Purification and characterization of a ferulic acid esterase (FAE-III) from *Aspergillus niger*: specificity for the phenolic moiety and binding to microcrystalline cellulose

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An inducible ferulic acid esterase (FAE-III) has been isolated, purified and partially characterized from *Aspergillus niger* after growth on oat spelt xylan. The purification procedure utilized ammonium sulphate precipitation, hydrophobic interaction and anion-exchange chromatography. The purified enzyme appeared almost pure by SDS-PAGE, with an apparent M_r of 36 000. A single band, corresponding to a pI of 3.3 was observed on isoelectric focusing. With methyl ferulate as substrate, the enzyme had a specific activity of 67 IU (mg protein) $^{-1}$, pH and temperature optima of 5 and 55–60 °C, respectively, and a K_m of 2.08 mM and a V_{max} of 175 μ mol min $^{-1}$ (mg protein) $^{-1}$. The enzyme was also active upon methyl sinapinate, methyl-3,4-dimethoxy cinnamate and methyl *p*-coumarate, but not benzoic acid methyl esters or methyl caffeate. Similarly, *Streptomyces olivochromogenes* FAE showed activity against methyl ferulate, methyl sinapinate and methyl *p*-coumarate, but at a level 420-fold less (on methyl ferulate) than the *A. niger* esterase. No activity was detected against the benzoate methyl esters. For both enzymes, this shows the necessity for C-3 on the phenol ring to be methoxylated and the aliphatic region of the substrate to be unsaturated. The specific activity of FAE-III on de-starched wheat bran was 31 U (mg protein) $^{-1}$ in the presence of *Trichoderma viride* xylanase and 3 U (mg protein) $^{-1}$ in the absence. Apparent pH dependent binding of *A. niger* FAE-III to microcrystalline cellulose was also demonstrated.

Keywords: *Aspergillus niger*, ferulic acid esterase, xylanase, *Streptomyces olivochromogenes*

INTRODUCTION

Plant cell wall polysaccharides are degraded by micro-organisms provided they produce the necessary enzymes. The more complex the polymer, the greater the number of enzymes required, in most cases, to degrade it. Polysaccharide-degrading enzymes act synergistically to achieve total bioconversion of cell wall material whether they exist as a multi-enzyme complex, as with the *Clostridium thermocellum* cellulosome (Lamed & Bayer, 1988), or as individual enzymes interacting with each

other. Groups of enzymes are arranged into 'systems', such as xylan-degrading (Tuohy *et al.*, 1993; Myburgh *et al.*, 1991; Johnson *et al.*, 1988) and cellulose-degrading (Beguín *et al.*, 1992; Wood, 1992). The nature of these enzyme–substrate interactions and cooperativity between enzymes are presently the subject of extensive research, especially concerning the breakdown of cellulose and hemicellulose (Gilbert & Hazlewood, 1993), because of possible exploitation in agricultural and industrial processes (Gilbert & Hazlewood, 1991).

Complex polysaccharides, such as the arabinoxylans of wheat bran (Smith & Hartley, 1983), barley straw (Mueller-Harvey *et al.*, 1986), maize (Kato & Nevins, 1985) and sugar-cane bagasse (Kato *et al.*, 1983), and the pectins of sugar beet (Rombouts & Thibault, 1986) and spinach (Fry, 1982) are highly branched and require a battery of enzymes to break down the polymer. A further complication in the hydrolysis of these polysaccharides

Abbreviations: FAE, Ferulic acid esterase; MCA, methyl caffeate (3,4-dihydroxycinnamate); MCC, microcrystalline cellulose; MdMCA, methyl 3,4-dimethoxycinnamate; MFA, methyl ferulate (3-methoxy-4-hydroxycinnamate); MpCA, methyl *p*-coumarate (4-hydroxycinnamate); MSA, methyl sinapinate (3,5-dimethoxy-4-hydroxycinnamate); MSyA, methyl syringate (3,5-dimethoxy-4-hydroxybenzoate); MVA, methyl vanillate (3-methoxy-4-hydroxybenzoate).

