The *Aspergillus niger faeB* gene encodes a second feruloyl esterase involved in pectin and xylan degradation and is specifically induced in the presence of aromatic compounds

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The *faeB* gene encoding a second feruloyl esterase from *Aspergillus niger* has been cloned and characterized. It consists of an open reading frame of 1644 bp containing one intron. The gene encodes a protein of 521 amino acids that has sequence similarity to that of an *Aspergillus oryzae* tannase. However, the encoded enzyme, feruloyl esterase B (FAEB), does not have tannase activity. Comparison of the physical characteristics and substrate specificity of FAEB with those of a cinnamoyl esterase from *A. niger* [Kroon, Faulds and Williamson (1996) Biotechnol. Appl. Biochem. **23**, 255–262] suggests that they are in fact the same enzyme. The expression of *faeB* is specifically induced in the presence of certain aromatic compounds, but not in the presence of other constituents present in plant-cell-wall polysaccharides such as arabinoxylan or pectin. The expression profile of *faeB* in the presence of aromatic compounds was compared with the expression of *A. niger faeA*, encoding feruloyl esterase A (FAEA), and *A. niger bphA*, the gene encoding a benzoate-*p*-hydroxylase. All three genes have different subsets of aromatic compounds that induce their expression, indicating the presence of different transcription activating systems in *A. niger* that respond to aromatic compounds. Comparison of the activity of FAEA and FAEB on sugar-beet pectin and wheat arabinoxylan demonstrated that they are both involved in the degradation of both polysaccharides, but have opposite preferences for these substrates. FAEA is more active than FAEB towards wheat arabinoxylan, whereas FAEB is more active than FAEA towards sugar-beet pectin.

Key words: caffeic acid, *p*-coumaric acid, ethyl ferulate, gene regulation.

INTRODUCTION

Feruloyl esterases are enzymes involved in the degradation of xylan and pectin, from which they release ferulic acid and other aromatic acids (e.g. *p*-coumaric acid) [1]. In xylan, ferulic acid is linked to O^5 of terminal arabinose residues attached to the xylan backbone [2]. Ferulic acid is also a component of the side chains of the pectic hairy regions, where it is attached to O^2 of terminal arabinose residues or O^6 of terminal galactose residues [3]. Feruloyl esterases have been purified from both bacteria and fungi [4–12]. The physical characteristics of these enzymes differ significantly, with a variation in molecular mass between 11 [11] and 112 kDa [4] and in pI between 3.3 [9] and 7.9 [12]. Detailed studies have been predominantly performed with the feruloyl esterases from the filamentous fungus *Aspergillus niger*. So far, two feruloyl esterases have been identified in *A. niger*, feruloyl esterase A (FAEA) and cinnamoyl esterase (CinnAE) [6,9], but to date only the FAEA-encoding gene (*faeA*) has been cloned [9] and its regulation of expression studied [13,14]. A previous report described the characteristics of two feruloyl esterases purified from a commercial *A. niger* preparation [15]. Based on their characteristics, it is likely that these two enzymes, feruloyl esterase-I and -II (FAE-I and FAE-II) correspond to CinnAE and FAEA. Counterparts of *A. niger faeA* have been cloned from *Aspergillus tubingensis* [9] and *Aspergillus awamori* (GenBank® accession number AB032760).

FAEA and CinnAE have different substrate specificities. Using methyl esters of several aromatic acids, it was demonstrated that FAEA 'prefers' substrates containing an aromatic ring substituted by a methoxy group at C^3 [17]. Additional methoxy groups attached to the aromatic ring increased FAEA activity. The same study demonstrated that CinnAE prefers substrates containing an aromatic ring substituted with hydroxy groups [17]. The activity of FAEA and FAE-I (CinnAE) towards feruloylated oligosaccharides derived from xylan and pectin has been studied previously [18]. FAEA released ferulic acid linked to O^5 of arabinose (as found in xylan) and O^6 of galactose (as found in pectin), but not linked to O^2 of arabinose (also found in pectin). FAE-I was active towards all oligosaccharides tested, but its activity was highest towards arabinose-linked feruloyl residues. Hydrolysis of wheat arabinoxylan and sugar-beet pectin (SBP) by a number of polysaccharide-degrading enzymes from *Aspergillus* (including *A. niger* FAEA) was studied recently [19], demonstrating that FAEA is important for the efficient degradation of both polysaccharides by *A. niger*.

We have now cloned the gene (*faeB*) encoding the second feruloyl esterase from *A. niger* and studied its expression. We have also purified the corresponding protein and designated it

Abbreviations used: ABFB, arabinofuranosidase B; AXHA, arabinoxylan arabinofuranohydrolase A; *bphA*, gene encoding benzoate-*p*-hydroxylase A; CinnAE, cinnamoyl esterase [probably identical with FAEB (feruloyl esterase B)]; CM, complete medium; CREA, carbon catabolite repressor protein; F_{crit} statistical value above which the difference between two conditions is significant; F, statistical value for the difference between two conditions; FAE-I, feruloyl esterase I (probably identical with FAEB); FAE-II, feruloyl esterase II [probably identical with FAEA (feruloyl esterase A)]; *faeA*, gene encoding FAEA; *faeB*, gene encoding FAEB; FERA, feruloyl esterase A; LACA (based on *lacA*), β-galactosidase A; MFA, methyl ferulate; MM, minimal medium; RGAEA, rhamnogalacturonan acetylesterase A; RHGA, rhamnogalacturonan hydrolase A; RT-PCR, reverse transcriptase PCR; SBP, sugar-beet pectin; WIP, water-insoluble pentosan of wheat arabinoxylan; XLNA, endoxylanase A; XLNR, xylanolytic transcriptional activator protein.

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FAEB to maintain a uniform naming of feruloyl esterases from *A. niger* that complies with the gene and protein nomenclature of *Aspergillus*. We have further compared the activity of FAEA and FAEB in the hydrolysis of wheat arabinoxylan and SBP.

EXPERIMENTAL

Materials

Caffeic acid, cinnamic acid, *p*-coumaric acid, ferulic acid, 4-hydroxybenzoic acid, 3,4-dimethoxycinnamic acid, protocatechuic acid, sinapic acid, syringic acid, vanillic acid, veratric acid, veratryl alcohol, vanillyl alcohol, anisyl alcohol, and methyl gallate were obtained from Acros (Geel, Belgium). Methyl ferulate, ethyl ferulate, methyl cinnamate, methyl caffeate and methyl 3,5-dimethoxy-4-hydroxycinnamate were from Apin Chemicals Ltd (Abingdon, Oxon., U.K.). D-Glucose, D-fructose and D-xylose were obtained from Merck (Darmstadt, Germany). L-Arabinose was from Sigma (Zwijndrecht, The Netherlands). All other standard chemicals were obtained from either Sigma or Merck. 3-Methoxy-4-hydroxyphenylpropionic acid was a gift from Dr Gary Williamson, Institute of Food Research, Norwich, U.K.

