A non-modular type B feruloyl esterase from *Neurospora crassa* exhibits concentration-dependent substrate inhibition

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Feruloyl esterases, a subclass of the carboxylic acid esterases (EC 3.1.1.1), are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in the plant cell wall. The enzymes have been classified as type A or type B, based on their substrate specificity for aromatic moieties. We show that *Neurospora crassa* has the ability to produce multiple ferulic acid esterase activities depending upon the length of fermentation with either sugar beet pulp or wheat bran substrates. A gene identified on the basis of its expression on sugar beet pulp has been cloned and overexpressed in *Pichia pastoris*. The gene encodes a single-domain ferulic acid esterase, which represents the first report of a non-modular type B enzyme (*fae-1* gene; GenBank accession no. AJ29 3029). The purified recombinant protein has been shown to exhibit concentration-dependent substrate inhibition (K_m 0.048 mM, K_i 2.5 mM and V_{max} 8.2 units/mg against methyl 3,4-dihydroxycinnamate). The kinetic behaviour of the non-modular enzyme is discussed in terms of the diversity in the roles of the feruloyl esterases in the mobilization of plant cell wall materials and their respective modes of action.

Key words: enzyme, fungus, *Neurospora*, plant cell wall, protein expression, substrate inhibition.

INTRODUCTION

The plant cell wall is a complex architecture of polysaccharides. For the complete hydrolysis of these polysaccharides, micro-organisms require a battery of enzymes. Many plant cell walls contain phenolic acid residues that are ester-linked to the polysaccharide network. In grasses these phenolic compounds are mainly found esterified to arabinoxylans (5-*O*-feruloyl group). In dicotyledons, such as spinach and sugar beet, ferulic acid is esterified to the O-2 or O-3 position of arabinose and to O-6 position of galactose residues in pectin [1,2]. Cross-linking of ferulic acids to cell wall components influences the properties of the cell wall, such as extensibility, plasticity and digestibility. Feruloyl esterases (FAEs; also known as ferulic acid esterases, cinnamoyl esterases and cinnamic acid hydrolases; EC 3.1.1.73), a subclass of the carboxylic acid esterases (EC 3.1.1.1), are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in the plant cell wall [3]. The esterases act to enable and facilitate the access of hydrolases to the backbone wall polymers. Most of the FAEs have been shown to act synergistically with xylanases, cellulases and pectinases to break down complex plant cell wall carbohydrates [4,5]. Several members of the enzyme group have been purified and characterized from aerobic and anaerobic microbes that utilize plant cell wall carbohydrates [6–20]. These enzymes have been classified as either type A or type B, depending on their substrate specificity for aromatic moieties. The enzymes show a preference for the phenolic linkage to the primary sugar, and vary in their ability to release dehydrodiferulic acids from esterified substrates. Regarding specificity against synthetic substrates, type A FAEs are active against methyl ferulate (MFA), methyl sinapate (MSA) and methyl *p*-coumarate (M*p*CA), but not methyl caffeate (MCA), whereas type B are active against MCA, MFA and M*p*CA, but not MSA. Only type A FAEs are able to hydrolyse synthetic ferulate dehydrodimers and to release dimers from agro-industrial materials, as they have a preference for more hydrophobic substrates with bulky substituents on the benzene ring [21–23].

Esterases are novel enzymes with considerable potential for agri-food processing applications. For example, phenolic acids derived from plant cell walls have long been used as food preservatives to inhibit microbial growth. *Aspergillus niger* has been shown to transform ferulic acid into vanillic acid and, similarly, *Pycnopotus cinnabarinus* converts ferulic acid into vanillin, an essential flavour in the food industry. Ferulic acid is also an effective natural antioxidant with potential applications in the pharmaceutical and food industries [3,24–28]. There is therefore considerable scope to utilize FAEs to produce and exploit natural products that can be extracted from otherwise waste agri-food material.

The two major FAEs of *Aspergillus niger* have been the focus of several studies over the last decade. However, in recent years, our knowledge of the FAE family has expanded, with reports of new enzyme activities, the characterization of gene sequences and the first crystal structure [15–19,29]. Molecular analysis of FAE genes and their predicted protein sequences has revealed that many of these enzymes are modular, comprising a catalytic domain translationally fused to a non-catalytic cellulose-binding

Abbreviations used: Ara_2F, [2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1,5)-L-arabinofuranose; CBD, cellulose-binding domain; FAE, feruloyl esterase (ferulic acid esterase; cinnamoyl esterase; cinnamic acid hydrolase: EC 3.1.1.72); Fae-1, feruloyl esterase from *Neurospora crassa*; MCA, methyl caffeate (methyl 3,4-dihydroxycinnamate); MFA, methyl ferulate (methyl 4-hydroxy-3-methoxycinnamate); M*p*CA, methyl *p*-coumarate (methyl 4-hydroxycinnamate); MSA, methyl sinapate (methyl 3,5-dimethoxy-4-hydroxycinnamate); ORF, open reading frame; SBP, sugar beet pulp; WB, wheat bran.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number AJ293029.

domain (CBD) [15,16,19,30], or are produced as monomeric enzyme units pre-designated to be assembled into longer cohesive units, such as the cellulosome of *Clostridium* [31].

In the present paper, we report the ability of the filamentous fungus *Neurospora crassa* to produce alternative FAE activities in response to the availability of plant cell wall carbohydrates, and the identification of a gene (*fae-1*) specifically induced on sugar beet pulp (SBP) that encodes the first example of a non-modular type B FAE (Fae-1) to be reported. The *N. crassa* gene has been overexpressed in *Pichia pastoris* [32] and the purified recombinant protein was shown to exhibit concentration-dependent substrate inhibition. The kinetic behaviour of this non-modular enzyme is discussed in terms of the structural diversity of the FAEs and their respective catalytic roles in the mobilization of plant cell wall materials.

EXPERIMENTAL

Materials

The methyl esters of ferulic acid, caffeic acid, *p*-coumaric acid and sinapic acid were obtained from Apin Chemicals Ltd (Abingdon, Oxon, U.K.). The corresponding free acids, chlorogenic acid, naphthyl acetate and all other standard chemicals were obtained from Sigma-Aldrich Chemical Co. (Poole, Dorset, U.K.).

Strains, vectors and media

Escherichia coli TOP10 (Invitrogen) was used as the bacterial host for DNA manipulations with the pCR®2.1-TOPO® cloning vector. *E. coli* TAP90 was used as the host for lambda [33] and *E. coli* TG1*rec*F$^-$ was used for propagation of plasmids and as a recipient in transformation. The vector pEMBL18 [34] was used for subcloning the putative *fae-1* gene. *Pichia pastoris* (*his4*) GS115 was used to produce recombinant Fae-1 with the expression vector pPIC3.5K (Invitrogen). *Neurospora crassa* wild-type ST *A* (74 *A*) was maintained on minimal Vogel's agar (1.5 %, w/v) plus 2 % (w/v) sucrose [35]. Liquid cultures were grown in minimal Vogel's supplemented with 2 % (w/v) carbon sources [sucrose, SBP or wheat bran (WB)].