occurs when some sugar residues are esterified to phenolic compounds, such as ferulic and *p*-coumaric acids. These phenolic acids limit the digestibility of the plant cell wall material by microbes and can even be toxic to growth (Borneman *et al.*, 1986; Chesson *et al.*, 1982; Jung & Sahl, 1986). Only a few species have been reported to produce esterases which cleave the phenolic compounds from the sugar moiety, leaving the remainder of the polysaccharide open for further hydrolytic attack by other enzymes. Recently, these esterases have been purified and partially characterized from *Streptomyces olivochromogenes* (Faulds & Williamson, 1991, 1993a), *Neocallimastix* (Borneman *et al.*, 1991, 1992), *Pseudomonas fluorescens* (Ferreira *et al.*, 1993), *Penicillium pinophilum* (Castaneres *et al.*, 1992) and several from *Aspergillus* (Tenkanen *et al.*, 1991; Faulds & Williamson, 1993b). The specificities of the enzymes for methyl esters of cinnamic acids, acting as model substrates, show that the *Pseudomonas* esterase and two isoforms from *Aspergillus niger* (Faulds & Williamson, 1993b) are specific for compounds with different substitutions on the phenolic ring of the cinnamic acid. Furthermore, these enzymes could not hydrolyse methyl esters of the benzoic acids (vanillate and syringate) showing the importance of the C = C bond in the recognition of substrates.

The *Pseudomonas* esterase has been sequenced and shown to contain a cellulose-binding domain located at the N-terminus (Ferreira *et al.*, 1993). This enzyme did not bind to xylan. The cellulose binding domain exhibited almost complete homology with the corresponding regions of a xylanase and an arabinofuranosidase, and is related to the cellulose binding domain of the cellulases from the same organism (Ferreira *et al.*, 1991, 1993). Fungal cellobiohydrolases also contain a cellulose binding domain (Tomme *et al.*, 1988; Ong *et al.*, 1989) but there have been no reports of non-cellulase enzymes containing a cellulose binding domain in fungi, unlike the bacterial ferulic acid esterase of *P. fluorescens*.

In this paper, we report the isolation, purification and characterization of an inducible ferulic acid esterase from *Aspergillus niger* CBS 120.49, which we have called FAE-III, as the activities and properties differ from FAE-I and -II from *A. niger* (Faulds & Williamson, 1993b). The specificity for methyl esters of cinnamic and benzoic acids are discussed and compared to the specificity of *S. olivochromogenes* FAE. We also show that FAE-III is able to bind to microcrystalline cellulose.

METHODS

Growth of *A. niger*. *A. niger* CBS 120.49 was grown as described by Archer *et al.* (1990), without the addition of peptone and Casamino acids. Carbon sources were added to this *Aspergillus* Minimum Medium as described in Results. Cultures, containing approximately 10^6 spores per 100 ml medium, were incubated at 25 °C and shaken at 150 r.p.m. in 250 ml Erlenmeyer flasks, containing 100 ml medium, over a 4 d period.

Purification of FAE-III. 1. Ammonium sulphate precipitation. Culture filtrate, obtained by filtering through muslin, was subjected to 50 % and 80 % saturation $(\text{NH}_4)_2\text{SO}_4$ fractionation.

2. Hydrophobic interaction chromatography. The 80 % pellet was resuspended in 50 mM sodium phosphate (pH 7)/1 mM EDTA/0.6 M $(\text{NH}_4)_2\text{SO}_4$, syringe filtered, and fractionated on an FPLC hydrophobic interaction chromatography column (Phenyl-Superose, Pharmacia) as described previously (Faulds & Williamson, 1991). Fractions of 0.5 ml were collected. Aliquots of the pooled active fractions in 0.6 M $(\text{NH}_4)_2\text{SO}_4$ (2 ml) were reloaded, and fractions of 0.5 ml were collected.

