The purification and characterization of 4-hydroxy-3-methoxycinnamic (ferulic) acid esterase from *Streptomyces olivochromogenes*

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A 4-hydroxy-3-methoxycinnamic acid (ferulic acid) esterase has been purified from the extracellular broth of cultures of *Streptomyces olivochromogenes* after growth on oat spelt xylan. The purification procedure utilizes ion exchange on DEAE-BioGel A, anion exchange on Mono Q, gel filtration and hydrophobic interaction chromatography. The purified enzyme appeared as a single band on SDS-PAGE, with an apparent M_r of 29000. Two bands, at pI 7.9 and 8.5, were observed on isoelectric focusing. With methyl ferulate as substrate, the pH and temperature optima were 5.5 and 30 °C respectively, with a K_m of 1.86 mM and V_{max} of 0.3 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. The purified enzyme released ferulic acid from de-starched wheat bran only in the presence of xylanase.

Introduction

Plant cell walls are composed in part of lignocellulose, in which the complex lignin structure is covalently linked to hemicellulose and pectin. The crosslinking of lignin and arabinoxylans is mediated by phenolic acids, similar in structure to the phenylpropanoid units 4-hydroxy-3-methoxycinnamic acid (ferulic acid, FA) and 4-hydroxycinnamic acid (*p*-coumaric acid) of the lignin polymer (Scalbert *et al.*, 1985; Watanabe *et al.*, 1989). Ferulic acid and *p*-coumaric acid are common constituents of forage fed to animals, and may represent up to 2.5% by weight of the cell walls in temperate grasses (Hartley & Jones, 1977). Ferulic acid has been shown to be mainly associated with hemicellulose, where it is ester-linked (Mueller-Harvey *et al.*, 1986; Smith & Hartley, 1983), and many authors have isolated feruloylated compounds after treatment of cell wall polymers with 'cellulases' (Hartley *et al.*, 1990; Fry, 1982; Borneman *et al.*, 1990a). Feruloylated pectin has also been identified in spinach and the 'hairy region' of sugar beet pectins (Fry, 1982; Rombouts & Thibault, 1986). Cross-linkage has also been reported between ferulic acid from water-soluble pentosans and cysteine residues in proteins during wheat flour dough formation (Hoseney & Faubion, 1981). It has been postulated that ferulic acid may cross-link with

other phenolic acids to play a role in cell wall growth and stabilization (Iiyama *et al.*, 1990; Ishii & Hiroi, 1990).

Certain phenolic acids are indigestible and even toxic to many soil and other micro-organisms such as ruminal bacteria which come into contact with decaying plant materials (Borneman *et al.*, 1986; Chesson *et al.*, 1982; Jung & Sahlu, 1986). Certain bacteria on the other hand can survive in the presence of phenolic acids and can even utilize them as carbon sources (McCarthy, 1987).

Recently, ferulic acid esterase (FAE) activity has been reported in culture supernatants of *Streptomyces olivochromogenes* (Johnson *et al.*, 1988b), *Schizophyllum commune* (MacKenzie & Bilous, 1988) and *Aspergillus niger* (Johnson *et al.*, 1988a). In addition, a *p*-coumaric acid esterase was reported from *Streptomyces viridosporus* (Deobald & Crawford, 1987; Donnelly & Crawford, 1988) and from *Neocallimastix* MC-2 (Borneman *et al.*, 1990b), and both enzymes have been detected in anaerobic ruminal fungi (Borneman *et al.*, 1990a).

We have purified an FAE to homogeneity for the first time. The enzyme, from *S. olivochromogenes*, interacts synergistically with xylanase to release ferulic acid from wheat bran.

Methods

Bacterial strain. *Streptomyces olivochromogenes* (NRCC 2258), was obtained from the National Research Council of Canada Culture Collection, and grown in a proteose peptone/yeast extract/mineral salts medium designated IAF (Ishaque & Kleupfel, 1980). The trace element solution was as described by Hopwood *et al.* (1985). Inocula for such

Abbreviations: DSWB, de-starched wheat bran; FA, ferulic acid; FAE, ferulic acid esterase; MFA, methyl ferulate; PNA, *p*-nitrophenyl acetate; PNB, *p*-nitrophenyl butyrate; PN β X, *p*-nitrophenyl β -D-xylopyranoside.

cultures were obtained from 48 h cultures grown in Trypticase Soy Broth (BBL).

