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SHORT CONTRIBUTION

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Identification of a type-D feruloyl esterase from *Neurospora crassa*

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Abstract Feruloyl esterases constitute an interesting group of enzymes that have the potential for use over a broad range of applications in the agri–food industries. In order to expand the range of available enzymes, we have examined the presence of feruoyl esterase genes present in the genome sequence of the filamentous fungus *Neurospora crassa*. We have identified an orphan gene (contig 3.544), the translation of which shows sequence identity with known feruloyl esterases. This gene was cloned and the corresponding recombinant protein expressed in *Pichia pastoris* to confirm that the enzyme (NcFaeD-3.544) exhibits feruloyl esterase activity. Unusually the enzyme was capable of *p*-coumaric acid release from untreated crude plant cell wall materials. The substrate utilisation preferences of the recombinant enzyme place it in the recently recognised type-D sub-class of feruloyl esterase.

Introduction

Feruloyl esterases (E.C. 3.1.1.73) are a subclass of the carboxylic acid esterases (E.C. 3.1.1.1) that are able to hydrolyse the ester bond between the hydroxycinnamic acids and sugars present in plant cell walls (Williamson et al. 1998). These enzymes were initially classified as types A or B based on their substrate specificity for aromatic moieties (Kroon et al. 1999, 2000). This classification has been extended to recognise types A, B, C and D in the accompanying paper on the basis of the functional properties of the enzymes and supported by phylogenetic comparisons of protein sequences (Crepin et al. 2003c).

The filamentous fungus *Neurospora crassa* grows well on plant cell wall materials, having the ability to secrete hydrolytic enzymes to digest cell wall carbohydrate polymers. The production of enzymes of the cellulase complex by *N. crassa* grown on cellulosic substrates such as wheat straw has been investigated (Romero et al. 1999; Yazdi et al. 1990). *N. crassa* has also been shown to produce multiple feruloyl esterase activities depending upon the time of fermentation with either sugar beet pulp or wheat bran substrates (Crepin et al. 2003a). The presence of feruloyl esterase sequence identities extracted from the genome sequence (Galagan et al. 2003) reflect the diversity and the multiplicity of esterase activities produced by *N. crassa*, and offer a number of genes to investigate in terms of the substrate specificities of the enzymes so encoded. Here we report the cloning and expression of a type-D feruloyl esterase from the filamentous fungus *N. crassa*. The classification of this enzyme based on substrate preferences is discussed in comparison with previously characterised feruloyl esterases.

Materials and methods

Materials

The methyl esters of ferulate (MFA), *p*-coumarate (M*p*CA), caffeate (MCA) and sinapate (MSA) were obtained from Apin Chemicals (Oxfordshire, UK). Ferulic (FA), *p*-coumaric (*p*CA), sinapic and caffeic acids were obtained from Sigma (Dorset, UK). Ferulic acid dehydrodimers (diFA) were quantified according to the method of Waldron and colleagues (Waldron et al. 1996) using the following response factors (RFs) against trans cinnamic acid at 280 nm: RF=0.21 for 5,5′ diFA, RF=0.14 for 8-*O*-4′ diFA, RF=0.18 for 8,5′ diFA (open form) and RF=0.12 for 8,5′ diFA (benzofuran form). Brewer's spent grain (BSG) was provided by Scottish Courage (Edinburgh, Scotland) and de-starched wheat bran (WB) was obtained from Agro-industrie Recherches et Developpements (ARD, Pomacle, France). Alcohol insoluble residues (AIR) were prepared by the method of Waldron and Selvendran (1990) and dried overnight in acetone.

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Strains, vectors and media

Escherichia coli strain TOP 10F′ (Invitrogen) was used for vector construction work using the pCR2.1-TOPO cloning vector (Invitrogen). *Pichia pastoris* strain GS115 (*his*4) (Invitrogen) was used to produce the recombinant enzyme with the expression vector pPIC3.5 K (Invitrogen). The wild type *N. crassa* strain ST *A* (74 *A*; Fungal Genetics Stock Center) was maintained on minimal Vogel's agar (1.5% w/v) plus 2% sucrose.