3. Anion-exchange chromatography. Pooled active fractions from the second hydrophobic interaction chromatography column, after buffer exchange using a Sephadex G-25 PD-10 column (Pharmacia) into 20 mM Tris/Cl (pH 8.8), were further fractionated on an anion-exchange chromatography column (Mono Q, Pharmacia), under conditions previously described (Faulds & Williamson, 1993b). Fraction of 0.5 ml were collected.

Enzyme assays. FAE activity was measured against methyl ferulate (MFA), methyl sinapinate (MSA), methyl *p*-coumarate (MpCA), methyl caffeate (MCA), methyl vanillate (MVA) and methyl syringate (MSyA) as described previously (Faulds & Williamson, 1993b). Release of the cinnamic acids was monitored at 310 nm and at 280 nm for the benzoic acids. Activity against methyl-3,4-dimethoxycinnamic acid (MdMCA) was monitored by a change in absorbance at 335 nm using a Beckman DU-70 thermostated spectrophotometer under the same conditions as the HPLC assay. The spectrophotometric technique utilizes the relative differences at pH 6 of the absorbance at 335 nm between the free acid and the corresponding methyl ester. The same method was used for the other methyl esters using estimated ϵ values. Incubations for both spectrophotometer and HPLC assays were carried out at 37 °C. Activity on de-starched wheat bran was measured both in the absence and in the presence of 2 U *Trichoderma viride* xylanase [Megazyme (Aust) Pty Ltd] as described previously (Faulds & Williamson, 1991). Xylanase activity was detected using standard methodology by determining the release of reducing sugar (Miller, 1959) from 1 % (w/v) soluble oat spelt xylan (Kellert *et al.*, 1990), and was expressed as xylose equivalents: one unit of enzyme activity released $1 \mu\text{mol}$ sugar min^{-1} at 37 °C, pH 6.5. Acetyl esterase and acetyl xylan esterase activity were determined as described previously (Faulds & Williamson, 1993b). One unit of enzyme released $1 \mu\text{mol}$ product min^{-1} under the relevant assay conditions for all the enzyme activities measured. Protein was estimated using the Coomassie Protein Assay Reagent from Pierce.

M_r determination. SDS-PAGE was performed using a Pharmacia PhastSystem by the method of Laemmli (1970), using a 12.5 % (w/v) homogeneous PhastGel. Gels were run in accordance with the optimized method for a homogeneous SDS-PAGE gel given in the manufacturer's techniques file no. 111. Samples, extensively dialysed against distilled water, freeze-dried and subsequently resuspended in distilled water, were loaded using applicator strips, and protein bands were determined by Coomassie staining. M_r values were estimated from a plot of $\log M_r$ versus mobility using the following standards: bovine serum albumin (66 000), egg albumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (20 100) and α -lactalbumin (14 200).

Gel filtration. Pooled active fractions from the second hydrophobic interaction chromatography column were also fractionated on an FPLC gel filtration column (Superose 6, Pharmacia), previously equilibrated with 50 mM sodium phosphate/0.15 M NaCl (pH 7), and calibrated with a MW-GF-200 kit (Sigma). Fractions of 0.2 ml were collected.

Fig. 1. Structures of the seven phenolic acids used in the present investigation.

Isoelectric point determination. The pI of FAE-III was determined by the method described previously (Faulds & Williamson, 1993b). Samples, extensively dialysed against water, were loaded using 6/4 applicator strips (Pharmacia). Gels were fixed and the protein bands visualized by Coomassie staining. pI values were determined from a plot of migration distance versus pI values of the following standards: patent blue (VF) sodium salt (2.4), glucoamylase (3.65), acetylated cytochrome C (3.95), glucose oxidase (4.25), C. phytoerythrin (4.75 and 4.85), β -lactoglobulin A (5.25), β -lactoglobulin B (5.35) and azurin (5.65), obtained from BDH ('Electran' pI calibration kit, range 2.4–5.65).