Growth. IAF medium containing 1% (w/v) oat spelt xylan (Fluka) was inoculated (5%, v/v) with a 48 h *S. olivochromogenes* culture, and incubated for 72 h at 37 °C in a 5-litre New Brunswick fermenter, with an agitation speed of 200 r.p.m., an air flow rate of 5 l min⁻¹, and the addition of 20 ml polyethylene glycol as antifoam prior to sterilization.

Harvesting. Cultures were harvested by ultrafiltration through a 0.45 µm Pelicon cassette filter (Millipore).

Concentration. Samples were concentrated using a Minitan (Millipore) ultrafiltration unit with a 10 kDa cut-off membrane. Filtrate was assayed for residual protein to check for correct membrane fraction.

Anion-exchange chromatography. The concentrated culture supernatant was passed down a DEAE-BioGel A column equilibrated with 10 mM-potassium phosphate (pH 7.0), supplemented with 1 mM-CaCl₂, 1 mM-MgCl₂, 0.2 mM-DTT, 1 mM-EDTA, 0.02% sodium azide and 0.05% aprotinin. The enzymes of interest do not adsorb to this column (Johnson *et al.*, 1988b) but are separated from dark pigments, which strongly bind to the gel.

The eluate from the BioGel column was concentrated and then 2 ml samples were fractionated by ion-exchange (Mono Q, Pharmacia) using a 0–1 M-NaCl gradient in 10 mM-Tris/HCl (pH 8.0) supplemented with the above additives. Fractions of 1 ml were collected and enzyme activities were measured as described below.

Gel filtration. Five millilitres of pooled and concentrated FAE-active fractions from the second anion-exchange chromatography step were passed down a gel filtration column (Sephadex S200HR) (90 × 2.6 cm) equilibrated with 10 mM-Tris/HCl (pH 8.0) supplemented with the above additives. Fractions of 5 ml were collected.

Hydrophobic interaction chromatography. The pooled and concentrated FAE-active fractions from the gel filtration column were fractionated on an FPLC hydrophobic interaction chromatography column (Phenyl-Superose, Pharmacia) using a 100–0% (saturation) (NH₄)₂SO₄ gradient in 50 mM-sodium phosphate (pH 7.0) and 1.7 M-(NH₄)₂SO₄ supplemented with the above additives (without aprotinin) at 0.5 ml min⁻¹. Fractions of 0.5 ml were collected.

Enzyme assays. FAE was assayed as described by Johnson *et al.* (1988b). De-starched wheat bran (DSWB) (Johnson *et al.*, 1988b) and methyl ferulate (MFA) (Fry, 1982) were prepared in MOPS buffer (pH 6.0), and used as substrates. One unit (U) of activity was defined as the amount of enzyme releasing 1 µmol ferulic acid (FA) min⁻¹ at pH 6.0 and 50 °C. 4-Hydroxy-3-methoxycinnamic acid (ferulic acid) obtained from Aldrich was used as a reference standard for HPLC.

Xylanase activity was assayed by measuring the release of reducing groups from 1% (w/v) 'purified' xylan (Adams, 1965) using dinitrosalicylic acid (Miller, 1959). One unit of activity was defined as the amount of enzyme releasing 1 µmol xylose min⁻¹ at pH 5.3 and 50 °C. Xylan (from larchwood) was obtained from Koch-Light.

β-Xylosidase was determined by measuring (ΔA₄₀₀) *p*-nitrophenol release from *p*-nitrophenyl β-D-xylopyranoside (PNβX) (Sigma). A solution of 5 mM-PNβX in 50 mM-sodium citrate buffer (pH 4.0) was incubated at 50 °C for 10 min in the presence of enzyme.

Extracellular esterase was measured spectrophotometrically by the method of Donnelly & Crawford (1988) using *p*-nitrophenyl butyrate (PNB, 0.4 mM, dissolved in acetonitrile) as the substrate. Acetyl esterase activity was measured using a similar method except that *p*-nitrophenyl acetate (PNA, 1 mM, dissolved in dimethyl sulphoxide) was used as substrate. One unit is defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol min⁻¹ at pH 7.0 and 25 °C for PNB, and at pH 6.0 and 30 °C for PNA. Both substrates were obtained from Sigma.

