



CHEMISTRY & BIOLOGY INTERFACE

An official Journal of ISCB, Journal homepage; www.cbijournal.com

An efficient assay for ferulic acid esterase and *para*-coumaric acid esterases in plants at physiologically relevant activities

Ifat Parveen,^{a,*} Iain S. Donnison,^a Joshua Insole,^a Ana L. Winters,^a Susan J. Dalton,^a Timothy J. Woodman,^b Michael D. Threadgill^b

^aInstitute of Biological, Environmental and Rural Sciences, Aberystwyth University, Gogerddan, Aberystwyth SY23 3EB, United Kingdom.

^bMedicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, United Kingdom.

*Corresponding author: Tel: +44 (0)1970 823216; Fax: +44 (0)1970 828357; E-mail: ifp@aber.ac.uk Received 14 August 2016; Accepted 17 October 2016

Abstract: 4-Nitrophenyl ferulate and 4-nitrophenyl coumarate have been synthesised as chromogenic substrates for measurement of the activity of feruloyl esterases. These enzymes have been isolated from a number of fungi and are found in micro-organisms involved in the degradation of plant material. Ferulic and *para*-coumaric acid were coupled with 4-nitrophenol using water-soluble carbodiimide to yield 4-nitrophenyl ferulate and 4-nitrophenyl coumarate, respectively. Maize, tall fescue and *Miscanthus* varieties, transformed with an *Aspergillus niger* ferulic acid esterase (FAE) gene, were analysed for activity using these synthetic substrates. This technique gave a rapid measurement of enzymic activity when compared with the conventional HPLC assay. This colorimetric assay was also more sensitive and accurate for determining rates of activity. A rapid high-throughput assay, based on substrates described here, would greatly facilitate identification of enzymes with potential to increase the efficiency of sugar release from lignocellulosic biomass and thereby reduce costs of processing.

Keywords: *para*-nitrophenyl ferulate; *para*-nitrophenyl coumarate; feruloyl esterase; coumaroyl esterase; rapid screen; HPLC-PDA

Introduction

The lack of sustainable fossil fuels has kindled worldwide interest in plant biomass as a renewable source of biofuels, bioenergy and chemicals. Cellulose / lignocellulose is the world's most abundant source of renewable material [1,2]; approximately 180 million tons of agricultural feedstocks and forest litter are available *per annum*[3]. Nevertheless, plant cell walls represent a resource which is largely untapped, owing to the cross-linking of hydroxycinnamic acids (specifically ferulic and *para*-coumaric acids) to lignin and polysaccharide polymers. This cross-linking alters the physical and chemical properties of the cell wall, for example, extensibility, plasticity, and digestibility, by limiting the access of hydrolytic enzymes [4-6].

The use of plant cell walls as a renewable resource is not only sustainable but also reduces emissions of CO₂ and diminishes agricultural waste sent to landfill. Nevertheless, hydrolysis of highly recalcitrant cellulose and cellwall polysaccharides requires many enzymes, including hemicellulases, β -glucanases, xylanases, pectinases, xyloglucanases and esterases [4,6]. The latter group of enzymes, cinnamoyl ester hydrolases, include ferulic / para-coumaric acid esterases [6]. Moreover, ferulic acid esterases (FAEs; EC 3.1.1.73) and microorganisms have been shown to release ferulic acid by hydrolysing ferulate esters involved in the cross-links between hemicelluloses and between hemicelluloses and lignin [6]. Recently, this family of enzymes has been further divided into four functional classes A-D [6,7]. Monomeric ferulate and coumarates are present as esters with arabinose; esterified dehydrodimers of ferulates are also observed in monocot cell walls [4,8].Ferulate dimers have been shown to cross-link cell-wall polymers covalently, providing structural stability and making the cell walls resistant to enzymecatalysed hydrolysis [4,9].