Detection of esterase activities in *Neurospora crassa* culture supernatants

N. crassa mycelia were grown in 100 ml of Vogel's sucrose medium for 2 days at 30 °C on a flat-bed shaker (200 rev./min). Cultures were vacuum-filtered through a sterile Buchner funnel, washed with distilled water and transferred aseptically to 500 ml of Vogel's supplemented with 2 % (w/v) SBP or 2 % (w/v) WB. Samples of 5 ml of supernatant were removed every 24 h for a period of 9 days. FAE activities in supernatant samples were assayed spectrophotometrically at 335 nm by the method of Ralet and co-workers [1]. Activity was measured against MFA (methyl 4-hydroxy-3-methoxycinnamate), MCA (methyl 3,4-dihydroxycinnamate), MSA (methyl 3,5-dimethoxy-4-hydroxycinnamate) and M*p*CA (methyl 4-hydroxycinnamate) at 0.1 mM final concentration in 100 mM Mops buffer (pH 6.0), using 20 μl of culture supernatant. Protein concentration was determined by the Bradford method [36] using the Coomassie Protein Assay Reagent from Pierce.

Genomic DNA extraction

DNA was extracted in duplicate experiments using the ethanolic perchlorate method of Stevens and Metzenberg [37], after growth of *Neurospora crassa* on minimal Vogel's sucrose medium.

RNA extraction

Neurospora crassa ST *A* (74 *A*) was grown at 30 °C in a shaking incubator (200 rev./min) in 100 ml of minimal Vogel's supplemented with 2 % (w/v) sucrose. After 48 h, cultures were vacuum filtered through a sterile Buchner funnel washed with sterile distilled water and transferred aseptically to 100 ml of Vogel's supplemented with the selected carbon source (SBP or WB). After 3–4 days of growth, cultures were filtered through muslin and filtrates (pre-digested medium) were sterilized by autoclaving before inoculation with a fresh mycelial mat. Mycelia were incubated for 6 h to induce FAE expression before harvesting. Total RNAs were extracted in two independent experiments from mycelia following growth on pre-digest supplemented media [Vogel's plus 2 % (w/v) SBP or WB] or minimal Vogel's plus 2 % (w/v) sucrose, using the method described by Sokolovsky et al. [38]. Poly-adenylated RNAs were selected from total RNAs using oligo(dT) chromatography.

RNAs were extracted every 48 h in independent duplicate experiments during the direct fermentation of SBP and WB by *N. crassa*, as described above for the detection of esterase activities.

Screening of the genomic library

Plaques of the *N. crassa* genomic lambda library λJ1-74A [39] were lifted in duplicate and probed with cDNA synthesized from poly(A)$^+$ RNA extracted from mycelia cultured on either sucrose or SBP. Superscript™ (Gibco BRL) was employed to reverse transcribe poly(A$^+$) mRNA using [α-^{32}P]dCTP ($\sim$ 3000 Ci $\cdot$ mmol^{-1}; Amersham Pharmacia Biotech) to label the cDNAs. The cDNAs were precipitated with 2.5 M ammonium acetate and propan-2-ol, resuspended in water and boiled before use as a hybridization probe against plaque lifts.

Southern and Northern blots

*Eco*RI restriction digests of *N. crassa* genomic DNA were electrophoresed in 0.8 % (w/v) agarose gels and blotted by capillary transfer on to Hybond-N$^+$ (Amersham Pharmacia Biotech) nylon membranes before fixation with UV light. The blots were probed with a DNA fragment specific for the *fae-1* gene, labelled using the PCR DIG-labelling system (Boehringer Mannheim) according to the manufacturer's instructions. Colorimetric detection was performed in the presence of Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates, according to the manufacturer's instructions (Boehringer Mannheim).

RNAs were electrophoresed in a formaldehyde denaturing agarose gel according to the method described by Sambrook and colleagues [40] with 1 $\times$ Mops, blotted by capillary transfer on to a Hybond-N$^+$ nylon membrane and hybridized to a labelled DNA probe specific for the *fae-1* gene. Colorimetric detection was performed as described above.

Overlapping PCR

Overlapping-PCR amplification was used in order to amplify cDNA of the *Neurospora crassa* FAE gene (*fae-1*) from genomic DNA. Two specific primers were designed on either side of a putative intron (ssN1F and I1R primers 5′ of the intron, and I1F and C1R primers 3′ of the intron) (Table 1). The reverse primer (I1R) has a tail, the sequence of which complements the forward primer (I1F), and vice versa. Two PCR reactions were performed to amplify separately the sequences upstream and downstream of the intron using the primer combinations ssN1F/I1R and I1F/C1R. DNA amplification was carried out through 30 cycles

Table 1 Names and sequences of oligonucleotides used to amplify the *N. crassa fae-1* cDNA

Specific restriction sites were incorporated into the PCR primer sequences: *Bam*HI at the 5′ end of the ssN1F primer and *Bam*HI–*Not*I at the 5′ end of the C1R primer (in bold). †indicates the stop codon.

Primer name	Primer sequences with their translations											
ssN1F	5′-AAAA**GGATCC**AT	ATG	TTG	CCC	AGA	ACA	TTG	C-3′				
		M	L	P	R	T	L	L				
C1R	5′-AAAA**GGATCCGCGGCCG**	**C**GA	CAT	CTA	GTT	GAT	CAA	CCC-3′				
				†	N	I	L	G				
I1F	5′-C	GCC	ATT	ATT	GTG	GCT	CTC	CAC	GGC	TGT	GGC	GG-3′
		A	I	I	V	A	L	H	G	C	G	
I1R	5′-GCC	ACA	GCC	GTG	GAG	AGC	CAC	AAT	AAT	GGC	GGG	C-3′
	G	C	G	H	L	A	V	I	I	A	P	

of denaturation (30 s at 94 °C), annealing (1 min at 60 °C) and extension (1 min at 72 °C). Amplified fragments were gel-purified and equal quantities of each were mixed together. The DNA mixture was denatured at 100 °C for 10 min and the reaction was allowed to cool down slowly at room temperature. dNTPs (0.5 μl, 25 mM) (Promega), 10 × Klenow buffer (2 μl), Klenow enzyme (1 μl; 2 units/μl) (Boehringer Mannheim) and water were added to make the reaction up to 20 μl (final volume). The reaction mixture was incubated at 37 °C overnight to allow synthesis of double-stranded DNA. DNA was precipitated with 0.1 vol. of 3 M sodium acetate, pH 5.2, and 2 vol. of 100 % (v/v) ethanol, recovered in water and amplified by PCR with primers designed on the 5′ and 3′ ends of the *fae-1* cDNA (ssN1F and C1R; Table 1). DNA amplification was carried out through 30 cycles of denaturation (30 s at 94 °C), annealing (1 min at 60 °C), and extension (1 min at 72 °C). The resulting PCR product was cloned into the pCR®2.1-TOPO® vector (TOPO TA Cloning® kit from Invitrogen) and sequenced using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser.

Construction of expression vector

The cDNA fragment (879 bp) containing the *N. crassa fae-1* native signal sequence was isolated from the cloning vector by restriction endonuclease digestion with *Bam*HI–*Not*I and ligated into *Bam*HI–*Not*I-digested pPIC3.5K expression vector.