All assays were performed in duplicate, with blanks to correct for background reactions in enzyme and substrate samples. Protein was estimated using the Coomassie Protein Assay Reagent from Pierce.

M_r determination. M_r values were estimated by SDS-PAGE by the method of Laemmli (1970) using a 10% (w/v) acrylamide gel. Proteins were detected with the silver staining kit from Sigma. M_r values were estimated from a plot of log M_r versus mobility using the following standards: bovine serum albumin (66000), egg albumin (45000), glyceraldehyde-3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20100) and α-lactalbumin (14200).

Isoelectric point determination. The isoelectric point of FAE was determined using a pH 3.5–9.5 PAGplate (Pharmacia). Samples were dialysed against distilled water prior to focusing. pI values were determined from a plot of migrated distance versus pI values of standards obtained from BDH ('Electran' pI calibration kit, range 4.7–10.6).

Enzyme kinetics. Freshly prepared enzyme (10 µg ml⁻¹) was incubated with methyl ferulate (MFA) in a concentration range of 0–1416 nmol ml⁻¹ for 30 min at 50 °C and then assayed for release of FA as described above. Data were analysed by the method of Wilkinson (1961).

Temperature optimum. Freshly purified enzyme (0.1 ml) was incubated with 236 nmol MFA ml⁻¹ at a temperature range of 20–70 °C using a thermostatically controlled rotating incubator. The optimum activity was calculated from the total µmol product released after 30 min incubation.

pH optimum. Freshly prepared enzyme (10 µg ml⁻¹) was incubated at 50 °C with 590 nmol MFA ml⁻¹ in MOPS buffer titrated to a pH in the range 4.0–8.0 at 0.5 unit increments. The optimum activity was calculated as for the temperature optimum.

Effect of xylanase. Freshly prepared xylanase and FAE were added together in various amounts and incubated in the presence of 25 mg DSWB and MOPS buffer.

Results and Discussion

Purification of ferulic acid esterase

Table 1 summarizes a typical purification of FAE from *S. olivochromogenes*. FAE was purified from material secreted into the supernatant. Removal of dark pigments by the first anion-exchange chromatography step (Bio-Gel A) resulted in a slight increase in specific activity with MFA as the substrate. The yield and specific activity decreased upon fractionation by the second anion-exchange step (Mono Q), even though FAE-active fractions did not appear to bind to this column, and a large amount of protein with no FAE activity was eluted with 1 M-NaCl. Gel filtration chromatography resulted in the late elution of FAE as a peak with a shoulder (Fig. 1), referred to as GF I and GF II respectively. SDS-PAGE showed a mixture of proteins (15–43 kDa) in each peak (results not shown). GF I contained the highest activity and was fractionated with a hydrophobic interaction chromatography column. SDS-PAGE

Fig. 1. Elution profile of the pooled material from the second anion-exchange chromatography column on a gel filtration column. Material was eluted in 10 mM-Tris (pH 8.0) with a flow rate of 1 ml min⁻¹. Fractions of 5 ml were collected. —, A_{280} ; ●, FAE activity.

showed the FAE-containing fractions to be a sharp, single band after silver staining. A 47.5-fold purification, with a yield of 5.5% and a specific activity of 0.19 U (mg protein)⁻¹, were obtained after these steps.

Purification of FAE using DSWB as substrate

Table 2 summarizes a typical purification of FAE, by the same steps as in Table 1, when DSWB rather than MFA was used as the assay substrate. The ability to release FA from DSWB was detected in the MFA-hydrolysing sample, until fractionation by hydrophobic interaction chromatography. Higher apparent yield and purification were achieved when DSWB was used as substrate than when using MFA up until Phenyl-Superose separation. The loss of activity was due to the separation of xylanase from FAE fractions (Fig. 2) and we believe the same enzyme releases FA from both MFA and DSWB (see later).

Properties of the enzyme

The enzyme lost activity over 24 h in the pure form at 4 °C or -20 °C and pH 7.0, even in the presence of EDTA, DTT, Mg²⁺ and Ca²⁺. These additives greatly stabilized the enzyme during purification, and it is possible that FAE may be part of a protein complex which is required for enzyme stability. Note that a peak containing both FAE and xylanase activity was located between the two main enzyme-containing peaks after Phenyl-Superose fractionation (Fig. 2).