Strains, libraries and plasmids

All strains, except *A. niger* CMICC (CAB International Mycological Institute culture collection) 298302, were derived from *A. niger* wild-type N400 (= CBS 120.49). N402 is a mutant with short conidiophores (*csp*A1). The CreA mutant NW200 (*bio*A1, *csp*A1, *cre*Ad4, *pyr*A13::pGW635, *are*A1::pAREG1) was selected in an *are*A1 background [20] and subsequently co-transformed with pAREG1 (containing the *A. niger areA* gene) [21] and pGW635 (containing the *pyrA* selection marker) to restore the *areA* wild-type. *Escherichia coli* DH5αF′ was used for plasmid propagation. *E. coli* LE392 was used as a host for phage EMBL3. Subcloning was performed using pBluescript [22] and pGEM-T (Promega, Madison, WI, U.S.A.). The genomic library of *A. niger* has been described previously [23].

Media and growth conditions

Minimal medium (MM) and complete medium (CM) were described previously [13]. Liquid cultures were inoculated with 10^6 spores/ml, unless otherwise indicated, and incubated at 30 °C in an orbital shaker at 250 rev./min. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

Transfer experiments were performed by pre-growing the strains for 16 h in CM containing 2 % (w/v) fructose as carbon source, after which the mycelium was harvested and washed with MM without carbon source. Aliquots (1.5 g) of wet mycelium were then transferred to 50 ml of MM containing carbon sources as indicated in the text. After 2 or 4 h of incubation in a rotary shaker at 250 rev./min and 30 °C, mycelium was harvested, dried between tissue paper and frozen in liquid nitrogen and stored at −70 °C.

Purification of FAEB

For the purification of FAEB, *A. niger* CMICC 298302 was grown in MM containing 1.5 % (w/v) SBP and 0.02 % (w/v) caffeic acid. The medium was inoculated with 2×10^5 spores/ml and incubated for 65 h at 30 °C in an orbital shaker at 250 rev./min. Culture fluid was harvested by filtration using nylon gauze and diluted twice with water, after which the pH was adjusted to 7.5. During the purification procedure, fractions containing feruloyl esterase activity were determined by activity measurements using methyl caffeate as a substrate as described below. The enzyme was retrieved from the solution by batchwise adsorption to DEAE-Sephadex A-50 (50 g wet weight/litre). Bound protein was eluted with a pulse of 0.01 M Tris/HCl (pH 7.5)/1 M NaCl. Feruloyl esterase-containing fractions were pooled, and solid $(NH_4)_2SO_4$ was added to a concentration of 1 M. The solution was loaded on a Phenyl-Sepharose CL-4B column (2.5 cm × 12 cm; Pharmacia Biotech, Uppsala, Sweden) equilibrated in 50 mM sodium phosphate buffer (pH 7.0)/1 M $(NH_4)_2SO_4$. Elution was performed with a 500 ml linear gradient [1–0 M $(NH_4)_2SO_4$ in the same buffer], and fractions were collected. Feruloyl esterase-containing fractions were pooled and loaded on a 1 ml HiTrap Phenyl-Sepharose 6 Fast Flow column (high substitution; Pharmacia Biotech) in 50 mM sodium phosphate buffer (pH 7.0)/1 M $(NH_4)_2SO_4$. Bound protein was recovered by elution with a pulse of 50 mM sodium phosphate, pH 7.0. Further purification was achieved by gel-permeation chromatography on a Superdex 200 Prep Grade column (2.5 cm × 60 cm; Pharmacia Biotech) in 50 mM sodium phosphate (pH 7.0)/0.1 M NaCl. Feruloyl esterase-containing fractions were pooled, diluted 5-fold with water and loaded on a 1 ml Resource Q column (Pharmacia Biotech). Elution was performed with a 30 ml linear gradient (0–0.4 M NaCl) at a flow rate of 2 ml/min.

Characterization of FAEB

Deglycosylation of pure FAEB was performed with N-glycosidase F (Boehringer-Mannheim, Almere, The Netherlands) according to the procedure recommended by the manufacturer, with denaturation of the protein before addition of N-glycosidase F. The molecular masses of the mature and deglycosylated FAEB were determined by SDS/PAGE. The pH optimum was determined spectrophotometrically at 37 °C in McIllvaine buffers (pH range 4–7) using 0.1 mM methyl caffeate as a substrate.

SDS/PAGE and Western-blot analysis

Electrophoresis of proteins was performed under denaturing conditions on 10 % (w/v) polyacrylamide gels using the method of Laemmli [24] in a Mini-V system (Life Technologies B. V., Breda, The Netherlands). Western-blot analysis was performed as described previously [9].

PCR cloning of a specific fragment of *faeB*

N-terminal and internal peptide sequences of FAEB were determined as previously described [25], resulting in ATDPFQSRPNEFQNKIDIA for the N-terminal sequence and QCDLVDGALDGIIEDP as an internal sequence. Two partially overlapping degenerate oligonuclotides (5′-CARAAYAARATHGAYATHGC-3′ and 5′-CCNAAYGARTTYCARAAYAA-3′, with R, Y, H and N being A/G, C/T, A/C/T and A/C/G/T respectively) were designed on the N-terminal peptide sequence and one degenerate oligonucleotide (5′-GGRTCYTCDATDATNCCRTC-3′, with D, R, Y and N being A/G/T, A/G, C/T and A/C/G/T respectively) on the internal peptide sequence and used in PCRs under the following conditions: 1 min of denaturing at 95 °C, 1 min of annealing at 50 °C and 2 min of amplification at 72 °C, 30 cycles. Chromosomal DNA of *A. niger* CMICC 298302 was used as a template. This resulted in a fragment of approx. 800 bp which was cloned in pGEM-T easy (Promega). Sequence analysis was performed as described below.