Enzyme kinetics. Enzyme was incubated with 0–1.2 mM MFA, 0–1.2 mM MdMCA or 0–3 mM MSA for 15 min at 37 °C, and free acid determined as a change at 335 nm on a Beckman DU-70 spectrophotometer. Data were analysed by the method of Wilkinson (1961).

Temperature optimum. FAE-III was incubated with 0.94 mM MFA over a temperature range of 20–70 °C. The optimum temperature was calculated from the total μ mol product released after a 30 min incubation, as described in the assay procedure (Faulds & Williamson, 1991).

pH optimum. FAE-III was incubated with 0.94 mM MFA in 100 mM McIlvaine buffer over the pH range 3.0–7.0 and in 100 mM phosphate buffer over the pH range 7.0–8.0 in 0.5 unit increments. The pH optimum was calculated as for the temperature optimum.

Preparation of the methyl esters. Methyl derivatives of the phenolic acids shown in Fig. 1 were prepared by the method of Borneman *et al.* (1990). The absorption spectrum of each derivative was compared in dilute NH_3 (pH 10), MOPS (pH 6)

and dilute acetic acid (pH 3), with that of the free acid to check its purity (Fry, 1982). The absorption coefficient of each compound was calculated from these values using the Beer–Lambert law.

The absorbance maxima (λ) and absorbance coefficient (ϵ) values for each methyl ester compound and its corresponding free acid (Fig. 1) at three pH values are given in Table 1. The values for ferulic acid, methyl ferulate and *p*-coumaric acid at pH 3 and 10 are similar to the values obtained by Fry (1982). From this table, a measurement of the rate of hydrolysis at pH 6 may be obtained as a change in absorbance at a determined wavelength. This measurement is dependent on the pH values of the substrate and the pH range of the enzyme. For purified *S. olivochromogenes* FAE, this was determined to be 5.5 (Faulds & Williamson, 1991). Hatfield *et al.* (1991) used a spectrophotometric method to measure FAE activity from some commercially available preparations using a chemically synthesized substrate (methyl 5-*O*-*trans*-feruloyl- α -L-arabinofuranoside). By altering the pH at the end of a 20 min incubation at 25 °C from pH 8 to pH 11.5, a shift in absorbance at 375 nm was measured. By using this method, the absorbance difference between a free acid and its methyl ester could be measured for the esters prepared in this study. However, as *S. olivochromogenes* FAE activity was lost above pH 7.5, it is not suitable for all enzymes. A measurement at a lower pH would be more desirable. At 335 nm, the spectral differences between the free acid and the corresponding methyl ester allow direct measurements to be made, and at pH 6, the enzymes are active.

To check the purity of the methyl esters, ^1H -NMR spectra were recorded on a Hitachi R-200 Rapid Scan Correlation (Gupta *et al.*, 1974) NMR spectrophotometer (Nissei Sangyo Co. Ltd) at 60 MHz. All samples were dissolved in 1 ml of D_2O /acetone d_6 (1:1, v/v) at a concentration of approximately 5 mM. A

Table 1. Comparison of the absorption maximum (λ) and absorption coefficient (ϵ) for each phenolic acid and its methyl ester at three pH values

pH 3, dilute acetic acid; pH 6, 100 mM MOPS buffer; pH 10, dilute ammonia.