The protein from the hydrophobic interaction column migrated on SDS-PAGE as a single band with an M_r of 29000. A comparable result from gel filtration chromato-

graphy could not be obtained due to insufficient amounts of the purified enzyme. Using gel filtration to determine the M_r during the purification step also proved inadequate since GF I appeared to be retarded by Sephacryl. Similar retardation on Sephadex, which is based on the same dextran matrix, had been reported for xylanases (Paice *et al.*, 1978). It could be that an FAE/xylanase complex exists at that stage, giving rise to late elution owing to retention of the xylanase. The M_r of the enzyme is different from that of an extracellular esterase (M_r 36000), capable of hydrolysing PNB, reported from *Streptomyces scabies* (McQueen & Schottel, 1987); it also differs from that (M_r 25000) of another enzyme from the same organism capable of hydrolysing PNB and cutin (Lin & Kolattukudy, 1980).

Two forms of FAE were observed, at pI 7.9 and 8.5, by isoelectric focusing. They appeared in the same pI region as several acetyl esterases from *S. olivochromogenes* (MacKenzie *et al.*, 1987). Johnson *et al.* (1988b) reported three distinct forms of FAE from their crude culture broth elution profiles, which suggests the presence of a number of forms of FAE in the organism.

The FAE had optimum activity (with MFA as substrate) at pH 5.5 and 30 °C, although smaller peaks at pH 4.5 and 60 °C existed in the activity curves (results not shown). It is possible that these secondary peaks may involve the two isoenzymes detected by IEF. Both these pH values are lower than the optimum of pH 9.0 for the esterase from *S. viridosporus* T7A (Donnelly & Crawford, 1988), which hydrolysed PNB and lignin-esterified *p*-coumaric acid, again suggesting that FAE is a different enzyme. The optimum pH and temperature also differ from the values of pH 7.5 and 50 °C for the *p*-coumaric acid esterase from *Neocallimastix* MC-2 (Borneman *et al.*, 1990b). Presumably both FAE and *p*-coumaric acid esterase are present in *S. olivochromogenes*, and their specificities differ for the phenolic acids.

The K_m and V_{max} values for the hydrolysis of MFA were determined as 1.86 (SD $\pm$ 0.58) mM and 0.3 (SD $\pm$ 0.06) U mg⁻¹, respectively. The only published results we have found using methyl cinnamic acid esters as an assay substrate were for the crude supernatant of *Neocallimastix*, and no kinetic data were given (Borneman *et al.*, 1990a).

Associated enzyme activity during FAE purification

Throughout the purification steps, the FAE-active samples were assayed for other enzyme activities associated with either xylan hydrolysis or general esterase activity (butyryl esterase, acetyl esterase, xylanase and β -xylosidase) to determine whether FAE was a unique FA de-esterifying enzyme, a general esterase, or inherent activity of a xylanase enzyme.

Fig. 2. Elution profile of GF I on a hydrophobic interaction chromatography column. Material was eluted with a 100–0% $(\text{NH}_4)_2\text{SO}_4$ gradient, and 0.5 ml fractions were collected. ---, $(\text{NH}_4)_2\text{SO}_4$; —, A_{280} ; ●, FAE activity; ▲, xylanase activity.

Table 1. *Purification of FAE from S. olivochromogenes*

The results are from a typical purification using MFA as substrate.

Step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude culture supernatant	1024	4.00	0.004	1.00	100
DEAE BioGel A 1st anion exchange	218	2.95	0.014	3.47	74
Mono Q 2nd anion exchange	96	0.42	0.004	1.11	10
Sephacryl S200HR gel filtration	3	0.16	0.054	13.5	4
Phenyl-Superose hydrophobic interaction	0.46	0.22	0.190	47.5	5.5

Table 2. *Purification of FAE using DSWB as a substrate*

Step	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude culture supernatant	18.5	0.018	1.00	100
DEAE BioGel A 1st anion exchange	10.2	0.047	2.59	55
Mono Q 2nd anion exchange	6.78	0.071	3.92	37
Sephacryl S200HR gel filtration	4.57	1.51	83.8	25
Phenyl-Superose hydrophobic interaction	0	0	0	0

Table 3. Xylanase activity, on 1% 'purified' xylan, associated with each step in FAE purification

Step	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Purification (-fold)	Yield (%)
Crude culture supernatant	77.8	75.9	1	100
DEAE BioGel A 1st anion exchange	48.1	221	2.9	62
Mono Q 2nd anion exchange	16.7	174	2.3	21
Sephacryl S200HR gel filtration	1.2	398	5.2	1.5
Phenyl-Superose hydrophobic interaction	0	0	0	0

Butyrate esterase activity was present in FAE-active preparations only until hydrophobic interaction chromatography fractionation. GF II contained 16% of total PNB-hydrolysing activity (48 U mg⁻¹). The separation of FA- and PNB-hydrolysing activities suggests that FAE is a completely separate enzyme from those esterases reported previously.