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Table 1 Enzymes used for the hydrolysis of SBP and/or WIP

Enzyme	Origin	Amount used (μg/ml)	Reference or supplier
ABFB	*A. niger*	20.2	[39]
AXHA	*A. tubingensis*	5	Danisco-Cultor
FAEA	*A. niger*	0.5	[9]
FAEB	*A. niger*	0.5	The present paper
LACA	*A. niger*	8.4	[40]
RGAEA	*A. niger*	33.4	[19]
RHGA	*A. aculeatus*	33.4	[41]
XLNA	*A. tubingensis*	2.0	Danisco-Cultor

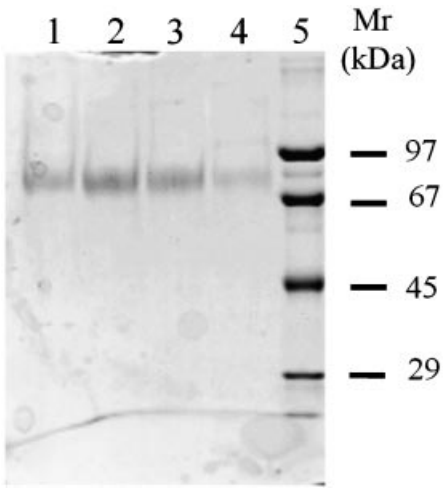

Figure 1 SDS/PAGE to demonstrate the purity of the FAEB preparation

Lanes 1–4, samples of the four fractions containing FAEB obtained after the last purification step, which were pooled to give the enzyme preparation used in the present study. Lane 5 and 'Mr', marker proteins to determine the molecular mass of FAEB.

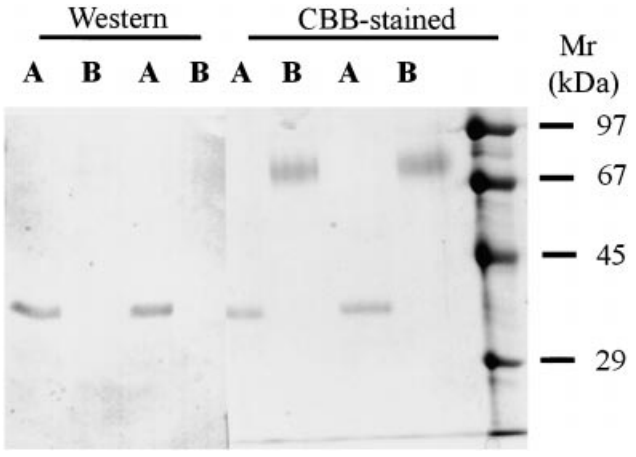

Figure 2 Western-blot analysis of purified FAEA and FAEB with an antibody against FAEA

A, FAEA; B, FAEB; Mr, marker proteins of known molecular mass; CBB, Coomassie Brilliant Blue.

Cloning and characterization of *faeB*

Plaque hybridizations were performed as previously described [26]. Hybridizations were performed overnight at 65 °C by using the *faeB* PCR fragment as a probe. Filters were washed with $0.2 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.6)/0.5 % (w/v) SDS. Positive plaques, identified on duplicate replicas after autoradiography, were recovered from the original plates and purified by re-screening at low plaque density. Standard methods were used for other DNA manipulations, such as Southern-blot analysis, subcloning, DNA digestions, and lambda phage and plasmid DNA isolations [27]. Chromosomal DNA was isolated as previously described [28]. Sequence analysis of the reverse transcriptase (RT-)PCR products was performed on both strands of DNA by using the Cy5 AutoCycle Sequencing kit (Pharmacia Biotech). The reactions were analysed with an ALFred DNA Sequencer (Pharmacia Biotech). Sequence analysis of the genomic clone of *faeB* was performed at EuroSequence Gene Service (Génopole, Evry, France) on an ABI 377 sequencer using an ABI PRISM™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS (PerkinElmer Applied Biosystems, Foster City, CA, U.S.A.). Nucleotide sequences were analysed with computer programs based on those of Devereux et al. [29]. RT-PCRs were performed using the Enhanced Avian RT-PCR kit (Sigma) according to the supplier's instructions using *faeB*-specific oligonucleotides (5′-ACATC-GCCAATGTCACCG-3′ and 5′-CCAGAACCTCCGAGTTG-TTG-3′). Northern-blot analysis was performed as previously described [13].

Sequence alignments

Amino acid sequence alignments were performed by using the BLAST programs [30] at the server of the National Center for Biotechnology Information (Bethesda, MD, U.S.A.).

Enzyme assays

Activity of FAEA and FAEB using methyl ferulate, ethyl ferulate, methyl caffeate, methyl cinnamate, methyl 3,5-dimethoxy-4-hydroxy-cinnamate and methyl gallate as substrates was determined as described previously for methyl ferulate [9]. All assays were performed in duplicate. Separation of substrate and product was achieved as described previously for methyl ferulate [9], except for methyl gallate, for which separation was achieved using isocratic elution (1 ml/min) with aq. 28 % (v/v) methanol containing 0.3 % (v/v) acetic acid. Detection of substrates and corresponding products was performed at 325 nm, for methyl ferulate, ethyl ferulate and methyl 3,5-dimethoxy-4-hydroxy-cinnamate, 280 nm for methyl *p*-coumarate and methyl cinnamate, and at 260 nm for methyl gallate. K_m and k_{cat} values using methyl ferulate were determined as described previously [17].

Pectin and xylan incubations

Incubations using SBP and water-insoluble pentosan (WIP) from wheat arabinoxylan as a substrate were performed in duplicate as described previously [19], using the enzymes listed in Table 1. For the present study only SBP pre-treated with *A. aculeatus* rhamnogalacturonan hydrolase A (RHGA) and *A. niger* rhamnogalacturonan acetylesterase A (RGAEA), and WIP pre-treated with *A. tubingensis* endoxylanase A (XLNA) were used [19]. Ferulic acid concentrations were determined as described previously [19].

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Statistical analysis

Statistical analysis of the pectin and xylan incubations was performed using the ANOVA data analysis tool of EXCEL 97 SR-2 (Microsoft). An α value of 0.05 was used ($P = 95\%$).

RESULTS

Purification and characteristics of FAEB

FAEB was purified to electrophoretic homogeneity as described in the Experimental section (Figure 1). A sample of 1 μg of the purified protein was deglycosylated using N-glycosidase F, and this preparation, as well as 1 μg of intact protein, were analysed by SDS/PAGE. This gave a molecular mass of approx. 74 kDa for intact FAEB and approx. 60 kDa for the deglycosylated protein. The pH optimum of FAEB using methyl caffeate as a substrate was 6.0. A Western analysis was performed using a specific antibody against FAEA [9], which demonstrated that the FAEB preparation does not contain detectable amounts of FAEA, nor does FAEB itself react with the FAEA antibody (Figure 2).