Phenolic compound	pH 3.0		pH 6.0		pH 10.0	
	$\lambda_{\max}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)	$\lambda_{\max}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)	$\lambda_{\max}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)
Ferulic acid	320	17000	310	15900	343	22400
Methyl ferulate	322	49000	321.5	27500	369	35500
<i>p</i> -Coumaric acid	308	20000	285	17800	331	18600
Methyl coumarate	309	58900	310	23400	353	35500
Sinapinic acid	320.5	11500	306	12900	354.5	12600
Methyl sinapinate	321.5	19100	321.5	18200	382.5	24000
Caffeic acid	321	18200	286	14500	341	15800
Methyl caffeate	323	41700	322.5	25100	365	43700
3,4-Dimethoxycinnamic acid	318.5	8100	309	8100	285	7600
Methyl dimethoxycinnamic acid	320.5	69200	320.5	75900	320.5	66100
Vanillic acid	260	11000	251	9800	294	12300
Methyl vanillate	261	12300	261	12000	312	20000
Syringic acid	273	11200	261.5	12000	312	20000
Methyl syringate	274	9100	274	10200	322	13800

10 p.p.m. scan width was used with a sweep rate of 20 s; 10 scans were co-added before correlation. The HOD peak at 5.1 p.p.m. was used as an internal standard. The methyl esters were compared to the published spectra of the free acids (The Aldrich Library of NMR Spectra, 1974), and the methoxy peak confirmed synthesis of the methyl esters.

Binding studies. FAE-III (approx. 40 U) was incubated with 0–100 mg microcrystalline cellulose (MCC; Sigmacell, Type 20; Sigma) ml⁻¹ at 4 °C using a thermostatically controlled rotating incubator, for 2 h at various pH values. Samples were then centrifuged at 10000 r.p.m. for 10 min and the supernatant assayed for remaining FAE activity.

***S. olivochromogenes* FAE.** Ferulic acid esterase from *S. olivochromogenes* was purified as described previously (Faulds & Williamson, 1991). Assays were carried out at 50 °C with 0.94 mM substrates in 100 mM MOPS (pH 6).

RESULTS

Inducibility of FAE-III

A. niger CBS 120.49 was grown on Aspergillus Minimum Medium containing 1% (w/v) glucose, 1% (w/v) oat spelt xylan or 0.1% glucose/1% xylan. Samples of the medium were removed every 24 h over a 4 d incubation and assayed for FAE and xylanase activity (Fig. 2). Xylanase was produced by the fungus under all three growth conditions, levels of activity increasing with incubation time. Onset of activity appeared to be delayed 24 h when xylan/glucose served as the carbon source. Highest activity was recorded after 4 d growth when glucose alone served as the carbon source. Ferulic acid esterase activity was also detected under all three con-

ditions, but the level of activity was clearly dependent upon the carbon source. When 1% glucose or xylan alone served as the carbon source, activity was detected after 48 h incubation, but then decreased. Activity in the xylan cultures was 10-fold higher than that in the glucose cultures. In the xylan/glucose-grown cultures, FAE activity was produced after 72 h growth, simultaneously with xylanase, and levels increased over time. This appeared to be the most suitable carbon source for FAE production.

The fungus was also grown for 4 d on mixtures of plant cell wall material and glucose (0.1%). Highest activity [1.25 U (mg protein)⁻¹] was recorded when oat spelt xylan (1%) served as the second carbon source. When grown on wheat bran (4%)/distillers spent grain (1%), orange peel pectin (1%) and sugar beet pulp (4%), specific activities of 0.44, 0.01 and 0.005 U (mg protein)⁻¹, respectively, were obtained. These results show that the carbon source clearly influences the induction of FAE from *A. niger* and that FAE and xylanase expression are not under the same control.

Purification of FAE-III

Table 2 summarizes a typical purification of FAE-III from *A. niger*. The esterase was purified from material secreted into the medium by the fungus. Note all the oat spelt xylan was degraded by the fungus during growth. Non-solubilized substrate was centrifuged as a brown pellet along with protein after addition of (NH₄)₂SO₄ to 50% saturation. The resuspended pellet from this step was re-centrifuged and both the resulting (second) pellet and the

Fig. 2. Induction of FAE-III and xylanase from *A. niger* CBS 120.49 grown on *Aspergillus* Minimum Medium (see Methods) with added carbon source. FAE activity was measured using methyl ferulate. ○, Oat spelt xylan (1%, w/v); □, glucose (1%, w/v); ●, oat spelt xylan (1%) + glucose (0.1%).

clear supernatant exhibited FAE activity, but at very low levels compared to the initial pellet (results not shown). The location of the remaining activity has yet to be determined.