Transformation of *P. pastoris* and selection of secreting transformants

The transformation of *Pichia pastoris* GS115 was performed using the electroporation method [41]. For transformation, 1–5 μg of vector with or without insert (pPIC3.5K/*fae-1* and pPIC3.5K respectively) was linearized with *Dra*I restriction enzyme to allow gene replacement at the *Pichia pastoris AOX1* gene. Transformants were selected by their ability to grow at 30 °C on histidine-deficient regeneration medium (RDB: Regeneration Dextrose Base; Invitrogen). To assess the His^+ transformants for methanol utilization (Mut) phenotype, colonies were picked and patched on to minimal methanol plates and minimal dextrose plates (Invitrogen) in a regular pattern. After 5 days at 30 °C, normal growth on minimal dextrose plates and slow growth on minimal methanol plates is indicative of the Mut^s (Mut-sensitive) phenotype. Mut^s colonies were used to inoculate 10 ml of buffered complex glycerol medium, pH 6.0 (Invitrogen) and incubated at 30 °C with vigorous shaking (> 200 rev./min) until $D_{600} = 10–20$ (2 days). Cells were harvested by centrifugation at 4000 ***g*** for 10 min at room temperature and resuspended in 2 ml of buffered complex methanol medium, pH 6.0 (Invitrogen) and incubated at 30 °C with vigorous shaking for 3 days. Methanol (100 %) was added to a final concentration of 0.5 % methanol every 24 h to maintain induction. After 3 days of induction, cells were then pelleted by centrifugation at 13000 ***g*** for 3 min at room temperature and supernatants were analysed with SDS/PAGE [12 % (w/v) bisacrylamide] for protein expression [40]. To confirm that the positive clones were FAE-producing transformants, culture supernatants were assayed for activity against MCA and MFA using reverse-phase HPLC [6].

The N-terminal amino acid sequence of the recombinant protein was determined after separation by SDS/PAGE (12 % bisacrylamide) and Western transfer on to a PVDF membrane (Boehringer Mannheim). The membrane was stained with Coomassie Blue R250 and the candidate band was extracted followed by N-terminal amino acid sequencing using a Sequencer Model 473A (Applied Biosystems).

Purification of recombinant *N. crassa* Fae-1 expressed in *P. pastoris*

Large-scale culture was performed in 2-litre Erlenmeyer flasks. Cells were grown in 500 ml of buffered complex glycerol medium, pH 6.0, at 30 °C for 48 h (D_{600} 20–25). The recovered cells were resuspended in 200 ml of buffered complex methanol medium, pH 6.0, and grown for a further 5 days at 30 °C (200 rev./min). The culture was centrifuged at 13000 ***g*** for 15 min at room temperature and 196 ml of supernatant was recovered. Solid ammonium sulphate was added to 1 M and clarified by centrifugation at 10000 ***g*** for 15 min at 4 °C. Aliquots of 30 ml were loaded on to a butyl-Sepharose 4 Fast Flow hydrophobic interaction chromatography column (2.6 cm × 10 cm) membrane (Amersham Pharmacia Biotech). Unbound material was eluted (5 ml · min^{-1}) with 50 mM sodium phosphate buffer, pH 7.0, 1.0 M ammonium sulphate (buffer A), followed by elution of the bound proteins with a gradient of buffer A/buffer B (50 mM sodium phosphate buffer, pH 7.0). Fractions were collected and assayed for activity against MCA using the spectrophotometric method [1]. Active fractions were pooled and desalted through a NAP-5 column membrane (Amersham Pharmacia Biotech) into water and a 240–400 nm absorbance spectrum was recorded. The molar absorption coefficient for Fae-1 was determined from its amino acid content, and this value (51790 $M^{-1} \cdot cm^{-1}$) was used to determine the concentration of the sample, using the Beer–Lambert law.

Fae-1 was concentrated 13-fold through a 10000 Da cut-off ultrafiltration membrane (Amicon), and 3 ml of sample was dialysed against 5 litres of MilliQ water using a Slide-A-Lyzer

cassette (10000 Da cut-off; Pierce Chemical Co.). Electrospray ionization-MS of Fae-1 was performed using a Quattro II instrument (Micromass, Manchester, U.K.).

Enzyme assays

Assays for FAE activity were performed by incubating methyl esterified substrates (MFA, MCA, MSA or M*p*CA) [6] or the feruloylated oligosaccharide Ara_2F {[2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1,5)-L-arabinofuranose} [42], in a final volume of 0.5 ml for 15 min in 100 mM Mops buffer, pH 6.0, at 37 °C. Agro-industrial waste such as SBP, WB or brewer's (spent) grain (10 mg) was incubated in the presence of FAE for 3 h in 100 mM Mops buffer, pH 6.0, at 37 °C. Reactions were terminated by the addition of acetic acid (0.2 ml), and 0.2 ml samples were assayed for phenolic acids by reverse-phase HPLC [11]. One unit of activity is defined as the amount of enzyme (mg) or ml of culture supernatant releasing 1 μmol of free ferulic acid per min under the defined conditions.

Kinetic constants (k_{cat}, K_m, V_{max}) were calculated from initial-rate data using the Michaelis-Menten equation: $v = (V_{max}[S])/(K_m + [S])$. Due to substrate inhibition, the kinetic constants were determined at low values of substrate (from 0.02 mM to 0.4 mM), which is permissible when K_i is large compared with K_m. The K_i was estimated from initial-rate data at various substrate concentrations (from 0.02 mM to 3 mM), using the equation with respect to a substrate inhibition: $v = (V_{max}[S])/\{K_m + [S] + ([S]^2/K_i)\}$. Acetylesterase activity was determined using α-naphthyl acetate (0–2.5 mM) in a 1 ml reaction volume in 100 mM sodium phosphate buffer, pH 6.0, at 37 °C, by following the increase in absorbance at 235 nm over 5 min. Acetylesterase activity was calculated from the initial rate of change of A_{235} using a molar absorption coefficient for α-naphthyl acetate of 24000 $M^{-1} \cdot cm^{-1}$ [43]. One unit of activity is defined as the amount of enzyme forming 1 μmol of product per min at pH 6.0 and 37 °C.

Temperature–activity and pH–activity profiles

To determine the pH optimum, recombinant Fae-1 was incubated for 15 min at 37 °C with MFA (0.1 mM final concentration in a 500 μl reaction) in McIlvaine's buffer titrated to a pH within the range 3.0–7.5, and in phosphate buffer titrated to a pH within the range 6.0–8.0. To determine the temperature optimum, recombinant Fae-1 was incubated for 15 min with MFA (0.1 mM final concentration in a 500 μl reaction) in 100 mM Mops, pH 6.0, at temperatures ranging from 20 °C to 80 °C. The temperature stability of the recombinant Fae-1 was determined at 60 °C. The enzyme was incubated for up to 60 min at 60 °C, and aliquots were removed every 10 min and assayed for activity against MFA (0.1 mM final concentration in a 500 μl reaction), for 15 min in 100 mM Mops, pH 6.0, at 37 °C. The reactions were stopped by the addition of 0.2 ml of acetic acid. Controls containing the reaction mixture plus acetic acid were incubated to eliminate interference. Samples and blanks were centrifuged at 13000 ***g*** for 5 min prior to HPLC analysis of the released ferulic acid.