Cloning and characterization of *faeB*

On the basis of the N-terminal and internal amino acid sequences of FAEB, degenerate oligonucleotides were designed and used in PCRs, resulting in a specific fragment of *faeB*, as described in the Experimental section. Screening of a genomic library of *A. niger* using this fragment as a probe resulted in the isolation of three *faeB*-containing phage λ clones. From one of these clones a 5 kb *Bam*HI and a 2.8 kb BamHI fragment were cloned in pBluescript, resulting in plasmids pIM3284 and pIM3285 respectively. Double-stranded sequence was determined for regions of these constructs containing *faeB* and some of the flanking regions,

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FAEB      mkvasllslalpgaalaATDPFQSRCNEFQNKIDIANVTVRSVAYVAAGQNISQAEVASV  60
TANNASE    mrqhsrmavaalaaganaASFTDVCTVSNVKAALPANGTLLGISMLPSAVTANPLYNQS  59
consensus              AA A    F   C     K

FAEB      CKASVQASVDLCRVTMNISTS---DRSHLWAEAWLPRNYTGRFVSTGNGGLAGCVQETDL 117
TANNASE   AGMGSTTTYDYCNVTVAYTHTGKGDKVVIKYAFPKPSDYENRFYVAGGGGFSLSSDATGG 119
consensus          D C VT         D          P  Y  RF   G GG       T

FAEB      NFAANFGFATVGTNGGHDGDTAKYFLN-----NSEVLADFAYRSVHEGTVVGKQLTQLFY 172
TANNASE   LAYGAVGGATDAGYDAFDNSYDEVVLYGNGTINWDATYMFAYQALGEMTRIGKYITKGFY 179
consensus       G AT       D       L      N      FAY    E T  GK  T  FY

FAEB      DEGYN---YSYYLGCSTGGRQGYQQVQRFPDDYDGVIAGSAAMNFINLISWGAFLWKATG 229
TANNASE   GQSSDSKVYTYYEGCSDGGREGMSQVQRWGEEYDGAITGAPAFRFAQQQVHHVFSSEVEQ 239
consensus         Y YY GCS GGR G  QVQR    YDG I G  A  F        F

FAEB      LADDPDFISANLWSVIHQEIVRQCDLVDGALDGIIEDPDFCAP-------VIERLICDG- 281
TANNASE   TLD--YYPPPCELKKIVNATIAACDPLDGRTDGVVSRTDLCKLNFNLTSIIGEPYYCAAG 299
consensus   D            I       CD  DG  DG     D C           E   C

FAEB      --TTNGTSCITG-------------------------AQAAKVNRALSDFYG--PDGTVY 312
TANNASE   TSTSLGFGFSNGKRSNVKRQAEGSTTSYQPAQNGTVTARGVAVAQAIYDGLHNSKGERAY 359
consensus   T  G     G                          A    V  A  D          Y

FAEB      YPRLNYGGEADSASLYFTGS-------------MYSRTEEWYKYVVYNDTNWNSSQWTLE 359
TANNASE   LSWQIASELSDAETEYNSDTGKWELNIPSTGGEYVTKFIQLLNLDNLSDLNNVTYDTLVD 419
consensus           D    Y                                  D N

FAEB      SAKLALEQNPFNIQAFDPNITAFRDRGGKLLSYHGTQDPIISSTDSKLYYRRVANALN-- 417
TANNASE   WMNTGMVRYMDSLQTTLPDLTPFQSSGGKLLHYHGESDPSIPAASSVHYWQAVRSVMYGD 479
consensus              Q   P  T F   GGKLL YHG  DP I    S  Y   V

FAEB      ----AAPSELDEFYRFFQISGMGHCGDGTGASYIGQGYGTYTSKAPQVNLLRTMVDWVEN 473
TANNASE   KTEEEALEALEDWYQFYLIPGAAHCGTNS-----------LQPGPYPENNMEIMIDWVEN 528
consensus      A   L   Y F  I G  HCG                     N    M DWVEN

FAEB      GKAPEYMPGNKLNANGSIEYMRKHCRYPKHNIHTGPGNYTDPN------SWTCV          521
TANNASE   GNKPSRLN-ATVSSGTYAGETQMLCQWPKRPLWRGNSSFDCVNDEKSIDSWTYEFPAFKVPVY 590
consensus G  P                    C  PK     G       N      SWT
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Figure 3 Alignment of the amino acid sequences of *A. niger* FAEB and *A. oryzae* tannase

The (putative) signal peptides are in lower-case letters. Identical amino acids are indicated in the consensus. Putative N-glycosylation sites are indicated in **bold**.

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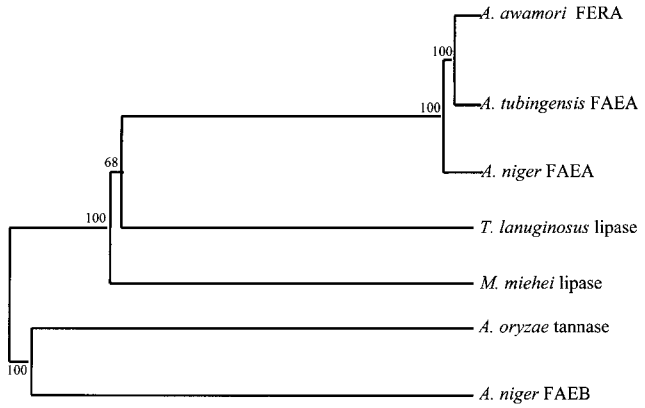
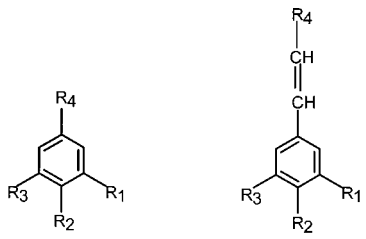


Figure 4 Phylogenetic tree of the feruloyl esterases from *Aspergillus*, and the enzymes with the highest sequence similarity to them

The numbers given in the Figure are bootstrap values (bootstrap values are an indication of the certainty of splitting the enzymes into different branches; a value of 100 means that the split is certain; the lower the value, the higher the probability that they actually belong in one branch).