There are many applications for FAEs; for example, they can be used in the pharma-

ceutical industry for production of medicinal compounds. agro-industrial in processes for fine / speciality chemicals, for treatment of pulp and for production of biofuels [6]. Moreover, FAEs are used to degrade cellulosic biomass and provide tools to facilitate a greater understanding of the intricate network and linkage patterns within the plant cell wall. Of the cinnamoyl esterases, the most commonly studied have been FAEs [10]. Ferulic acid esterases of Type A (FAEA) from Aspergillus niger and Fusarium oxysporum have been shown to hydrolyse preferentially the $(1 \rightarrow 5)$ ester bond in arabinose ferulates, whereas Type B and Type C ferulic acid esterases are less substrate-selective [11]; these enzymes have demonstrated high specificity for 4'-hydroxy-3'-methoxycinnamate and 3-(4-hydroxy-3methoxyphenyl) propanoate esters. Several cinnamic acid esterases have been identified. which show a higher affinity for coumaric acid esters, compared with ferulic acid esters [12-14]. This is noteworthy, since *p*-coumaroylated heteroxylan side chains have been reported in maize bran, indicating that esterases with a high specificity for coumaroyl linkages may enhance breakdown of fibre fractions [15]. However, a specific caffeoyl esterase has also been reported [16].

High-performance liquid chromatography (HPLC) with UV detection has been the predominant method used to date to quantify the enzymic activity of feruloyl esterases [3,10,17]. Other methods have also been investigated, including capillary-zone electrophoresis and

[°]Abbreviations used: 5-5-DiFA, 5-5-diferulic acid diethyl ester; 8-O-4-DiFA, 8-O⁴-diferulic acid diethyl ester; BBSRC, Biotechnology and Biological Sciences Research Council; DCC, N,N'-dicyclohexylcarbodiimide; DIC, N,N'-diisopropylcarbodiimide; DMF, dimethylformamide; DMSO, dimethylsulfoxide; ESI, electrospray ionization; EtOAc, ethyl acetate; FAE, ferulic acid esterase; FAEA, ferulic acid esterase A; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; FW, fresh weight; HMBC, heteronuclear multiple bond correlation spectroscopy; HPLC, high-performance liquid chromatography; HPLC-PDA, high-performance liquid chromatography with photodiode array detection; HRMS, high-resolution mass spectrometry; HSQC, Heteronuclear single quantum coherence spectroscopy; ICR, ion cyclotron resonance; LC-ESI-MS/MS, liquid chromatographyelectrospray ionization-tandem mass spectrometry; LoD, limit of detection; MS², tandem mass spectrometry; WSR, nuclear magnetic resonance spectroscopy; SIM, selected ion monitoring; THF, tetrahydrofuran; UV, ultra-violet spectrometry; WSC, 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide

gas chromatography-mass spectrometry [7,10]. However, these methods do not provide a high-throughput platformfor screening a large number of samples. To overcome this, several chromogenic and fluorogenic compounds have been synthesised for the detection of hydrolytic activities of enzymes [7,10,11,17]. These have recently been reviewed [18]. Here, we report the straightforward synthesis of 4-nitrophenyl ferulate and 4-nitrophenyl coumarate as chromogenic substrates and demonstrate measurement of feruloyl-and para-coumaroyl esterase activity in transgenic plants transformed with a fungal FAE.

Materials and methods

General Experimental Procedures. Melting points were determined on a MEL-TEMP[®] capillary melting point apparatus with a thermocouple attached to a digital thermometer (Sigma-Aldrich, U.K.) and are uncorrected. ¹H, ¹³C and 2-D NMR spectra were recorded on a Bruker DRX500 instrument operating at 500 MHz (for ¹H) and 125 MHz (for ¹³C), using CD_3OD as the solvent and $SiMe_4$ as the internal standard. Chemical shifts (δ) are given in ppm relative to SiMe₄. Experiments were conducted at ambient temperature, unless otherwise noted. The brine was saturated. High resolution accurate masses (3,4,5 and 8) were recorded on a Nano – Flow (Triversa Nanomate; Advion Biosciences Limited, Norfolk, UK) linear trap quadrupole Fourier Transformation Ion Cyclotron Resonance Mass Spectrometry Ultra (FT-ICR-MS). Samples were dissolved in MeOH / H_2O (4:1) at 1.0 mg mL⁻¹. For each sample, 13 μL was injected by the Triversa Nanomate, with a 5 μ L aliquot being delivered to the ICR cell. Gas pressure was maintained at 0.5 psi, with an applied voltage of 1.5 KV, and the scan range was from m/z 50 to 500. When the FT-ICR-MS was operating in the SIM mode, the resolution was 100,000.