RESULTS

FAE activity in the culture supernatant

Neurospora crassa ST *A* (74 *A*) was grown in minimal Vogel's medium supplemented with 2% (w/v) sucrose for 24 h, then transferred to Vogel's containing 2% (w/v) SBP or WB for 9 days. Culture supernatant samples were removed every 24 h and assayed for FAE activities against the methyl esters of caffeic (MCA), sinapic (MSA), ferulic (MFA) and *p*-coumaric (M*p*CA) acids (Figure 1). FAE activity was detected following growth on SBP and WB. The substrate activity profile, however, suggested the presence of two FAE activities. Samples removed in the first 3 days of growth on WB showed FAE activity against MFA, MSA and M*p*CA, but not MCA, whereas samples removed during the first 3 days of growth on SBP showed FAE activity mainly against MFA, MCA and M*p*CA, but not MSA. As FAEs are discriminated based on their ability to utilize MSA and diferulates [12,16,19–21,23], we can identify type A and type B FAE activities from *N. crassa* grown on WB or SBP. In addition, at days 7–8 and 8–9 of growth on SBP and WB respectively, FAE activity was detected against all four synthetic monomeric substrates. These data confirm that *N. crassa* is able to induce and adapt the production of FAEs according to the carbon source present in the culture medium, and as a function of growth time. Therefore *N. crassa* is a potential source of new FAEs.

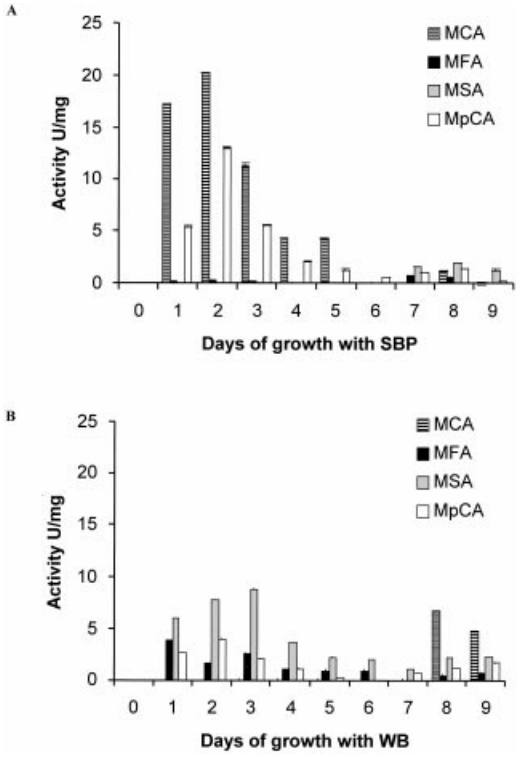

Figure 1 FAE activities detected in culture supernatants of *Neurospora crassa*

FAE activity (units/mg) was measured in culture supernatants of *Neurospora crassa* grown with SBP (**A**) or WB (**B**). FAE activities were measured with MCA, MFA, MSA and M*p*CA as a function of incubation time (0–9 days). Day 0 corresponds to the supernatant sample analysed after 24 h of growth with Vogel's medium plus 2% (w/v) sucrose.

Genomic library screen

Labelled cDNA reverse-transcribed from mRNA extracted from mycelia of *Neurospora crassa* grown on either sucrose or SBP was used to probe duplicate plaque lifts of a genomic library in order to identify genes differentially expressed on SBP, in particular the genes encoding FAEs [44]. The library screen revealed several positives clones. Restriction digests of the cloned DNAs were transferred to a Southern blot and hybridized with

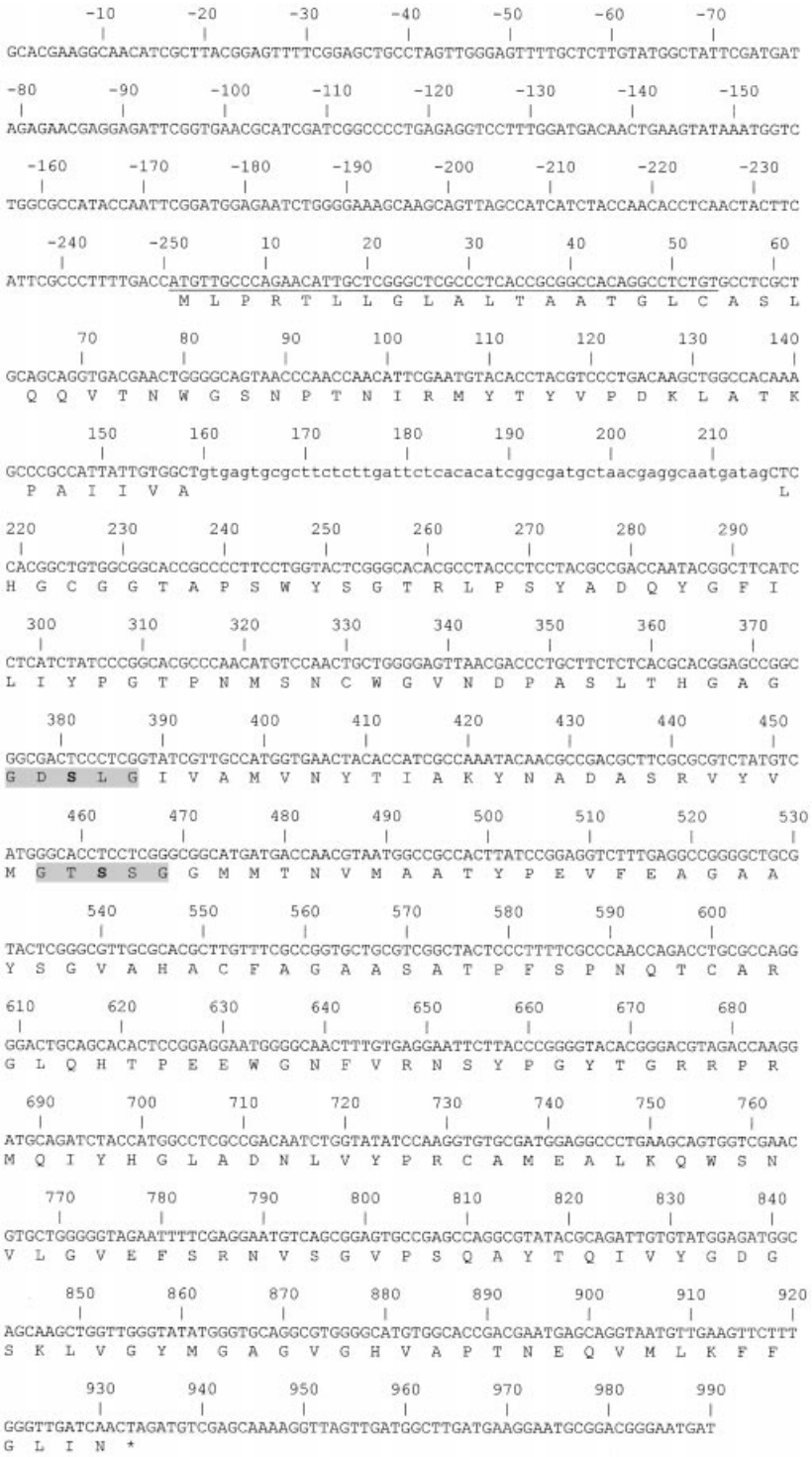

Figure 2 Nucleotide and deduced amino acid sequences of the *fae-1* gene from *Neurospora crassa*