Structure I **Structure II**

Compound	Structure	R_1	R_2	R_3	R_4
benzoic acid	I	H	H	H	COOH
caffeic acid	II	OH	OH	H	COOH
cinnamic acid	II	H	H	H	COOH
p-coumaric acid	II	H	OH	H	COOH
ferulic acid	II	OCH_3	OH	H	COOH
4-hydroxybenzoic acid	I	H	OH	H	COOH
3,4-dimethoxycinnamic acid	II	OCH_3	OCH_3	H	COOH
protocatechuic acid	I	OH	OH	H	COOH
sinapic acid	II	OCH_3	OH	OCH_3	COOH
syringic acid	I	OCH_3	OH	OCH_3	COOH
vanillic acid	I	OCH_3	OH	H	COOH
vanillyl alcohol	I	OCH_3	OH	H	CH_2OH
vanillin	I	OCH_3	OH	H	CHO
3-methoxy-4-hydroxyphenylpropionic acid	I	OCH_3	OH	H	C_2H_4COOH
coniferyl alcohol	II	OCH_3	OH	H	CH_2OH
veratric acid	I	OCH_3	OCH_3	H	COOH
veratryl alcohol	I	OCH_3	OCH_3	H	CH_2OH
anisyl alcohol	I	H	OCH_3	H	CH_2OH

Figure 5 Aromatic compounds tested as inducers of *faeA*, *faeB* and *bphA* transcription

The compounds can be divided into two groups with respect to their basic structure. The groups attached to the aromatic ring are listed in the tabular part of the Figure.

resulting in the genomic sequence of *fae*B (EMBL accession number AJ309807). The presence of one putative intron was confirmed by RT-PCR using total RNA of *A. niger* mycelium, transferred to CM containing 0.03 % caffeic acid, as a template. The *faeB* gene has a length of 1644 bp and encodes a protein of 521 amino acids. Computer analysis predicted a eukaryotic signal sequence of 17 amino acids, which is in agreement with the N-terminal amino acid sequence of the mature protein. The mature enzyme has a calculated pI of 4.89, a calculated molecular mass of 55551.4 Da and contains 18 putative glycosylation sites. BLAST analysis of the deduced amino acid sequence of FAEB did not identify enzymes with high sequence similarity to FAEB. The only significant similarity was found with an *A. oryzae* tannase [31], which shares 24 % identity with FAEB (Figure 3). Construction of a phylogenetic tree of FAEB, the *A. oryzae* tannase, FAEA and two lipases with amino acid sequence similarity to FAEA demonstrated that FAEA and FAEB clearly belong to different classes of enzymes (Figure 4). It also demonstrated that the *A. awamori* feruloyl esterase A (FERA) is more similar to *A. tubingensis* FAEA than to *A. niger* FAEA.

Sequence analysis of the promoter region of *faeB* identified several sequences possibly involved in the regulation of *faeB* transcription. A TATA box was found at position −101 from the ATG codon. Twelve putative binding sites for the carbon catabolite repressor protein (CREA) that mediates carbon catabolite repression [32] were identified at positions −155, −193, −233, −311, −321, −374, −391, −395, −783, −818, −837 and −858.

Expression of *faeB* on aromatic compounds

An experiment was performed in which mycelium of *A. niger* CBS 120.49 was transferred to CM containing 0.03 % of different aromatic compounds (Figure 5), CM containing 0.03 % fructose (control) and CM containing no carbon source (blank). Mycelium was harvested after 2 h of incubation, after which RNA was isolated and a Northern-blot analysis was performed as described in the Experimental section. Expression of *faeB* was compared with the expression of *faeA* and *bphA*, the gene encoding benzoate-*p*-hydroxylase that was previously reported to be induced by benzoic acid and 4-hydroxybenzoic acid [33,34].

The highest level of *faeB* transcript was observed in the presence of caffeic and *p*-coumaric acid (Figure 6), but ferulic acid, 3-methoxy-4-hydroxyphenylpropionic acid, protocatechuic acid and 4-hydroxybenzoic acid also induced expression of *faeB*. Transcription of *faeA* was observed in the presence of ferulic acid, vanillic acid, vannilyl alcohol, vanillin, 3-methoxy-4-hydroxyphenylpropionic acid and veratric acid (Figure 6) [13]. The *bphA* gene was transcribed at low levels in the presence of benzoic acid and cinnamic acid, at intermediate levels in the presence of *p*-coumaric acid and 4-hydroxybenzoic acid, and at a high level in the presence of anisyl alcohol (Figure 6).

Influence of other carbon sources on the expression of *faeB*

To determine whether, apart from aromatic compounds, other carbon sources also stimulate the expression of *faeB*, or whether they modulate the ferulic acid induction, an experiment was performed in which mycelium from *A. niger* CBS 120.49 or NW200 was transferred to several carbon sources with and without 0.03 % (w/v) ferulic acid. The transcription profile of *faeB* was compared with that of *faeA*, which is known to be regulated by the xylanolytic transcriptional activator protein (XLNR) [14] and CREA [13]. Mycelium was harvested after 4 h of incubation, after which RNA was isolated and a Northern-blot analysis was performed as described in the Experimental section.

Xylose and arabinose specifically induced the expression of *faeA*, but neither these nor any of the other compounds resulted

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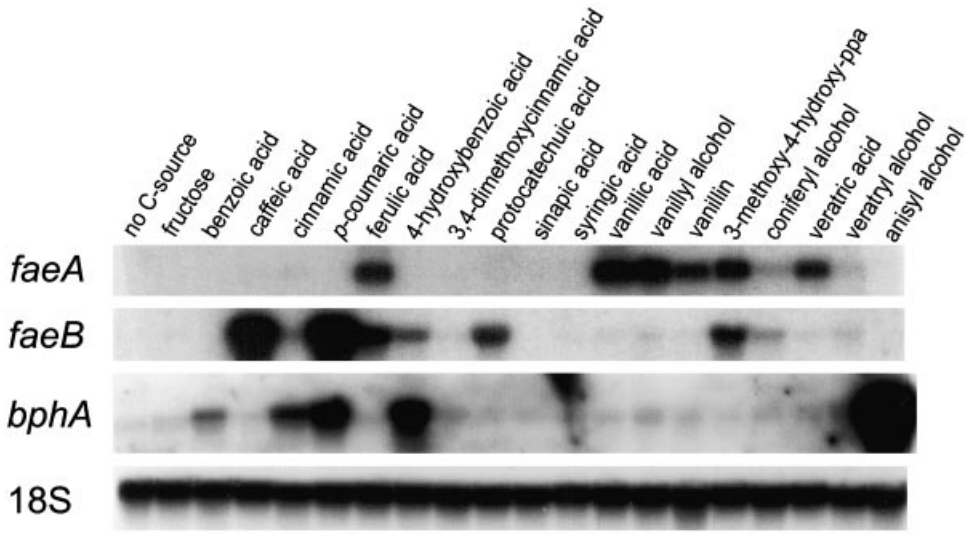

Figure 6 Comparison of the transcription profiles of *faeA*, *faeB* and *bphA* in the presence of a number of aromatic compounds

Mycelium, obtained from an overnight culture of *A. niger* CBS120.49 in CM containing 2% (w/v) fructose, was incubated for 2 h in CM containing no carbon source, 0.03% (w/v) fructose or 0.03% (w/v) of the aromatic compounds indicated. A fragment of the 18 S rRNA gene [42] was used as an RNA loading control.