Where only integer masses are given (2 and 6), these data were obtained on a Thermo-Finnigan HPLC/MSⁿ system (Thermo Electron



Scheme 1. Synthetic approaches to nitrophenyl esters **5** and **8**. *Reagents*: i, Bu'Me₂SiCl, Prⁱ₂NEt, CH₂Cl₂; ii, K₂CO₃, THF; iii, (COCl)₂; DMF, CH₂Cl₂; iv, 4-nitrophenol, Et₃N, CH₂Cl₂; v, fluoride ion, various conditions; vi, DCC, 1,4-dioxane; vii, WSC, EtOAc.

Chemistry & Biology Interface

Corporation, USA). Other mass spectra were acquired using a Finnigan LTQ linear trap quadruple instrument with an ESI source (Thermo Electron Corporation, USA). N₂ was used as the sheath and auxiliary gas and He was used as the collision gas. Spectra obtained in negative-ion mode used interface parameters: sheath gas, 30 arbitrary units; auxiliary gas, 10 U; spray voltage, 4.0 KV; capillary temperature 320°C; capillary voltage, -31 V; tube lens offset, -63 V. Spectra obtained in negative-ion mode used interface parameters: sheath gas, 30 arbitrary units; auxiliary gas, 30 arbitrary voltage, -31 V; tube lens offset, -63 V. Spectra obtained in negative-ion mode used interface parameters: sheath gas, 30 arbitrary units; auxiliary gas, 5 U; spray voltage, 4.0 KV; capillary temperature 320°C; capillary voltage, 23 V; tube lens offset, 50 V.

Ethyl *E*-3-(4-hydroxyphenyl)propenoate / ethyl coumarate was synthesised by the method of Cernerud *et al* [19].

tert-Butyldimethylsilyl E-3-(4-(tertbutyldimethylsilyloxy)-3-methoxyphenyl) propenoate / bis-silylated ferulate (2). Bu'Me₂SiCl (6.09 g, 40.5 mmol) was added to $Pr_{2}^{i}NEt(6.24 \text{ g}, 48.4 \text{ mmol}) \text{ and } E-3-(4-hydroxy-$ 3-methoxyphenyl)propenoic acid (ferulic acid; 1) (3.13 g, 16.1 mmol) in CH₂Cl₂(20 mL) and the mixture was stirred for 48 h at 20°C and at reflux for 30 h. The evaporation residue, in EtOAc (100 mL), was washed with H₂O (20 mL), aq. HCl (1.0 M, 2×50 mL) and brine $(2 \times 50 \text{ mL})$ before being dried (MgSO₄). Evaporation gave 2 (6.72 g, quant.) as a waxy pale yellow solid: ¹H NMR (500 MHz, CDCl₂) δ 0.17 (6 H, s, SiMe₂), 0.33 (6 H, s, SiMe₂), 0.99 $(18 \text{ H}, \text{ s}, 2 \times \text{Bu}^{t}), 3.84 (3 \text{ H}, \text{ s}, \text{OMe}), 6.27 (1$ H, d, J = 15.8 Hz, propenoate 2-H), 6.84 (1 H, $d, J = 8.6 Hz, Ar 5-H), 7.02 (2 H, m, Ar 2, 6-H_2),$ 7.55 (1 H, d, J = 15.8 Hz, propendate 3-H); ¹³C NMR (125 MHz, CDCl₂) δ -4.5 (2 × SiMe₂), $18.0 (SiCMe_2), 18.6 (SiCMe_2), 25.8 (2 \times CMe_2),$ 55.6 (OMe), 111.0 (Ar 2-C), 118.0 (propenoate 2-C), 121.2 (Ar 5-C), 122.5 (Ar 6-C), 128.5 (Ar 1-C), 145.3 (propenoate 3-C), 147.6 (Ar 4-C),

151.4 (Ar 3-C), 167.3 (C=O).