The signal peptide is underlined. The 56 bp intron is shown in lower case. The two sites containing the consensus motif (G-X-S-X-G) for the active-site serine are shown by grey boxes, with the putative active-serine in bold. The stop codon is indicated by *.

the reverse-transcribed cDNA to identify the fragments containing the genes of interest, which were in turn subcloned into the pEMBL18 vector and sequenced. The translation of one of these clones showed 50% identity with a type B FAE from *Pe. funiculosum* [19] and 45% identity with an acetylesterase reported from *Aspergillus awamori* [45]. Comparative analysis of the sequence indicated that a reading frame of 876 bp was present, but interrupted by a single intron. A cDNA for this gene was constructed by overlapping PCR from *N. crassa* genomic DNA. Specific primers were designed based on the genomic sequence in

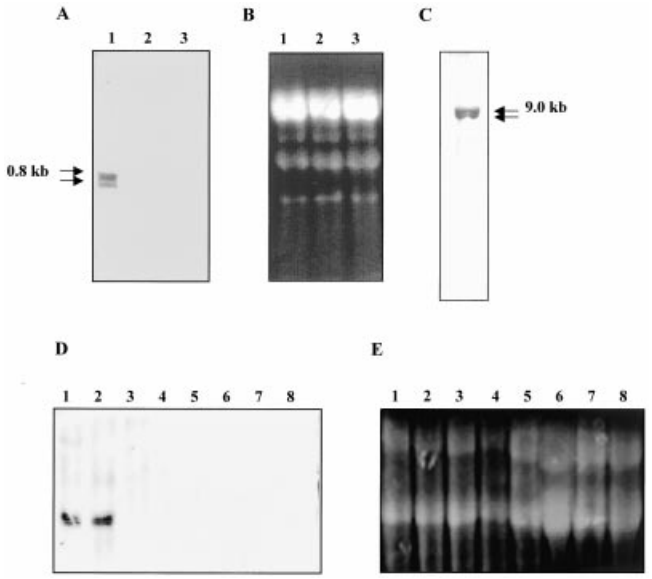

Figure 3 Northern and Southern blots

(**A**) Northern blot of RNAs extracted from *N. crassa* following 6 h of growth on predigested selective SBP (lane 1) or WB (lane 2) media, and following 48 h of growth on minimal Vogel's medium plus 2% (w/v) sucrose (lane 3). The membrane was hybridized with the *N. crassa* genomic clone coding for a type B FAE. (**B**) Ethidium bromide stain of the membrane shown in (**A**), demonstrating equal loading of the RNA samples. (**C**) Southern blot of *N. crassa* genomic DNA digested with the restriction enzyme *Eco*RI, known to cut once in the genomic clone. The relative molecular sizes of the main hybridization products are recorded alongside the blots. (**D**) Northern blot of RNAs extracted from *N. crassa* following 2, 4, 6 and 8 days of fermentation with 2% (w/v) SBP (lanes 1–4 respectively) or 2% (w/v) WB (lanes 5–8 respectively). (**E**) Ethidium bromide stain of the membrane shown in (**D**) demonstrating equal loading of the RNA samples. Southern and Northern blots were repeated using independent DNA and RNA preparations, as indicated in Experimental section.

order to independently amplify the sequences upstream and downstream of the intron that were joined as overlapping PCR fragments. After cloning in the pCR®2.1-TOPO® vector, the nucleotide sequence resulting from the overlapping-PCR products was sequenced. The open reading frame (ORF) encoded a protein of 292 amino acids, including an 18-amino-acid signal peptide (Figure 2). The calculated average mass of the mature protein is 29286 Da, and the theoretical pI is 8.26. Inspection of the protein sequence reveals the presence of two motifs characteristic of the serine esterase family (Gly-Xaa-Ser-Xaa-Gly) [19,46,47]. The first motif (Gly-Asp-Ser-Leu-Gly) is centred on serine-91, but is not conserved within the *Pe. funiculosum* cinnamoyl esterase sequence or the acetylesterase sequence from *Aspergillus awamori*. The second motif (Gly-Thr-Ser-Ser-Gly), centred on serine-118, is conserved in the *Pe. funiculosum* cinnamoyl esterase and the *Aspergillus awamori* acetylesterase sequences, as well as in other members of the serine esterase family [19]. Serine-118 is therefore a candidate for a component of the catalytic triad featured in all the family members. The translated sequence, however, does not feature a recognizable CBD, as has been observed with the type B FAE reported from *Pe. funiculosum* [19].

Northern and Southern blots

To confirm the differential expression of the putative FAE gene, RNAs were extracted after growth on SBP, WB or sucrose and analysed by Northern blot (Figures 3A and 3B). Two transcripts specific to growth on SBP were detected by hybridization with a DNA probe. These data are consistent with the expression profile of type B FAEs observed in other fungal species [12,19,20]. In order to determine whether the two transcripts arise from two independent genes or are the product of a single gene, a Southern blot of genomic DNA was performed (Figure 3C). *N. crassa* genomic DNA was digested with *Eco*RI (known to cut once in the genomic sequence) and hybridized with the genomic sequence to produce two bands, as might be anticipated for a single-copy gene. The transcripts are therefore likely to be products of the same gene. The mRNA expression of the putative FAE gene was also analysed by Northern blot at 48 h intervals over the period of fermentation with raw particulate SBP and WB substrates that correspond to the detection of the esterase activities in the culture supernatants (Figures 3D and 3E). The putative type B FAE mRNA was expressed during the early stages of growth on SBP, and its expression decreased later in the fermentation, in parallel with the type B enzyme activity (Figure 1A). During growth on WB, expression of the mRNA was barely detectable throughout the course of the fermentation (Figure 1B).

Overexpression in *Pichia pastoris*

In order to confirm the function of the putative gene product and to provide a ready source of the enzyme for study, the *N. crassa* cDNA was overexpressed in *Pichia pastoris*. The *P. pastoris* system was selected as it had already been proven to efficiently secrete active FAE [32]. The cDNA of the FAE from *N. crassa*, containing the native signal sequence, was cloned into the expression vector pPIC3.5K under the control of the alcohol oxidase 1 gene promoter (*AOX1*). The resulting expression plasmid (pPIC3.5K/*fae-1*) and the parent vector were linearized with *Dra*I restriction enzyme, allowing gene replacement at *AOX1*, and used to transform *P. pastoris*. Both transformations generated hundreds of His^+ transformants. The His^+ transformants were scored for their ability to grow on minimal methanol media. A total of 13 Mut^s pPIC3.5K/*fae-1* transformants were screened for expression in small-scale cultures along with two colonies transformed with the parental vector as a control for background protein secretion levels. Culture supernatants were analysed by SDS/PAGE for secreted protein products. Five pPIC3.5K/*fae-1* transformants produced a major secreted protein band of approx. 35 kDa. No protein was detected with the vector controls. To confirm that these cultures were FAE-producing transformants, culture supernatants were assayed for activity against MCA, MFA and MSA. The recombinant protein was found to be active as a FAE, and showed the characteristics of a type B FAE, in that is was inactive against MSA. We therefore have named the *N. crassa* protein Fae-1, as the product of the *fae-1* gene (GenBank® accession number AJ293029). The most active clone (clone 10) was retained for large-scale expression in order to purify and characterize the recombinant enzyme (Figure 4A).