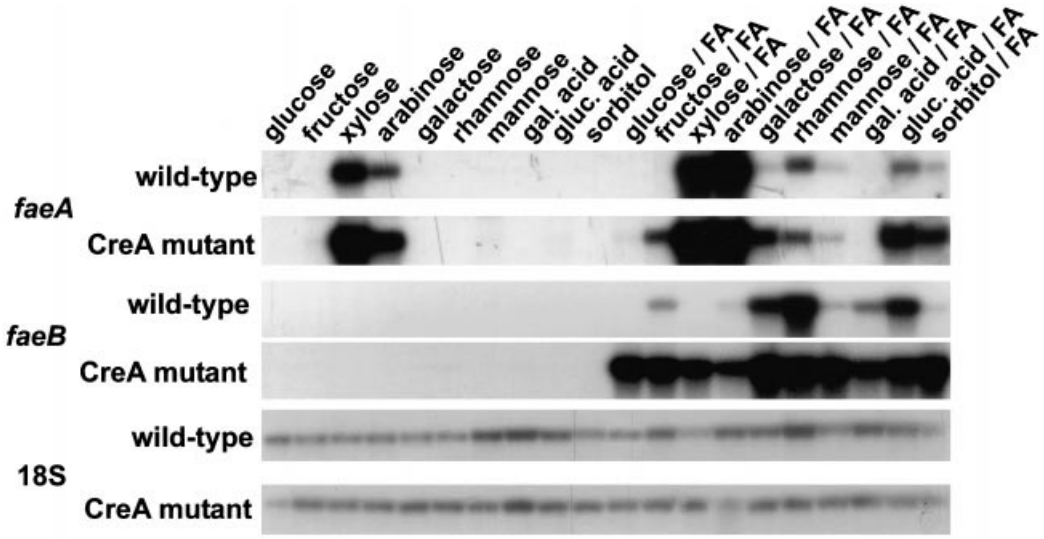

Figure 7 Comparison of the transcription profiles of *faeA* and *faeB* in the presence of different carbon sources

Mycelium, obtained from an overnight culture of *A. niger* CBS120.49 or NW200 in CM containing 2% (w/v) fructose, was incubated for 4 h in CM containing 1% (w/v) of the carbon source as indicated alone or in combination with 0.03% (w/v) ferulic acid. A fragment of the 18 S rRNA gene [42] was used as an RNA loading control.

in specific induction of *faeB* expression (Figure 7). Prolonged exposure of the autoradiogram demonstrated that *faeB* has a low constitutive expression level (results not shown). This was not the case for *faeA*, where no transcription was detected under non-inducing conditions. The level of transcription of both *faeA* and *faeB* is increased in the CREA-derepressed mutant, but this effect was stronger for *faeB* (Figure 7).

Activity towards synthetic substrates

The activity of FAEA and FAEB on several synthetic substrates was determined. Both FAEA and FAEB were able to release ferulic acid from menthyl ferulate and ethyl ferulate (Figure 8), but the activity of FAEA was higher using these substrates. Using methyl cinnamate and methyl caffeate as substrates, only FAEB was active (results not shown). The opposite was found when using methyl-3,5-dimethoxy-4-hydroxycinnamate as a substrate. Release of 3,5-dimethoxy-4-hydroxycinnamate release was only observed in incubations with FAEA (results not shown). Incubations with methyl gallate (a synthetic substrate to measure tannase activity) revealed that neither FAEA nor FAEB was able to hydrolyse this compound (results not shown). Using methyl ferulate as a substrate, the K_m values for FAEA and FAEB were 0.76 and 1.31 mM respectively. The k_{cat} values for FAEA and

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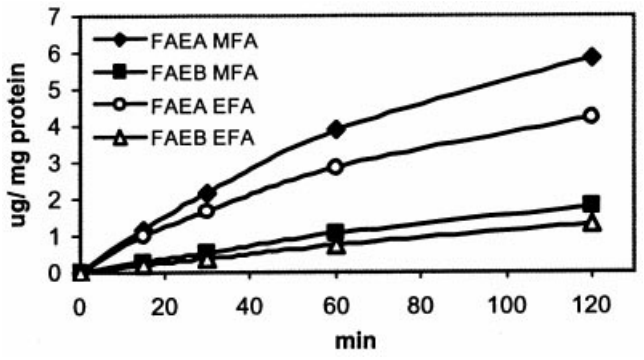

Figure 8 Activity of FAEA and FAEB on methyl ferulate (MFA) and ethyl ferulate (EFA)

The activity is given as the amount of ferulic acid (μg) released/mg of enzyme from 5 mM substrate.

FAEB using this substrate were 104 and 58 kat/mol of protein respectively.

Comparison of the activity of FAEA and FAEB towards pre-treated SBP

A 1% (w/v) SBP suspension was incubated for 24 h with *A. aculeatus* RHGA and *A. niger* RGAEA to obtain partial deacetylation and hydrolysis of the pectin main chain. This substrate was then used in incubations with FAEA and FAEB, alone and in combination, and in incubations in which *A. niger* arabinofuranosidase B (ABFB) and/or *A. niger* β-galactosidase A (LACA) were also added. Samples were taken after 30 min, 1.5 h, 4 h, 8 h and 24 h, and the amount of free ferulic acid was determined. Data from the 4 and 24 h samples are given (in Figure 9), since they best represent the trend observed for the incubations. FAEB had a higher activity towards SBP than did FAEA, both acting alone or in combination with LACA and/or ABFB. Statistical analysis indicated that the addition of FAEA to FAEB did not significantly increase the amount of ferulic acid released, unless the incubation mixture also contained LACA. When comparing incubations with FAEB and LACA with incubations with FAEB, LACA and FAEA, a significant increase in ferulic acid release was observed after 24 h [F (statistical value for the difference between two conditions) = 102.18; F_{crit} (statistical value above which the difference between two conditions is significant) = 18.51]. The presence of ABFB positively influenced the activity of FAEB at both time points ($F = 370.99$, $F_{crit} = 18.51$ for 4 h; $F = 19.02$, $F_{crit} = 18.51$ for 24 h), but only had a significant effect at the amount of ferulic acid released by FAEA after 24 h ($F = 19.84$, $F_{crit} = 18.51$). Addition of LACA to the incubations only resulted in a significant increase of the amount of ferulic acid released by FAEA after 24 h ($F = 85.98$, $F_{crit} = 18.51$) and by FAEB after 4 h ($F = 46.70$, $F_{crit} = 18.51$). The highest release of ferulic acid was detected when FAEB, LACA and ABFB were present. The addition of FAEA did not significantly increase the amount of ferulic acid released in this incubation.