Vector construction and transformation

The *N. crassa* open reading frame (ORF) *faeD*-3.544 was PCR amplified from genomic DNA using specific primers designed at the 5′ (5′-AAA A*TA CGT A*TG GCA GGC CTT CA-3′) and 3′(5′-AAA A*GC GGC CGC* TAG TTG AAC CTG CTA AAG AAC-3) termini to append respective *Sna*BI and *Not*I restriction sites (italics). DNA amplification was carried out through 30 cycles of denaturation (30 s at 95°C), annealing (45 s at 62°C) and extension (1 min 50 s at 72°C), followed by 10 min of further extension at 72°C. The specific PCR product was cloned into the pCR2.1-TOPO vector and the sequence determined using a Perkin-Elmer ABI Prism 310 fluorescent DNA analyser. The *N. crassa faeD*-3.544 fragment was subcloned into *Sna*BI–*Not*I digested pPIC3.5 K vector, to produce the plasmid pPIC3.5 K/*faeD*-3.544. *P. pastoris* was electroporated as described by Becker and Guarente (1991) using 1–5 μg of plasmid DNA linearised with *Dra*I restriction enzyme to allow gene replacement at the *AOX1* gene (Crepin et al. 2003b). Transformants were analysed for secreted expressed products by separation on 12% SDS-PAGE as described previously (Crepin et al. 2003a) from which candidate protein bands were excised for tryptic cleavage before spray ionisation and internal protein sequence determination by matrix desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry using a Micromass Q-TOF-2 instrument.

Enzyme assay

Feruloyl esterase activity was determined against methyl esters of hydroxycinnamic acids using the HPLC assay method, as described by Juge et al. (2001). One unit of esterase activity was defined as the amount of enzyme required to release hydroxycinnamic acid at the rate of 1 μmol min^{-1} at 37°C, pH 6.0. Enzymatic release of FA and diferulates from plant cell wall material was determined in 100 mM 3-(*N*-morpholino)propanesulphonic acid buffer (pH 6.0) in a thermostatically controlled rotating incubator at 37°C. Plant cell wall material (10 mg) was incubated with 1 mU *N. crassa* feruloyl esterase (as determined against MFA) and 2 U *Trichoderma viride* xylanase (Megazyme) in a final volume of 500 μl for 3 h. Incubations were terminated by the addition of 200 μl glacial acetic acid. After centrifugation (20,000 *g*, 5 min, 15°C), samples were filtered through a 0.45-μm filter and 40 μl was analysed by HPLC, as described by Juge et al (2001). The phenolic material released was quantified against standard curves. Controls containing the reaction mixture plus glacial acetic acid were incubated and analysed in identical manner to eliminate interference. The total alkali-extractable hydroxycinnamate content of BSG and WB was determined as described by Faulds et al. (2003). Xylanase activity against 1% (w/v) wheat arabinoxylan in 10 mM phosphate–citrate buffer, pH 5.5, was determined as described by Bailey et al. (1992).

Results

Identification of putative feruloyl esterase genes

Protein translations of gene sequences present in the *N. crassa* genomic database (http://www.genome.wi.mit.edu/) were searched for sequence identity to known feruloyl esterases. The sequences identified in the *N. crassa* genome were then conversely compared to the sequences available in the general databank (National Center for Biotechnology Information, NCBI) using the BLAST program at NCBI (Altschul et al. 1997). The translation of five ORFs from *N. crassa* genome database shows significant primary sequence identity with feruloyl esterases, which is consistent with the expectation that *N. crassa* is capable of producing more than one type of feruloyl esterase (Crepin et al. 2003a). Two ORFs encode putative esterases with 43–41% identity with FaeA from *Penicillium funiculosum* (AJ312296) and XYLD from *Pseudomonas fluorescens* (Ferreira et al. 1993). Three further ORFs encode putative enzymes showing 48–45% identity with Fae-1 from *N. crassa* (Crepin et al. 2003a) and 46–44% identity with FaeB from *P. funiculosum* (Kroon et al. 2000). One of these latter ORFs encodes a putative modular enzyme that features a fungal-type cellulose-binding domain (44% identity with FaeB from *P. funiculosum*). The ORF present in contig 3.544 (43% identity with FaeA from *P. funiculosum* and 41% identity with XYLD from *Ps. fluorescens*) was selected as a candidate type-D feruloyl esterase gene and provisionally named *faeD*-3.544.