Acetyl esterase activity was detected during all stages of FAE purification, although the activity in the FAE fraction after hydrophobic interaction chromatography separation amounted to only 0.008% of the total initial acetyl esterase activity. Thus purified FAE has associated general acetyl esterase activity (10.4 U mg⁻¹). Again most of the acetyl esterase activity was associated with GF II, and it may be that a general esterase, active against PNB and PNA, is present in that sample. Acetyl esterase was found to bind to a cation-exchange column by Johnson *et al.* (1988b) along with an FAE-active fraction, but separate from the main, unbound FAE sample. In the present work, only 25% of acetyl esterase activity was recovered after the second anion-exchange chromatographic separation, with a 0.44-fold purification after hydrophobic interaction chromatography, indicating that FAE is not the main PNA-hydrolysing enzyme in *S. olivochromogenes*.

Xylanase activity was detected at all stages until separated by hydrophobic interaction chromatography (Table 3). It was important to separate FAE from xylanase to observe effects between the two enzymes on DSWB (see Table 2). The xylanase in GF I accounts for only 3% of total xylanase activity in *S. olivochromogenes*, and thus was probably not the major endoxylanase of pI 6.8 mentioned by MacKenzie *et al.* (1987). The xylanase was clearly separated from the FAE-active fractions on the hydrophobic interaction column (Fig. 2).

Only β -xylosidase activity was found to correlate with ferulate and *p*-coumarate esterase activities in ruminal anaerobic fungi (Borneman *et al.*, 1990a). Virtually no β -xylosidase was detected in the crude culture supernatant of *S. olivochromogenes* in this study, and activity was not detected in the GF I sample. This suggests that cooperativity between FAE and xylanase may be important in *S. olivochromogenes*, as in *Schizophyllum commune* (MacKenzie & Bilous, 1988), while cooperativity between FAE and β -xylosidase may be more important in *Neocallimastix* and *Orpinomyces* species.

Interaction between FAE and xylanase on DSWB

FAE was unable to release FA from DSWB in the absence of xylanase (Table 2). This agrees with the results of MacKenzie & Bilous (1988), who reported that FAE and xylanase act synergistically in *Schizophyllum commune*. Xylanase was present in FAE-active samples up until Phenyl-Superose fractionation (Table 3), and adding back the xylanase fraction to the pure FAE sample induced release of FA (Fig. 3). No FA was released when FAE and xylanase were present on their own, or when one of the enzymes was boiled, showing that release of FA was solely due to the synergistic action of both enzymes. This suggests that FAE requires low- M_r substrates to act upon, and xylanase hydrolyses the arabinoxylose chain into smaller units on which FAE can act. Thus, it appears that FAE can release FA from plant cell wall material only in the presence of a polysaccharide-hydrolysing enzyme.

Similar synergistic interactions are common in xylan hydrolysis. Biely *et al.* (1986) found that xylanase cleaved a limited number of glycosidic bonds, but the extent and rate of breakdown increased when acetyl-xylan esterase

Fig. 3. Release of FA from DSWB by FAE and xylanase. Pooled xylanase-containing fractions, after hydrophobic interaction chromatography fractionation, were added to purified FAE together with DSWB (see Methods). The release of FA was measured as a function of xylanase activity. No FA was released when FAE or xylanase alone was incubated with DSWB.

was present. In addition, Johnson *et al.* (1989) found synergism between xylanase and 4-*O*-methylglucuronidase for the release of 4-*O*-methylglucuronic acid from lignocellulose. Neither enzyme independently released the acid. An arabinofuranosidase from *Ruminococcus albus* 8 was found to enhance the hydrolytic action of hemicellulolytic or pectinolytic enzymes on alfalfa cell wall material (Greve *et al.*, 1984). It is thus common to find synergism between plant cell wall degrading enzymes.

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