Comparison of the activity of FAEA and FAEB on pre-treated wheat arabinoxylan

A 0.5% (w/v) WIP suspension was incubated for 24 h with *A. tubingensis* XLNA to obtain partial hydrolysis of the xylan main chain. This substrate was then used in incubations with FAEA and FAEB, alone and in combination, and in incubations in which *A. niger* ABFB or *A. niger* arabinoxylan arabinofurano-

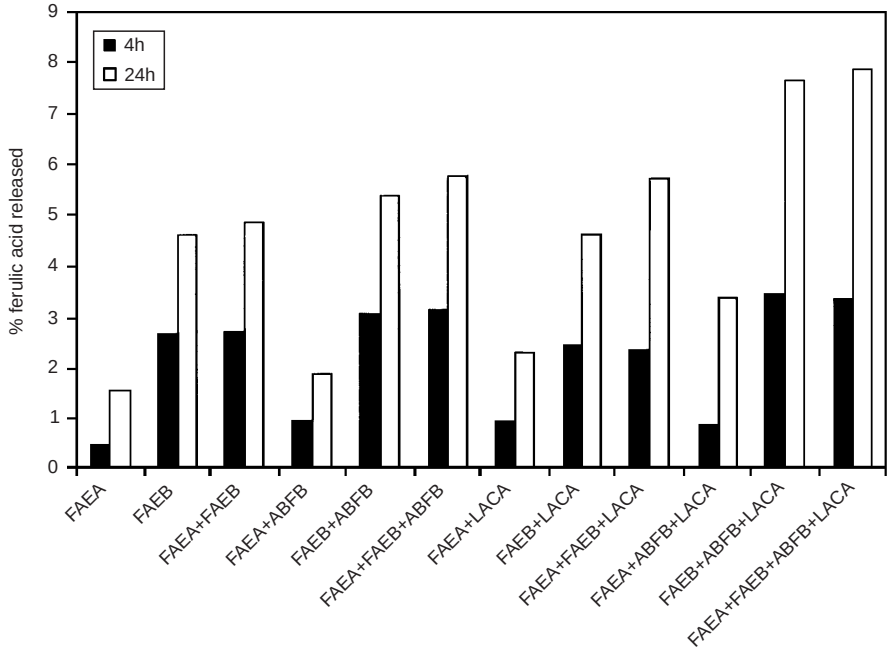

Figure 9 Comparison of the activity of FAEA and FAEB against pre-treated SBP

SBP was pretreated with RHGA and RGAEA to partially deacetylate and hydrolyse the pectin main chain. Activities are expressed as the percentage of the total amount of ferulic acid present in the substrate. Absolute variations between duplicate incubations were all between 0.1% and 0.9%.

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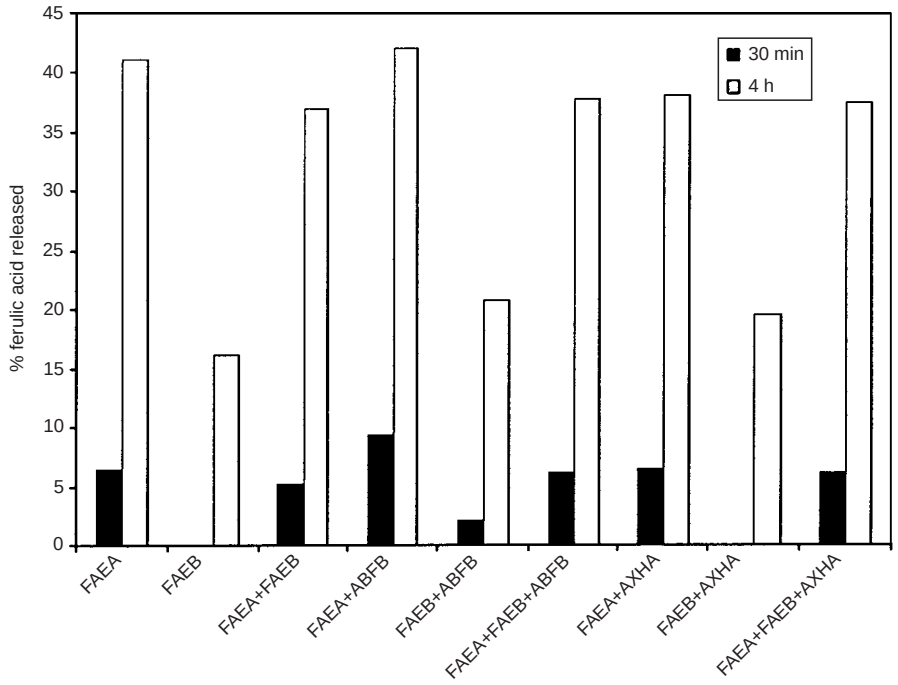

Figure 10 Comparison of the activity of FAEA and FAEB against pre-treated WIP

WIP was pretreated with XLNA to partially hydrolyse the xylan main chain. Activities are expressed as the percentage of the total amount of ferulic acid present in the substrate. Absolute variations between duplicate incubations were all between 0.1 and 1.2%.

hydrolase A (AXHA) were also added. Samples were taken after 30 min, 1.5 h, 4 h, 8 h and 24 h and the amount of free ferulic acid was determined. Data from the 30 min and 4 h samples are given (in Figure 10), since they best represent the trend observed for the incubations. FAEA had a higher activity than FAEB towards this substrate, both acting alone or in combination with ABFB or AXHA (Figure 10). Addition of FAEB to FAEA did significantly decrease the amount of ferulic acid released at both time points ($F = 29.46$, $F_{crit} = 18.51$ for 30 min; $F = 632.46$, $F_{crit} = 18.51$ for 4 h). This was also observed when incubations with FAEA and ABFB and incubations with FAEA, FAEB and ABFB were compared. After 30 min of incubation the presence of FAEB significantly decreased the amount of ferulic acid released ($F = 2159$, $F_{crit} = 18.51$).

DISCUSSION

The molecular mass determined for FAEB by SDS/PAGE was 74 kDa, which is similar to the value obtained previously for CinnAE (75.8) [6]. CinnAE has a pI of 4.8 [6], which is in good accordance with the calculated pI for FAEB (4.89). In addition, the activity of FAEB on methyl esters of a number of different aromatic compounds is identical with that reported for CinnAE [17]. Both FAEB and CinnAE are unable to hydrolyse methyl-3,5-dimethoxy-4-hydroxycinnamic acid, but are active against methyl ferulate, methyl caffeate and methyl cinnamate. All these data suggest that the two enzymes are, in fact, identical. This is confirmed by the nearly identical values for the K_m and k_{cat} of FAEB and CinnAE [17] using methyl ferulate as a substrate. The activity of both enzymes against ethyl ferulate was approx. 72% of their activity against methyl ferulate, indicating that the difference in the substrate had the same effect on the activity of both enzymes.