E-3-(4-(tert-Butyldimethylsilyloxy)-3methoxyphenyl)propenoic acid / monosilvlated ferulic acid (3). Bis-silvlated ferulate 2 (6.72 g, 15.9 mmol) was stirred with K_2CO_2 (3.30 g, 23.8 mmol) in THF (20 mL) for 5 h. The mixture was diluted with EtOAc (60 mL) and washed with H₂O (40 mL), aq. HCl (1.0 M, 30 mL) and brine (30 mL). Drying (MgSO₄) and evaporation gave 3 (4.84 g, 99%) as an off-white solid: mp 145-146°C; ¹H NMR (500 MHz, CDCl₂) δ 0.18 (6 H, s, SiMe₂), 1.00 (9 H, s, Bu^t), 3.85 (3 H, s, OMe), 6.32 (1 H, d, J = 15.8 Hz, propenoic acid 2-H), 6.86 (1 H, d, J = 8.7 Hz, Ar 5-H), 7.05 (2 H, m, Ar 2,6-H₂), 7.73 (1 H, d, J = 15.8 Hz, propenoic acid 3-H); ¹³C NMR (125 MHz, CDCl₂) δ -4.5 (SiMe₂), 18.6 (CMe₂), 25.8 (CMe₂), 55.6 (OMe), 111.2 (Ar 2-C), 118.0 (propenoic acid 2-C), 122.5 (Ar 5-C), 122.9 (Ar 6-C), 128.1 (Ar 1-C), 147.3 (propenoic acid 3-C), 148.2 (Ar 4-C), 151.4 (Ar 3-C), 172.9 (C=O); λ_{max} (nm) 295, 319. HRMS (ESI +ve) 309.1523 (M + H) ($C_{16}H_{24}O_{4}S$ requires 309.1522).

4-Nitrophenyl E-3-(4-(tertbutyldimethylsilyloxy)-3-methoxyphenyl) propenoate / mono-silylated para-nitrophenyl ferulate (4). The silvlated ferulic acid 3 (1.02) g, 3.25 mmol) was stirred with oxalyl chloride (500 µL, 762 mg, 6.0 mmol) and DMF (25 µL) in $CH_2Cl_2(20 \text{ mL})$ for 2 h. Evaporation gave the crude acid chloride. To this acid chloride, in CH₂Cl₂ (20 mL), was added 4-nitrophenol (440 mg, 3.2 mmol) and triethylamine (354 mg, 3.5 mmol) and the mixture was stirred for 16 h. The mixture was washed with water $(3 \times 50 \text{ mL})$ and brine $(3 \times 50 \text{ mL})$ and was dried (MgSO₄). Evaporation and column chromatography (silica gel, CH_2Cl_2 / hexane 4:1) yielded 4 (850 mg, 67%) as pale yellow crystals: mp 101-103°C; ¹H NMR (500 MHz, CDCl₂) δ 0.19 (6 H, s, SiMe₂), 1.02 (9 H, s, Bu^t), 3.87 (3 H, s, OMe), 6.47 (1 H, d, J = 15.9 Hz, propendate 2-H), 6.89 (1 H, d, J = 8.1 Hz, Ar 5-H), 7.10 (1 H, m,

6-H), 7.26 (1 H, s, Ar 2-H), 7.45 (2 H, m, NP 2,6-H₂), 7.84 (1 H, d, J = 15.9 Hz, propenoate 3-H), 8.30 (2 H, d, J = 9.1 Hz, NP 3,5-H₂); ¹³C NMR (125 MHz, CDCl₃) δ -5.3 (SiMe₂), 18.5 (CMe₃), 25.6 (CMe₃), 55.5 (OMe), 111.2 (Ar 2-C), 113.7 (propenoate 2-C), 121.2 (Ar 5-C), 122.5 (NP 2,6-C₂), 122.9 (Ar 6-C), 125.2 (NP 3,5-C₂), 127.6 (Ar 1-C), 145.2 (NP 4-C), 148.1 (Ar 3-C), 148.9 (propenoate 3-C), 151.4 (Ar 4-C), 155.8 (NP 1-C), 164.2 (C=O); HRMS (ESI +ve) 430.16856 (M - H) (C₂₂H₂₇NO₆Si requires 430.16859).