Protein sequence analysis

The ORF of *faeD*-3.544 encodes a protein of 291 amino acids. The computer program SignalP (Nielsen et al. 1997) predicts the putative FaeD-3.544 protein to contain secretory signal peptide of 25 amino acids. Based on these predictions the calculated mass of the mature protein is 28,598 Da. Analysis of the protein sequence reveals the presence of two motifs that are characteristic of the serine esterase family (G-X-S-X-G) (Brenner 1988). Only one of these motifs (G-F-S-Y-G) is centred on a serine (S132) within a conserved region also present in *P. funiculosum* FaeA (AJ312296) and *Ps. fluorescens* XYLD (Ferreira et al. 1993) sequences, as well as other members of the wider serine esterase family (Fillingham et al. 1999). The translated sequence, however, does not feature a recognisable cellulose-binding domain, unlike those observed for the *P. funiculosum* and *Ps. fluorescens* esterases. As representative type-A, -B, and -C feruloyl esterase genes have already been cloned and expressed in *P. pastoris* (Crepin et al. 2003a, b; Juge et al. 2001), we therefore undertook to amplify the nucleotide sequence encoding FaeD-3.544 from *N. crassa* DNA in order to express the corresponding protein in *P. pastoris*.

Cloning and expression of a type-D feruloyl esterase in *P. pastoris*

The uninterrupted ORF of *faeD*-3.544 was PCR-amplified from *N. crassa* genomic DNA using specific primers designed to introduce *Sna*BI and *Not*I restriction sites at 5′

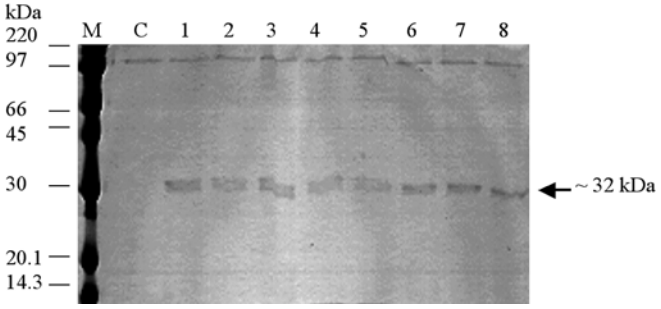

Fig. 1 SDS-PAGE to analyse the expression of FaeD-3.544 in *Pichia pastoris* small-scale cultures. *M* Standard protein molecular weight in kilodaltons; *C* parental vector transformant as a negative control; *lanes* 1–8: $His^{+}Mut^{S}$pPIC3.5 K/*faeD*-3.544 transformant clones 1–8

and 3′ termini of the gene sequence. The PCR product was cloned into the pCR2.1-TOPO for DNA sequence analysis and subcloned into *Sna*BI–*Not*I digested pPIC3.5 K expression vector under the control of the alcohol oxidase 1 promoter (*AOX1*). The resulting expression construct pPIC3.5 K/*faeD*3.544 and the parent vector were linearised with *Dra*I restriction enzyme, to allow gene replacement at the *AOX1* locus, and used to transform *P. pastoris* GS115 (*his*4). *P. pastoris* transformants were selected by their ability to grow on histidine-deficient medium and assessed for their methanol utilisation phenotype (Mut phenotype). Eight colonies ($His^{+}Mut^{S}$) were screened for protein expression and secretion under methanol induction. All eight *faeD*-3.544 transformants produced a major secreted protein product of approximately 32 kDa upon examination of culture supernatants by SDS-PAGE (Fig. 1), whereas no protein could be detected with the vector control. The additional mass to that predicted from the protein sequence would suggest that the FaeD-3.544 product, like other exogenous proteins expressed by *P. pastoris*, is probably glycosylated.

Feruloyl esterase activity of the recombinant FaeD-3.544

To confirm that the transformants were producing feruloyl esterase, culture supernatants of three independent clones were assayed for activity against 1 mM MFA at 37°C for 30 min using reverse-phase HPLC (Faulds and Williamson 1994). The clone expressing the highest enzyme activity against MFA was retained for further analysis (clone 4.29: 85 mU ml^{-1} of culture supernatant, clone 2.37: 79 mU ml^{-1}, clone 4.15:77 mU ml^{-1}). The amino acid sequences of three internal peptides of the protein product of clone 4.29 were determined by MALDI-TOF mass spectrometry of tryptic digests to confirm the identity of the polypeptide (LIFLW, AVAVI and VEYQG). The recombinant protein was then ultrafiltrated (>10 kDa cut-off) to remove contaminating peptides and assayed against the four synthetic methyl esters. The enzyme was found to be active against all four synthetic substrates (MFA: 566 mU mg^{-1} protein, MSA: 557 mU mg^{-1}, MCA: 297 mU mg^{-1} and M*p*CA: 304 mU mg^{-1}). These results confirm that the *faeD*-3.544 gene does indeed encode a feruloyl esterase.