The recombinant Fae-1 was transferred by electroblotting to a PVDF membrane for protein sequence analysis. The first 10 amino acids of the N-terminus of the expressed protein were determined to be Ala-Ser-Leu-Gln-Gln-Val-Thr-Asn-Trp-Gly. This sequence is identical to the sequence predicted for the mature Fae-1 product, indicating that *P. pastoris* was able to efficiently process the *N. crassa* native signal sequence. Four N-linked glycosylation sites can be predicted from the Fae-1 sequence. Glycosylation of the *P. pastoris* product was confirmed by electrospray ionization-MS, which explained the mass difference of 6 kDa between the calculated average mass of the mature protein and that estimated from SDS/PAGE of the

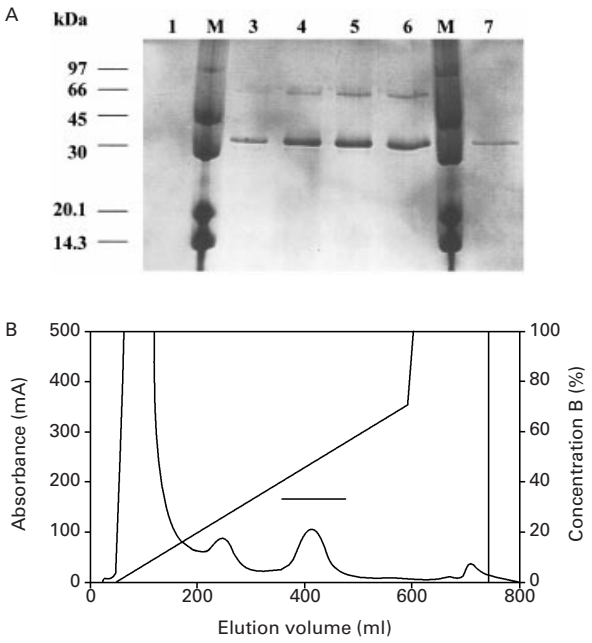

Figure 4 Purification of recombinant Fae-1

(**A**) SDS/PAGE on 12% (w/v) acrylamide. M, standard protein molecular mass markers. Lane 1, negative control; lanes 3–6, samples of culture supernatant removed daily (days 2–5 respectively) from a large-scale culture of *P. pastoris* transformant expressing recombinant Fae-1 (clone 10); lane 7, recombinant Fae-1 following purification. (**B**) Elution profile of recombinant *N. crassa* Fae-1 on a butyl-Sepharose Fast Flow 4 hydrophobic interaction chromatography column. The fractions active against MFA are marked with a horizontal line, eluting around 400 ml.

recombinant Fae-1. Six main peaks were apparent on MS, ranging between 34716 and 35523 Da. The main mass was calculated to be 35040 Da. The other peaks correspond to different levels of glycosylation of the enzyme, where each peak corresponds to the addition of one sugar unit. The unglycosylated protein has a calculated molecular mass of 29286 Da, and thus the main product can be estimated to contain 32 mannose residues.

Purification and characterization of recombinant Fae-1

The pPIC3.5K/*fae-1 Pichia* transformant (clone 10) was grown for 5 days in buffered complex methanol medium at 30 °C before harvesting. Aliquots of 30 ml of the culture supernatant were fractionated on a butyl-Sepharose Fast Flow 4 hydrophobic interaction chromatographic column. A single peak of activity against MCA was eluted (Figure 4B), and active fractions were pooled. The enzyme yield after purification was determined to be 73 %. The protein concentration of the recombinant Fae-1 was calculated from the absorbance of the desalted sample at 280 nm. The molar absorption coefficient of Fae-1 (51790 $M^{-1} \cdot cm^{-1}$) was determined from the amino acid sequence and the concentration of the purified esterase, determined to be 0.034 mg of protein/ml of sample. The yield of secreted recombinant Fae-1 protein from methanol-induced shake-flask culture was 210 $mg \cdot l^{-1}$ after 5 days.

Table 2 presents the kinetic constants (V_{max}, k_{cat}, K_m and k_{cat}/K_m) calculated from the initial-rate activity of recombinant *N. crassa* Fae-1 against hydroxycinnamic acid esters. Substrate concentrations between 0.02 mM and 0.4 mM were employed to estimate the kinetic constants from the Michaelis–Menten equation. As expected of a type B FAE, Fae-1 showed a high catalytic efficiency for the hydrolysis of MCA and M*p*CA, but no activity was detected against MSA. MCA and M*p*CA substrates were turned over at higher rates, but the catalytic efficiency with MFA was lower. The low rate recorded for the Ara_2F substrate is consistent with a requirement for a longer feruloylated oligosaccharide to produce optimal hydrolysis. The catalytic efficiency of *N. crassa* Fae-1 for the hydrolysis of Ara_2F (12.87 $mM^{-1} \cdot s^{-1}$) is 112-fold greater than that recorded for the

Table 2 Substrate specificity of *Neurospora crassa* Fae-1 with synthetic (A) and natural (B) substrates

Nd, not detected. FAEA is type A FAE from *A. niger*. Released metabolites are expressed as a percentage of total alkali-extractable phenolic acids, after 3 h of incubation with 100 m-units of Fae-1.

(A)

Substrate	V_{max} (μmol · min^{-1} · mg^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Methyl esters				
MFA	8.97	5.24	0.25	21
M*p*CA	20.87	12.19	0.021	580
MCA	8.20	4.80	0.02	100
MSA	Nd	Nd	Nd	Nd
Feryloylated oligosaccharides				
Ara_2F	10.34	6.04	0.46	13
Acetylated substrates				
α-Naphthyl acetate	6.65 (Fae-1)/11.64 (FAEA)	3.44 (Fae-1)/5.86 (FAEA)	1.13 (Fae-1)/5.45 (FAEA)	3 (Fae-1)/1 (FAEA)
Chlorogenated substrates				
Chorogenic acid	9.14	5.34	0.18	29

(B)

	Released metabolite (%)		
Substrate	Ferulic acid	*p*-Coumaric acid	Dimers
WB	1.2	0.7	Nd
Spent grain	2	0.7	Nd
SBP	2.5	0	Nd