N,N'-Dicyclohexyl-O-(*E*-3-(4-hydroxy-

3-methoxyphenyl)propenoyl)isourea (6). N,N'-Dicyclohexylcarbodiimide (1.30 g, 6.24 mmol) was added to 1 (1.10 g, 5.67 mmol)and 4-nitrophenol 4 (783 mg, 6.24 mmol) in dry 1,4-dioxane (15 mL) and the mixture was stirred for 24 h. The mixture was cooled on ice for 2 h and was filtered (Celite[®]). Evaporation and column chromatography (CHCl, / EtOAc 25:1) yielded 6 (980 mg, 43%) as a pale yellow oil: ¹H NMR (500 MHz, CDCl₂) δ 1.0-2.2 (20 H, m, $10 \times CH_2$), 3.85 (3 H, s, OMe), 4.13 (1 H, m, cHex 1-H), 4.30 (1 H, m, cHex 1-H), 5.95 (1 H,s, NH), 6.55 (1 H, d, J = 15.8 Hz, propenoyl 2-H), 6.90 (2 H, m, Ar 2,5-H₂), 7.05 (1 H, d, J = 8.6 Hz, Ar 6-H), 7.55 (1H, d, J = 15.8 Hz, propenoyl 3-H); LC-ESI-MS/MS m/z 399 [M -H]⁻, 384 [M – (H, CH₃)]⁻, λ_{max} (nm) 327.

4-Nitrophenvl E-3-(4-hydroxy-3methoxyphenyl)propenoate / paranitrophenyl ferulate (5). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide. hydrochloride (WSC; 1.10 g, 5.67 mmol) and Prⁱ₂NEt (1.46 g, 11.3 mmol) were added to 1(1.10 g, 5.67 mmol) and 4 (0.80 g, 5.67 mmol) in EtOAc (60 mL) and the mixture was stirred for 24 h. The mixture was taken up in EtOAc (100 mL) and was washed with aq. NaHCO, (2%, 50 mL), aq. citric acid (5%, 50 mL) and brine (50 mL). Drying (MgSO₄), evaporation and chromatography (CH₂Cl₂) gave 5 (0.85g, 48%) as off-white crystals: mp 183-184°C

(lit.[17] mp 176-178°C); ¹H NMR (500 MHz, $(CD_3)_3SO$ δ 3.84 (3 H, s, OMe), 6.75 (1 H, d, J = 15.8 Hz, propenoate 2-H), 6.84 (1 H, d, J =8.1 Hz, Ar 5-H), 7.24 (1 H, dd, J = 8.1, 2.0 Hz, Ar 6-H), 7.45 (1 H, d, J = 2.0 Hz, Ar 2-H), 7.52 $(2 \text{ H}, d, J = 9.1 \text{ Hz}, \text{NP } 2,6-\text{H}_2), 7.82 (1 \text{ H}, d, J)$ = 15.8 Hz, propenoate 3-H), 8.32 (2 H, d, J =9.1 Hz, NP 3,5-H₂), 9.78 (1 H, s, OH); ¹³C NMR (125 MHz, DMSO) δ 55.75 (OMe), 111.61 (Ar 2-C), 112.56 (propenoate 2-C), 115.59 (Ar 5-C), 123.15 (NP 2,6-C₂), 123.98 (Ar 6-C), 125.23 (NP 3,5-C₂), 125.29 (Ar 1-C), 144.85 (NP 4-C), 148.02 (Ar 3-C), 148.15 (propenoate 3-C), 150.12 (Ar 4-C), 155.63 (NP 1-C), 164.59 (C=O); HRMS (ESI -ve) 314.0666 (M - H) $(C_{16}H_{13}NO_{6} requires 314.0665).$