The pellet, after 80% (NH₄)₂SO₄ saturation, was then subjected to hydrophobic interaction chromatography. The enzyme eluted as a single active peak between 68 and

34 mM ammonium sulphate (not shown). This peak was rechromatographed. On the second hydrophobic interaction chromatography step, activity coincided with the sole A_{280} peak. After anion-exchange (Mono Q), FAE-III eluted as a single sharp peak between 0.37 and 0.42 M KCl (not shown), giving a final purification of 26-fold and an apparent yield of 117% activity using methyl ferulate. This high yield may signify the removal of an inhibitory factor. The second peak on the anion-exchange column had no FAE activity associated with it and contained no protein as shown using the Coomassie Protein Assay reagent. It was possibly low molecular mass material, as found after gel filtration, since the absorption maxima (at 260 nm) were identical.

Properties of FAE-III

FAE-III was stable for at least 2 months at 4 °C after hydrophobic interaction chromatography. Stability after further purification was not examined. On SDS-PAGE, FAE-III eluted as a band corresponding to $M_r = 36000$ (not shown). There was also a very faint band at $M_r \sim 33000$.

On gel filtration, FAE-III, after the second hydrophobic interaction chromatography step, eluted as a single active peak correlating to $M_r = 14500 (\pm 2300)$ (not shown), which is lower than the value obtained by SDS-PAGE. Retardation of FAE-III on the gel filtration column was also observed for *S. olivochromogenes* FAE (Faulds & Williamson, 1991). As the M_r of the native and the denatured enzyme differ, it is not possible to estimate the number of subunits associated with FAE-III. An absorption spectrum of FAE-III after gel filtration showed that there was a peak at 280 nm, with no additional chromophores. After gel filtration, a large peak was separated which showed no FAE activity, perhaps similar to the peak on the anion-exchange column. The spectrum of this peak exhibited $\lambda_{max} = 260$ nm and did not react in the Coomassie Protein Assay.

FAE-III has an isoelectric point of 3.3. No other bands were observed. This value is in the pI range of other

Table 2. Purification of FAE-III from *A. niger* as measured by MFA hydrolysis

Step	Vol. (ml)	Specific activity [U (mg protein) ⁻¹]	Total activity (U)	Purification (-fold)	Yield (%)
Crude culture supernatant	890	2.6	239	1	100
50% ammonium sulphate	20	ND	58	ND	24
80% ammonium sulphate	20	ND	165	ND	69
1. 1st hydrophobic interaction	40	28	104	10.6	43
2. 2nd hydrophobic interaction	60	36	82	13.7	34
3. Anion-exchange	63	67	279	25.7	117
Supernatant from ammonium sulphate	1110	ND	3.2	ND	1.3

ND, Not determined.

Table 3. Specificity of FAE-III for methyl esters of phenolic acids. Values in parentheses are $\pm$ SD.

Substrate	Specific activity [U (mg protein) ⁻¹]	K _m (mM)	V _{max} [U (mg protein) ⁻¹]	V _{max} /K _m
MFA	67.2	2.08 (0.19)	175 (14)	84.1
MVA	—	ND	ND	ND
MpCA	0.07	ND	ND	ND
MSA	156	1.44 (0.36)	286 (34)	199
MSyA	—	ND	ND	ND
MCA	—	ND	ND	ND
MdMCA	38.9	1.36 (0.30)	123 (18)	90.4

—, Not detected.

ND, Not determined.

Aspergillus esterases (3.0–3.8), and is the same as the major esterase previously identified, but not purified, from a wheat-grown culture of *A. niger* (McCallum *et al.*, 1991).