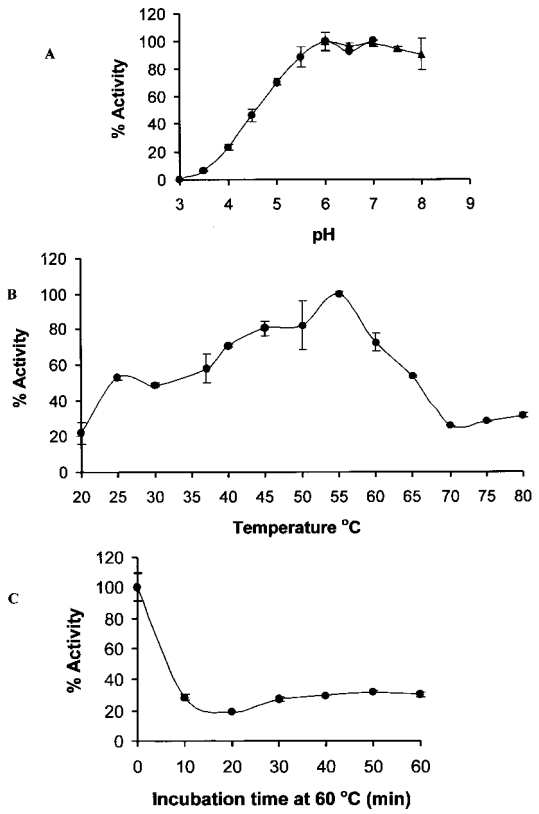

Figure 5 Temperature– and pH–activity profiles of recombinant Fae-1

(**A**) pH profile of recombinant Fae-1. Activities were determined against MFA (0.1 mM) over the pH ranges 3.0–7.0 using McIlvaine's buffer (●) and pH 6.0–8.0 using a phosphate buffer (▲). (**B**) Temperature profile of recombinant Fae-1 determined with MFA (0.1 mM) between 20 and 80 °C (5 °C increments). (**C**) Temperature stability of recombinant Fae-1. The temperature stability was estimated at 60 °C over 60 min. Samples were removed every 10 min and assayed for residual activity at 37 °C against MFA (0.1 mM).

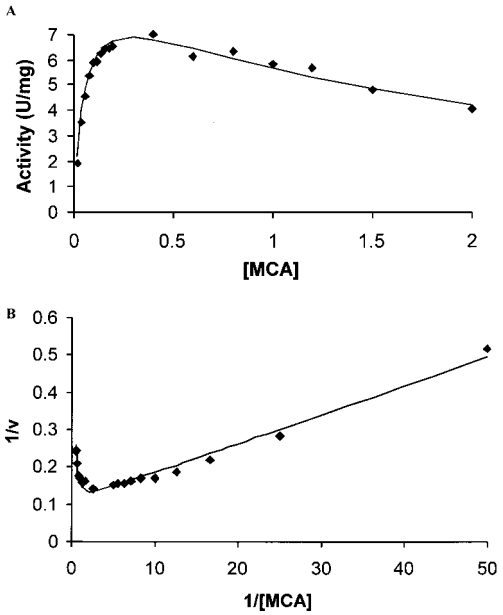

Figure 6 Fae-1 activity profile with MCA using a range of substrate concentrations between 0.02 and 2 mM

(**A**) Substrate concentration against activity (units/mg). (**B**) Double-reciprocal plot of substrate concentration against activity (units/ml).

Table 3 Kinetic constants for Fae-1 determined against synthetic methyl esters

Nd, not detected.

Substrate	K_m (mM)	K_i (mM)	χ^2
MFA	0.25	2.82	0.067
M*p*CA	0.021	2	0.082
MCA	0.048	2.5	0.003
MSA	Nd	Nd	Nd

A. niger cinnamic acid esterase CinnAE or the *Pir. equi* cinnamoyl ester hydrolase CEH (EstA') [5,16,42]. However, the rate of turnover of Ara_2F by Fae-1 is 40-fold lower than the catalytic efficiency of *Pe. funiculosum* FAEB (530 $mM^{-1} \cdot s^{-1}$) [19].

Fae-1 was also tested for its ability to release ferulic acid and *p*-coumaric acid from cereal-derived material. Fae-1 was shown to release ferulic acid from spent brewer's grain at a level similar to that reported for type A FAE from *A. niger* (Table 2). Fae-1, however, did not release ferulated dimers from any of the agro-industrial materials tested, which is consistent with the properties of other type B FAEs [12,19]. The acetylesterase activity of Fae-1 was investigated using α-naphthyl acetate as substrate. Fae-1 is able to efficiently hydrolyse α-naphthyl acetate with an activity in excess of that measured for FAEA from *A. niger* [48], but lower than the specificity of true acetylxylan esterases (Table 2). In addition, there are probably constraints on the access of the FAEs compared with the xylan esterases, as neither FAEA from *A. niger* nor Fae-1 can release acetate from cereal cell wall material.

The pH optimum of recombinant Fae-1 was determined against MFA for a range of pH values between 3.0 and 8.0 (Figure 5A). Maximum activity was recorded at pH 6.0, with enzyme activity stable between pH 6.0 and 7.5. The enzyme was generally compromised at acidic pH. The temperature optimum of recombinant Fae-1 was determined against MFA for a range of temperatures between 20 and 80 °C (Figure 5B). The recombinant enzyme had a temperature optimum of 55 °C for the hydrolysis of MFA. Above this temperature, the activity dropped significantly, to reach a plateau (24 % activity) between 70 and 80 °C. The temperature stability of recombinant Fae-1 was measured by incubating the enzyme at 60 °C for 60 min. Samples were removed every 10 min and assayed for residual activity against MFA at 37 °C (Figure 5C). After 10 min at 60 °C, the enzyme had lost 80 % of its activity. However, the residual activity was retained even after 60 min at 60 °C.

Substrate inhibition of Fae-1

At high concentrations of the synthetic monomeric substrates (MCA, MFA, MSA and M*p*CA), the enzyme activity of Fae-1 was shown to fall. The initial-rate data could be related to the rate equation derived for substrate inhibition: $v = (V_{max}[S])/\{K_m + [S] + ([S]^2/K_i)\}$. This equation was applied to first estimate the kinetic constants V_{max} and K_m, and to determine K_i values at substrate concentrations from 0.02 to 0.3 mM. As the K_i was large compared with K_m, at low concentrations of substrate (between 0.02 and 0.3 mM) the enzyme rates approximated to the Michaelis–Menten equation. Therefore, at low substrate

concentrations, the kinetic constants V_{max} and K_m were determined in the usual way from the Michaelis–Menten equation (see the Experimental section), using Lineweaver–Burk double-reciprocal plots. The kinetic curves obtained with MCA are shown in Figures 6(A) and 6(B), and kinetic constants determined for all four synthetic substrates are presented in Table 3.

DISCUSSION

We report the development of FAE activities in the utilization of plant cell wall materials by the filamentous mesophilic fungus *Neurospora crassa*. We show that *N. crassa* will produce either type A or type B FAE activity when grown on WB or SBP respectively. The substrate specificity profiles are similar to those reported for the *A. niger* esterases FAEA (type A) and CinnAE (type B) against synthetic methyl esters [11,12,19,49]. During the early stages of the growth period (days 2–5), *N. crassa* produces type A and type B carbon-source-dependent esterase activities; however, later in the fermentation of either WB or SBP (days 5–6), these activities become repressed, to be replaced by the expression of a new activity (days 7–9), characterized by its ability to hydrolyse all four synthetic substrates tested (MCA, MFA, MSA and M*p*CA). This later activity may represent a compound activity of replacement type A and B enzymes; alternatively the new activity may be the product of a single enzyme with a hitherto unrecognized ability to deal with a broad range of substrate structures. The induction of an enzyme with broad substrate activity could replace the specialized enzymes to complete the digestion of complex plant materials. The *fae-1* gene is only strongly expressed during the early stages of fermentation on SBP, and may only contribute residual activity at later stages. The *fae-1* gene is weakly expressed throughout fermentation on WB. Therefore the FAE type B enzyme substrate utilization observed in the later stages of growth on SBP and WB may arise due to the expression of an alternative gene or genes. A BLAST-X search performed on the *N. crassa* genomic database (http://www-genome.wi.mit.edu/) using Fae-1 and FAE sequences available from the NCBI database revealed five further ORFs exhibiting significant protein sequence identity. Two ORFs encode putative type A FAEs, and three encode putative type B enzymes related to the *fae-1* product reported here. The presence of these genes is consistent with the idea that *N. crassa* is able to produce more than one type of FAE, and our activity measurements, as well as the time-course mRNA expression analysis, would suggest that these act as either specialist or generalist enzymes, depending on the substrate and its degree of depolymerization. Thus *A. niger* is not the only fungus able to produce more than one type of FAE, and it is likely that most fungi will utilize a battery of such enzymes.