Table 1 Release of phenolic residues from agro-industrial waste materials. Values are expressed as percentages of the total alkali-extractible phenolic acids (Faulds et al. 2003). *FA* Ferulic acid, *pCA* *p*-coumaric acid, *diFA* diferulic acid, *ND*values not determined

	AnFaeA[a]			PfXYLD[a]			NcFaeD-3.544[b]		
	FA	*p*CA	diFA	FA	*p*CA	diFA	FA	*p*CA	diFA
Wheat bran	64	<2	19	67	ND	20	36	23	12
Spent grain	31	<2	18	27	ND	14	18	0.5	10

[a]13 mU AnFaeA or PfXYLD esterase was incubated with plant cell wall material in the presence of 2 U *Trichoderma viride* xylanase at 37°C for 7 h (Bartolome et al. 1997)

[b]1 mU NcFaeD-3.544 esterase was incubated with plant cell wall material in the presence of 2 U *T. viride* xylanase (Megazyme) at 37°C for 3 h

Release of phenolics from crude plant cell walls

The ability of FaeD-3.544 to release phenolic compounds from crude plant cell wall materials in the form of agro-industrial waste materials was tested. The enzyme was found to be able to release FA, *p*CA and diFA from WB and BSG in combination with xylanase from *T. viride* (Table 1).

Discussion

The recombinant *N. crassa* FaeD-3.544 protein is able to hydrolyse the ester bonds between all four synthetic substrates as reported for the genes encoding *Ps. fluorescens* type-D (PfXYLD) and *Talaromyces stipitatus* FaeC type-C esterases (Crepin et al. 2003b; Ferreira et al. 1993). Moreover, in the presence of 2 U *T. viride* xylanase, FaeD-3.544 is capable of releasing FA and 5-5’ DiFA from WB and BSG. The ferulate and diferulate levels released are similar to those released by AnFaeA and PfXYLD under similar conditions (Table 1). FaeD-3.544 also appears to release *p*CA from WB and BSG. Release of *p*CA from cell wall materials has been previously reported for *p*-coumaroyl esterases from *Aspergillus awamori*, *Penicillium pinophilum* and *Neocallimastix* strain MC-2 (Borneman et al. 1991; Castanares et al. 1992; McCrae et al. 1994). However, these measurements have been carried out on phenolic acid-substituted xylan polysaccharide isolated from wheat straw and rarely on untreated crude cell wall materials, with the exception of coastal Bermuda grass utilised to study the *p*CA release by *Neocallimastix* strain MC-2 *p*-coumaroyl esterase (Borneman et al. 1991). Therefore, this is the first report of an esterase able to release significant quantities of *p*CA from untreated crude cell wall materials such as WB and BSG. Feruloyl esterases such as FaeD-3.544 have the potential to remove

esterified phenolics from animal feed sources such as wheat straw, and thereby improve its nutritional value. As wheat straw is rich in *p*CA esterified to lignin (Scalbert et al. 1985), an enzyme able to release these *p*-coumarate residues could be attractive for the feed industry.

The ferulate and diferulate levels released from plant cell wall materials by FaeD-3.544 are similar to those released by AnFaeA under similar conditions. However, the broad substrate specificity against methyl hydroxycinnamates as well as the ability to release 5-5′ diFA and significant levels of *p*CA from untreated plant cell wall materials demonstrate that FaeD-3.544 is distinct from other sub-classes of feruloyl esterase [type-A enzymes are inactive against MCA (Faulds and Williamson 1994); type-B enzymes are inactive against MSA (Kroon et al. 1996); type-C enzymes do not release dimers (Crepin et al. 2003b)]. Therefore, based on these substrate utlilisation properties of FaeD-3.544, we propose that it is indeed a novel type-D feruloyl esterase as predicted from comparative sequence analysis.

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