4-Nitrophenvl E-3-(4-hydroxyphenyl) propenoate / para-nitrophenyl coumarate (8).1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide.hydrochloride (WSC; 2.40 g, 12.8 mmol) and Prⁱ₂NEt (2.48 g, 19.2 mmol) were added to 7 (2.10 g, 12.8 mmol) and 4-nitrophenol (0.890 g, 6.4 mmol) in dry THF (20 mL) and the mixture was stirred for 24 h. The evaporation residue, in EtOAc (100 mL), was washed with aq. NaHCO₂ (2%, 2×50 mL), aq. citric acid (5%, 50 mL) and brine (50 mL). The solution was dried (MgSO $_{4}$) and the solvent evaporated. Column chromatography was (CH_2Cl_2) yielded the ester 8 (0.866 g, 47%) as pale yellow crystals: mp 177-178°C (lit.[20] mp 174-175°C); ¹H NMR (500 MHz, DMSO) δ 6.66 (1 H, d, J = 15.9 Hz, propendate 2-H), 6.84 $(2 \text{ H}, d, J = 8.7 \text{ Hz}, \text{Ar } 3.5 \text{-H}_2), 7.51 (2 \text{ H}, d, J =$ 9.0 Hz, NP 2,6-H₂), 7.67 (2 H, d, J = 8.7 Hz, Ar 2,6-H₂), 7.83 (1 H, d, J = 15.9 Hz, propendate 3-H), 8.32 (2 H, d, J = 9.0 Hz, NP 3, 5-H₂), 10.19 (1 H, s, OH); ¹³C NMR (125 MHz, DMSO) δ 112.3 (propenoate 2-C), 115.9 (Ar 3,5-C₂), 123.2 (NP 2,6-C₂), 124.8 (Ar 1-C), 125.2 (NP 3,5-C₂), 131.0 (Ar 2,6-C₂), 144.85 (NP 4-C), 147.8 (propenoate 3-C), 155.6 (NP 1-C), 160.6 (Ar 4-C), 164.6 (C=O); HRMS (ESI-ve) 284.05645 (M - H) (C₁₅H₁₁NO₅ requires 284.055899).

Enzyme assays

Assays with a commercial preparation of cinnamoyl esterase

The approximate pH optimum for the enzymatic hydrolysis was estimated as follows. Stock solutions of 4-nitrophenyl ferulate 5 and 4-nitrophenyl coumarate 8 (1.5 and 1.4 mM, respectively) were prepared in dimethylsulfoxide. Reaction mixtures were assembled by adding substrate stock solutions (30 µL) to mixtures of commercial preparation of feruloyl esterase (Depol 740, Biocatalysts Ltd., UK) (10 μ L) and McIlvaine buffer (pH 4.5, pH 5.5 or pH 6.0) in duplicate. Triplicate aliquots (100 μ L) from each were added to wells in a 96-well plate. After 30 min incubation at 25°C, the absorbance was measured at 347 nm with a µQuant Microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, USA) plate reader. Calibration curves for 4-nitrophenol were established using known concentrations of 4-nitrophenol in the reaction buffers.

For the main assays, stock solutions of the nitrophenyl esters were prepared as follows. para-Nitrophenyl ferulate 5 (6.3 mg, 20 umol) was dissolved in dimethylsulfoxide (2.0 mL) in an Eppendorf tube to make a 10 Similarly, para-nitrophenyl mM solution. coumarate 8 (5.7 mg, 20 µmol) was dissolved in dimethylsulfoxide (2.0 mL). Reaction mixtures were prepared in triplicate in Falcon tubes comprising McIlvaine buffer (citric acid (100 mM), Na, HPO₄ (200 mM), 48.5:51.5, pH 5.0) (4.485 mL) and stock substrate solution (500 μ L). The enzymatic reactions were initiated by addition of ferulic acid esterase (FAE) (Depol 740 L Biocatalysts) (15 μ L). The tubes were incubated at 25°C. At each time point, an aliquot (500 μ L) was removed from each Falcon tube and Na₂HPO₄ (200 mM, 500 μ L) was added to each. Aliquots $(3 \times 150 \ \mu L)$ were taken from each sample and placed in the wells of a 96well plate. The absorbance of the contents of

each well was measured immediately using the plate reader at 410 nm. Blanks were prepared by omission of the enzyme.