The Fae-1 sequence contains two potential motifs that accord with the consensus Gly-Xaa-Ser-Xaa-Gly, which is highly conserved in serine esterases [47]. Based on the conservation of the sequence environment around these putative active-site serine residues with the *Pe. funiculosum* and the *A. awamori* esterase sequences, we predict the active site of the enzyme to be centred on serine-118 (Gly-Thr-Ser-Ser-Gly).

The *fae-1* cDNA, containing the native signal sequence, was cloned and expressed in *Pichia pastoris* using the pPIC3.5K expression vector. A representative transformant producing a high level of secreted recombinant protein was selected for production of recombinant Fae-1 and used in large-scale culture for Fae-1 purification and characterization. The yield of secretion reached up to 210 $mg \cdot l^{-1}$ in buffered complex medium, and the recombinant protein was recovered as a single peak following hydrophobic interaction chromatography. A clear advantage of the use of the *P. pastoris* expression system is the high yield of secreted recombinant protein against a background of very limited host protein secretion, which allows for rapid purification of the target protein [32].

The substrate recognition of recombinant Fae-1 was characterized. As expected for a type B FAE, Fae-1 shows high catalytic efficiency for the hydrolysis of MCA and M*p*CA, but no detectable activity against MSA. The catalytic efficiency of *N. crassa* Fae-1 for the hydrolysis of Ara_2F was low (12.87 $mM^{-1} \cdot s^{-1}$) compared with that of *Pe. funiculosum* FAEB (530 $mM^{-1} \cdot s^{-1}$) [19], but was 112-fold greater than that of *A. niger* cinnamic acid esterase (CinnAE) [42] and *Pe. equi* cinnamoyl ester hydrolase (CEH or EstA') [16]. Low turnover of Ara_2F may reflect the need for longer feruloylated oligosaccharides to ensure optimal catalytic activity. The acetylesterase activity of Fae-1 was investigated. Even though Fae-1 was defined as a FAE because of its ability to efficiency hydrolyse hydroxycinnamic esters, the enzyme is also able to hydrolyse acetylated substrates, but at a significantly lower efficiency. The ability of Fae-1 to hydrolyse α-naphthyl acetate appears to be the highest activity measured for a FAE on this substrate (3 $mM^{-1} \cdot s^{-1}$), although the catalytic efficiency is 30–100-fold lower than that recorded for a true acetylxylan esterase [50–54].

Kinetic studies performed with recombinant Fae-1 and the synthetic monomeric substrates MCA, MFA and M*p*CA revealed substrate inhibition at higher substrate concentrations. These data were related to the following equation with respect to substrate inhibition: $v = (V_{max}[S])/\{K_m + [S] + ([S]^2/K_i)\}$. It is therefore possible for a second substrate molecule to bind to the enzyme–substrate complex ES to produce an inactive complex, SES. The mechanism would be analogous to that usually considered for uncompetitive inhibition with a non-productive substrate-binding site in addition to the productive binding site. Certain fungal FAEs have been characterized as modular enzymes containing a catalytic domain linked to a non-catalytic domain, such as a specific carbohydrate-binding module [30]. Modular esterases from *Pe. funiculosum* [19], *Pir. equi* [16] and *Trichoderma reesei* [55] have been shown to contain a non-catalytic CBD. CBDs act to bring the catalytic site spatially closer to its substrate and to create a microenvironment where substrate is easily accessible. An enzyme subject to substrate inhibition cannot bear such a binding domain without compromising its catalytic activity, even when the substrate may be limiting in the wider environment. In the light of this information, it is perhaps not surprising that the Fae-1 sequence does not feature a recognizable CBD. In the absence of a CBD, concentration-dependent substrate inhibition could be utilized by micro-organisms to regulate substrate conversion. The enzyme activity would be attenuated in the presence of high environmental substrate concentrations, in order to prevent the accumulation of toxic products that could not be metabolized immediately. Indeed, phenolic acids are used to inhibit microbial growth. We may speculate that the *N. crassa* enzyme has evolved to feature concentration-dependent substrate inhibition in order to regulate the activity of a secreted esterase working remote from the organism. In contrast, concentration-dependent substrate inhibition would be an ineffective way for micro-organisms to regulate the activity of enzymes carrying CBDs, because of the very intimacy of the contact between the enzyme and its potential substrate. The *N. crassa* genome also contains an ORF encoding a modular enzyme which may be identified as a putative FAE with a fungal-type CBD (45 % identity with Fae-1 from *N. crassa*; 44 % identity with FAEB from *Pe. funiculosum*). *N. crassa* therefore has the genetic capacity to produce more than one type B FAE and, as a consequence of their modular structure,

the organism can tailor its response to substrate availability in the environment. In the present study, substrate inhibition has been detected with synthetic substrates that may be structurally similar to the products released by the action of other host enzymes. However, it would be of interest to know if higher-order structures released from plant cell walls will produce substrate inhibition, in which case the organism may have evolved its enzymes not only to avoid toxicity, but also to regulate the rate at which a complex substrate is dismantled. In microbial communities, the ability of an organism to tolerate the actions of its neighbours while maintaining its own nutritional requirement will determine species succession in the environment. It is notable that in this respect *N. crassa* is an opportunist, being the first to colonize a nutrient source and complete its life cycle before giving way to other species.

In conclusion, we have demonstrated the ability of the filamentous fungus *N. crassa* to produce multiple FAE activities. We have also shown that the expression of these enzymes is regulated in response to the initial substrate and the degree of substrate degradation as a function of time. Moreover, we have demonstrated transcriptional regulation of a gene encoding a non-modular FAE, and that the purified product of this gene is subject to substrate inhibition, which has not been reported previously for any FAE. A search of the *N. crassa* genome sequence has revealed three further ORFs with protein sequences similar to Fae-1, including a putative enzyme carrying a CBD. We suggest that FAEs have evolved to operate over a wide range of substrate availability, but at the same time have an innate ability to modulate their catalytic activity in respect of the catabolic requirements and the product sensitivities of the organism that secretes them.

We thank Dr Fred Mellon (Institute of Food Research, Norwich, U.K.) for performing the electrospray ionization MS on Fae-1. We acknowledge support from the Biotechnology and Biological Sciences Research Council (BBSRC), U.K., and the Department of Trade and Industry, U.K., through the LINK Applied Biocatalysts programme (grant no. BTL/27/12).

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Received 13 June 2002/6 November 2002; accepted 18 November 2002
Published as BJ Immediate Publication 18 November 2002, DOI 10.1042/BJ20020917

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