The esterase has a temperature optimum between 55 and 60 °C on MFA. The standard assay temperature of 50 °C (Faulds & Williamson, 1993b) was changed to 37 °C in this study, as 50 °C is probably too close to the temperature optimum to ensure stability of the enzyme over long incubations. A comparison of activities at both temperatures over each step of the purification revealed an average drop in activity of only 28% at the lower temperature (results not shown).

A pH optimum of 5.0 was obtained, with 50% of activity at pH 4 and 6.1. This may be due to aspartic or glutamic acids (pK 4.4) in the deprotonated state, and histidine (pK 6.5) in the protonated state. Ferulic acid esterases isolated so far appear to have a range of pH optima, from 4.5–7.0; those from *Aspergillus* exhibit optimum activities in the more acidic range.

Substrate specificity of *A. niger* and *S. olivochromogenes* FAE for phenolic acids

Table 3 summarizes the specificity of FAE-III for the cinnamic acids. No hydrolysis of the benzoic acid esters occurred, showing the necessity for the C = C on the aliphatic chain. The enzyme has a higher activity on MSA than on MFA or MdMCA. FAE-III has no activity on MCA and very little on MpCA, showing the necessity for C-3 on the phenol ring to be methoxylated. Although an absence of substitution can be tolerated at C-5, the presence of a methoxy group increases the hydrolytic rate. Substitution at C-4 by a methoxy group does not influence catalysis. The specificity for cinnamic acid methyl esters of the 50% (NH₄)₂SO₄ pellet was identical to the 80% pellet (FAE-III) suggesting only one isoenzyme. This purification step, however, was required to remove undigested xylan from the enzyme preparation.

S. olivochromogenes FAE showed almost equal activity against MFA [0.16 U (mg protein)⁻¹] and MSA [0.13 U

(mg protein)⁻¹] and very low activity against MpCA [0.003 U (mg protein)⁻¹]. The difference in levels of activity between the fungal esterase and the bacterial esterase for MFA is 420-fold, even though their substrate specificities are similar.

Associated enzyme activities

FAE-III from *A. niger* was assayed for associated hydrolytic activities. Xylanase activity was detected in FAE-containing samples after the first hydrophobic interaction chromatography column [35 U (mg protein)⁻¹], but no xylanase was detected after further purification. Three milliUnits of enzyme released 0.6% of ferulic acid from de-starched wheat bran after a 30 min incubation at 37 °C, assuming ferulic acid is 0.5% (w/w) of the dry weight of wheat bran (Ralet *et al.*, 1990), without the addition of

Fig. 3. Binding of FAE-III to microcrystalline cellulose (MCC) at different pH values. Activity is expressed as a percentage of activity bound to cellulose when compared to controls without cellulose under the same conditions. ●, 50 mM sodium acetate (pH 4); ○, 100 mM MOPS (pH 6); □, 50 mM sodium phosphate (pH 7).

Fig. 4. Comparison of esterases with activity on MFA against methyl esters of cinnamic acids. A, Methyl ferulate; B, methyl *p*-coumarate; C, methyl sinapinate; D, methyl caffeate; ND, not determined.

xylanase. Upon the addition of 2 U *T. viride* xylanase (FAE free), the same amount of FAE-III released 6.6 % of the total ferulic acid from de-starched wheat bran, an 11-fold increase in activity due to the synergistic interaction of the two enzymes. The specific activity of FAE-III on de-starched wheat bran was 31 U (mg protein)⁻¹ in the presence of *T. viride* xylanase and 3 U (mg protein)⁻¹ in the absence. This shows that FAE-III acts directly on plant cell wall material.

Upon acetylated xylan, without the addition of a xylanase, FAE-III had a specific activity of 4 U (mg protein)⁻¹. Acetylerase activity was measured as 93 U (mg protein)⁻¹ on *p*-nitrophenyl acetate.

