels. Second, the fact that the asparagine residue of the putative glycosylation site could not be determined experimentally indicates that the enzyme is probably glycosylated, which results in a higher molecular mass on SDS-PAGE gels.

Overexpression of FAEA showed that the *A. tubingensis* enzyme was more sensitive to degradation than the *A. niger* enzyme. This was clearly demonstrated in two different genetic backgrounds, and it can therefore be assumed that the difference is due to the nature of the two enzymes, even though they are nearly 93% identical in sequence. Whether this difference in stability is due to the primary structure of these enzymes or to other factors is not clear at this moment. In wild-type *A. niger* carrying the *A. tubingensis faeA* gene, hardly any ma-

TABLE 3. Comparison of FAE-III, FAEA from *A. niger*, and FAEA isolated from a commercial pectinase preparation

Enzyme	M_r as determined by:		pI	Optimum pH	Optimum temp (°C)	V_{max} with methyl ferulate	K_m with methyl ferulate
	SDS-PAGE	Mass spectrometry					
FAE-III	36,000	29,740 ± 16	3.3	5.0	55-60	147	0.72
FAEA from *A. niger*	36,000	29,738 ± 50	3.3	5.0	60	143	0.76
FAEA from pectinase	36,000	ND	3.4	5.0	60	86.0	1.00

[a] Total protein content was determined by measuring the A_{280} of the purified enzyme solutions and using the extinction coefficient for recombinant FAEA (43,660 M^{-1} cm^{-1}) calculated from the amino acid sequence and the molecular mass determined by mass spectrometry.

TABLE 4. FAEA activity on wheat arabinoxylan and sugar beet pectin[a]

Substrate	Amt of FAEA (μl)	Amt of xylanase 1 (μl)	Amt of pectin lyase (μl)	Amt of ferulic acid released (ng/ml)	
				1 h	24 h
WIP from wheat arabinoxylan	0	0		0	17
	5	0		0	155
	50	0		94	314
	500	0		324	1,356
	0	500		12	0
	5	500		29	597
	50	500		196	2,273
	500	500		1,065	4,211
Sugar beet pectin	0		0	20	16
	5		0	21	22
	50		0	63	95
	500		0	84	166
	0		500	14	15
	5		500	16	43
	50		500	31	102
	500		500	95	202

[a] The FAEA solution contained 0.6 U/ml (0.6 μmol of ferulic acid liberated from methyl ferulate · min^{-1} · ml^{-1}), the xylanase 1 solution contained 0.18 mU/ml (0.18 nmol of xylose liberated from oat spelt xylan/min/ml), and the pectin lyase solution contained 6 U/ml; 1 pectin lyase unit was the amount of activity which gave rise to a change in A_{235} of 0.01 U/min when the preparation was incubated with highly esterified pectin.

ture FAEA from *A. tubingensis* can be detected. Since *A. tubingensis* does not acidify the medium as strongly as wild-type *A. niger* acidifies the medium, this could indicate that FAEA from *A. tubingensis* is more sensitive to acid proteases than FAEA from *A. niger*. However, in the *A. niger prtF* mutant, which is strongly reduced in acid protease activity, the difference between the two gene products is also very obvious. In this strain the amount of degradation for both gene products is much lower than the amount of degradation in the *A. niger* wild-type strain, but degraded bands can still be observed. If the degradation of FAEA from *A. tubingensis* in the *prtF* strain is due to residual protease activity, this enzyme could be a useful target for further studies to decrease protease activity.

FAEA is able to release ferulic acid from wheat arabinoxylan without the aid of other xylan-degrading enzymes. Addition of xylanase from *A. niger* increased the amount of released ferulic acid two- to sevenfold. Bartolomé et al. (3) showed that the source of the xylanase has a strong effect on the level at which the liberation of ferulic acid is increased. They studied the effects of various xylanases on the liberation of ferulic acid from destarched wheat bran by FAE-III and showed that the *A. niger* xylanase had a relatively small effect compared to some other xylanases. Addition of other xylanases could therefore increase the amount of ferulic acid liberated even more.

FAEA is also able to release ferulic acid from sugar beet pectin, without the aid of other enzymes. With FAE-III, no activity was detected on sugar beet pulp. This is because of differences in the experiments. The hydrolysis with FAEA was performed with purified sugar beet pectin, whereas the FAE-III hydrolysis was performed with crude sugar beet pulp, which leads to a lower substrate concentration and low substrate accessibility. Second, due to the amount of enzyme available the amount of enzyme added was much lower in the FAE-III experiment, which may have resulted in activity which was below the detection limit. FAE-III was active on feruloylated galactose, but not on feruloylated arabinose isolated from sugar beet pulp (26). The activity on sugar beet pectin observed for FAEA was therefore probably activity on the ferulic acid linked to the galactose residues of sugar beet pectin. Addition of a pectin lyase from *A. niger* had only a minor effect on the amount of ferulic acid which was released. This could indicate that most of the feruloyl groups on which FAEA is active are already sufficiently accessible for FAEA to release them. Alternatively, this pectin lyase may not produce any oligomeric compounds with accessible ferulic groups, other than the ones that FAEA can already release from the polymer.

A region of FAEA has high homology to a putative lipase serine active site, which could be a result of similarities in substrate characteristics and reaction mechanism for these two types of enzymes. Both act on large molecules which contain polar and apolar residues. Additional indications that there is a lipaselike serine active site in FAEA are found when the spacing within the catalytic triade from lipases, which consists of a serine, an aspartic acid, and a histidine, is compared with the putative serine active site in FAEA. The spacing for the serine and the aspartic acid is between 55 and 59 residues, and the spacing for the aspartic acid and the histidine is between 53 and 60 residues for the four lipases in Fig. 3. FAEA has an aspartic acid 61 residues after the putative active-site serine and a histidine 53 residues after that aspartic acid, which is a spacing similar to that of the lipases. No significant homology was found with any other gene in the database, including *cinI*, a gene coding for a cinnamoyl esterase from the bacterium *Butyrivibrio fibrisolvens* (9).

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