The determined molecular mass of mature FAEB (74 kDa) is significantly higher than the calculated molecular mass based on the amino acid sequence (55.6 kDa). Treatment with N-glycosidase F resulted in a protein of approx. 60 kDa, demonstrating that this difference is predominantly caused by N-glycosylation. The remaining difference in molecular mass could be due to O-glycosylation or SDS/PAGE running effects.

The sequence similarity between FAEB and the *A. oryzae* tannase is interesting, since both enzymes are esterases liberating aromatic acids from polymeric compounds. Another similarity between FAEB and the *A. oryzae* tannase is that they both have high activity towards aromatic compounds containing hydroxy groups at C^3, C^4 and C^5 of the aromatic ring. However, FAEB does not posses tannase activity, indicating that the difference of the group attached to C^1 of the aromatic ring is sufficient to distinguish FAEB from enzymes with tannase activity. No significant similarity could be detected to *A. niger* FAEA, or any other enzymes with feruloyl esterase activity present in the database, indicating that these enzymes do not form a family of polysaccharide-degrading enzymes based on their primary sequence as was described for many other polysaccharide-degrading enzymes (http://afmb.cnrs-mrs.fr/~cazy/CAZY/). The phylogenetic tree clearly puts *A. niger* FAEB and the *A. oryzae* tannase in different groups from the *A. niger* and *A. tubingensis* FAEA, *A. awamori* FERA and the two lipases. Recently it was shown, for a number of *A. awamori* strains, that they are in fact strains of either *A. niger*, *A. tubingensis* or *A. foetidus*, suggesting that *A. awamori* is in fact not a separate species [35]. The greater similarity of *A. awamori* FERA to *A. tubingensis* FAEA than to *A. niger* FAEA could indicate that this *A. awamori* strain in fact belongs to the *A. tubingensis* group.

Transcription of *faeB* in the presence of aromatic compounds is different from that of *faeA* and *bphA*. For *faeA*, transcription

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is observed in the presence of aromatic compounds containing an aromatic ring that is substituted at C_3 with a methoxy group and at C_4 with a hydroxy group [13]. FAEA is able to hydrolyse the methyl esters of these compounds, indicating a direct relationship between induction of gene expression and substrate specificity of the corresponding enzyme. On the basis of the model for the degradation of aromatic compounds by *A. japonicus* [36], all the compounds that induce expression of *bphA* are catabolized via 4-hydroxybenzoic acid, suggesting that this compound may be the true inducer of the expression of *bphA*. The expression profile of *faeB* is more difficult to explain, since the structural requirements for the aromatic compounds to be able to act as inducers appear to be more complex. Highest levels of *faeB* transcription are observed in the presence of caffeic acid and *p*-coumaric acid, which have in common the property that the aromatic ring does not contain methoxy groups, but only hydroxy groups (two and one respectively) and that the substituent at C^1 is identical (—CH=CH—COOH). Cinnamic acid, which has a similar structure, but does not contain a hydroxy group, is not an inducer, suggesting that the presence of at least one hydroxy group is necessary. This hydroxy group is not required for activity as FAEB (CinnAE) was shown to be active towards methyl esters of caffeic acid, *p*-coumaric acid and cinnamic acid [17]. Of these three aromatic compounds, only caffeic acid and *p*-coumaric acid are both found in plant cell walls linked to polysaccharides, whereas cinnamic acid is not [37]. This might be related to the induction pattern of *faeB*, suggesting a very strong structural requirement for the inducing compounds.

The presence of one or two methoxy groups respectively decreases (ferulic acid) or abolishes (sinapic acid) *faeB* transcription. The absence of the —CH=CH— chain between C^1 of the aromatic ring and the carboxy group (4-hydroxybenzoic acid, protocatechuic acid) results in a strong decrease in *faeB* expression, and the presence of methoxy groups in such compounds abolishes transcription. In summary, for transcription of *faeB* the inducing aromatic compounds require at least one hydroxy group to be attached to the aromatic ring, and a —CH=CH— chain attached to C^1 of the aromatic ring. The presence of methoxy groups as substituents reduces or abolishes the ability to stimulate *faeB* transcription. The distinct expression profiles for the three genes studied in the present study demonstrate the presence of at least three different activating systems in *A. niger* responding to the presence of specific aromatic compounds.

Regulation of *faeB* transcription differs from that of *faeA*, since it is specific for aromatic compounds, whereas *faeA* is also under the control of XLNR [14]. The low constitutive level of *faeB* expression may enable *A. niger* to release small amounts of ferulic acid from xylan or pectin, which would then activate transription of both *faeA* and *faeB*. In the presence of xylan, *faeA* will also be expressed via XLNR, resulting again in a release of ferulic acid and subsequent further stimulation of both *faeA* and *faeB* transcription. Both *faeA* and *faeB* are regulated by CREA, which will prevent production of (high levels of) the corresponding enzymes in the presence of easily metabolizable compounds.

FAEB is more active towards pre-treated SBP than FAEA. Release of ferulic acid by FAEB is positively influenced by the presence of ABFB, a finding that is in accordance with a previous study using CinnAE [38]. The presence of β-galactosidase affected the activity of FAEB to a lesser extent than ABFB. Ferulic acid release by FAEA is more strongly increased in the presence of LACA than in the presence of ABFB, as was shown previously [19]. Incubations using a combination of FAEA and FAEB did not result in a significant increase in the amount of ferulic acid released compared with incubations with FAEB alone, unless LACA is present. This is also in accordance with the substrate specificity of FAEA and FAE-I (FAEB) in respect of feruloylated pectin oligosaccharides, as previously described [18]. FAEA was only able to release galactose-linked ferulic acid from pectin oligosaccharides, whereas FAE-I could release both arabinose- and galactose-linked ferulic acid, but had a higher activity towards arabinose-linked ferulic acid. Towards pre-treated WIP the activity of FAEB is much lower that the activity of FAEA. Surprisingly, addition of FAEB to incubations with FAEA significantly decreased the amount of ferulic acid released. The reason for this is not clear. Ferulic acid is present as individual terminal groups attached to the side chains of xylan. Removal of a part of the ferulic acid groups by a second esterase should not affect the accessibility of the remaining groups.

The present results demonstrate that the two feruloyl esterases identified in *A. niger* (FAEA and FAEB) are both involved in the degradation of xylan and pectin, but have different preferences for these polysaccharides. FAEA is most active towards xylan, which is in agreement with XLNR regulation of *faeA*. FAEB has the highest activity against pectin. However, since transcription of both *faeA* and *faeB* is induced by ferulic acid, the product of the enzymes, both enzymes will be present during growth of *A. niger* on xylan or pectin and will contribute to the release of ferulic acid from these polysaccharides.

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