Binding to microcrystalline cellulose

As activity was apparently associated with the undigested xylan after the 0–50 % (NH₄)₂SO₄ cut (Table 2), even after washing with buffer, the possibility that FAE-III might bind to microcrystalline cellulose (MCC) was examined. On incubation at 4 °C with MCC in 50 mM sodium acetate (pH 4) for 2 h, FAE-III activity in the supernatant upon centrifugation was found to be related to the amount of MCC present (Fig. 3). This may be due to FAE-III binding to MCC: 91 % of the activity was removed from the supernatant in the presence of 40 mg MCC per ml assay mixture, and so the binding reaches

saturation. The binding was pH dependent. The enzyme was not released from MCC after a 2 h incubation at room temperature in the presence of water.

DISCUSSION

Ferulic acid esterase from *Aspergillus niger* CBS 120.49 is inducible by growth on oat spelt xylan. We have already purified two ferulic acid esterases from *A. niger* which were present in a commercial preparation of pectinase (Faulds & Williamson, 1993b); these we termed FAE-I and FAE-II. We therefore propose that the ferulic acid esterase described in this paper is termed FAE-III. Fig. 4 compares the specificities of these enzymes with ferulic acid esterases from *Streptomyces olivochromogenes*, *Pseudomonas fluorescens* and *Penicillium pinophilum*.

The two bacterial enzymes show highest activity on methyl ferulate, but otherwise are very different. The fungal enzymes show highest activity on methyl cinnamates other than methyl ferulate. The relative activities do not allow classification of ferulic acid esterases into particular classes.

However, when the relative specificities of *A. niger* FAE-III are compared to those of FAE-II, the pattern methyl sinapinate > methyl ferulate > methyl *p*-coumarate > methyl caffeate is observed for both enzymes. Although the specific activity of FAE-II (Faulds & Williamson,

1993b) is lower than FAE-III, it is possible that FAE-II may be a proteolytically modified FAE-III, since the M_r of the former (29000) is lower than the latter (36000). This hypothesis awaits resolution.

Most ferulic acid esterases release the free acid from wheat bran only in the presence of a xylanase (Faulds & Williamson, 1991, 1992, 1993b; Tenkanen *et al.*, 1991; Ferreira *et al.*, 1993). Some, however, are able to act on plant cell walls in the absence of a xylanase (Borneman *et al.*, 1992; Faulds & Williamson, 1993b; Castanares *et al.*, 1992). FAE-III clearly fits into the latter category, and in fact has a substantial activity (3 U mg^{-1}) on de-starched wheat bran.

Bacterial plant cell wall degrading enzymes commonly contain a cellulose binding domain (see Henrissat, 1992, for a review). However, it is less common for fungal enzymes to possess this domain. Although it is usually cellulose-degrading enzymes that contain a cellulose binding domain, there are exceptions, such as two xylanases, an arabinofuranosidase and acetyl/ferulic acid esterase from *P. fluorescens* (Hazlewood & Gilbert, 1992). Thus, FAE-III from *A. niger* appears to be a member of a very small number of non-cellulose degrading enzymes able to bind cellulose specifically.

In summary, a ferulic acid esterase (FAE-III) from *A. niger* CBS 120.49 was purified to homogeneity and partially characterized. It was specific for methyl esters of cinnamic acids methoxylated at C-3 on the phenolic ring (ferulic, sinapinic, 3,4-dimethoxycinnamic and *p*-coumaric acid). It did not recognize methyl esters of caffeic, vanillic and syringic acids. The enzyme has a pH optimum of 5, a temperature optimum between 55 and 60 °C, and released nitrophenol from nitrophenyl acetate. FAE-III has a specific activity of $31 \text{ U (mg protein)}^{-1}$ on de-starched wheat bran in the presence of *T. viride* xylanase and $3 \text{ U (mg protein)}^{-1}$ in the absence. FAE-III appears to bind to microcrystalline cellulose, and this binding is influenced by pH.

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