Samples were prepared for HPLC analysis as follows. Samples (1.0 mL) were passed into C₁₈ Sep Pak cartridges (500 mg; previously cleaned with MeOH, then 5% aq. AcOH), which were then washed with 5% aq. AcOH (4.0 mL). The esters and hydrolysis products were eluted with MeOH (4.0 mL). Aliquots (2.0 mL) were transferred to Eppendorf tubes and the solvent was evaporated at ambient temperature with a centrifugal evaporator. The residues were reconstituted in aq. MeOH (0.35 mL MeOH, 0.15 mL H₂O) and transferred to Waters HPLC vials.

Assays with transgenic plant material transformed with a fungal FAE gene

Callus tissue of amenable genotypes of Miscanthus sinensis (S14), Zea mays (Hi-II) and Festuca arundinacea (20BN3) were cotransformed with FAE gene constructs, pIGB6, pINH1 Δ , pJQ5 and pINH1 Δ + pJQ5 and selection genes [19, 21-23]. Plants cultured on soil were harvested during vegetative growth and stem and leaf tissue were frozen in liquid N₂ and stored at -80°C. Plant material (approx. 0.5 g FW) was subsequently extracted with McIlvaine buffer (pH 5, 2.0 mL). Ferulic acid esterase activity was assayed with ethyl ferulate substrate (stock solution (25 mM) in ethanol). Assay mixtures were prepared by adding the substrate (30 μ L of the stock solution) to the extract (270 μ L) to give a final substrate concentration of 2.5 mM; the mixtures were incubated at 30°C for 20 h. Incubated mixtures were analysed for release of ferulic acid by C_{18} reverse-phase HPLC with diode array detection. HPLC analysis was carried out on a Waters system with a 996 photodiode array detector (PDA) and a Waters C₁₈ Nova-Pak radial compression column (4 μ m, 8 mm \times 100 mm) equilibrated with 100% solvent A (5%

aq. acetic acid) at a flow rate of 1.0 mL min⁻¹. Compounds were eluted using a linear gradient to 100% solvent B (100% methanol) during 50 min and the output was monitored from 240 to 400 nm.

For the analysis with 4-nitrophenyl ferulate, plant extract (270 μ L) was incubated with stock substrate solution prepared in DMSO (30 μ L, 2.8 mM) in micro-titre plate wells to give a final substrate concentration of 0.28 mM. Duplicate reaction mixtures were incubated for 1 h at 30°C, after which time the pH was raised to 8.0 by addition of aq.Na₂HPO₄ (2.0 M, 1.2 mL) and absorbance at 410 nm was measured with a spectrophotometer.

Results and discussion

The first target molecule for synthesis was 4-nitrophenyl ferulate (4-nitrophenyl E-3-(4-hydroxy-3-methoxyphenyl)propenoate) 5 (Scheme 1). Synthesis of 5 had previously been reported by a complex chemoenzymatic procedure [24]. Alternative methods for the synthesis of 5 and 4-nitrophenyl paracoumarate 8 were sought. Initially, the phenolic hydroxy group of ferulic acid 1 was protected, prior to conversion of the carboxylic acid to the acid chloride; this strategy parallels the route of Mastihubová et al. [24] but we sought a protecting group that could be removed from the coupled intermediate without rupture of the base-labile 4-nitrophenyl ester and without resort to the use of enzymes. As shown in Scheme 1, the phenol and carboxylate of ferulic acid 1 were silvlated using tert-butyldimethylsilyl chloride [25] to give 2. The silvl ester was cleaved with potassium carbonate to expose the carboxylic acid **3**. 4-Nitrophenoxide is a weak nucleophile, so the carboxylate was converted to the acid chloride with oxalyl chloride; subsequent reaction with 4-nitrophenol and triethylamine gave the ester 4 in 67% yield. Notably, MS² of the $[M + H]^+$ ion (*m*/*z* 430) gave a fragment ion m/z 291 corresponding to loss

of 4-nitrophenoxide. However, many attempts under different conditions to cleave the silyl protecting group with tetrabutylammonium fluoride and other standard desilylating reagents were unsuccessful, either failing to remove the silyl group or cleaving the ester.

The synthesis of 5 has also previously been reported by Hegde et al., in a one-step route using dicyclohexylcarbodiimide (DCC) as a coupling reagent [17]. However, when the conditions (addition of 4-nitrophenol to ferulic acid 1 in 1,4-dioxane, followed by addition of base and DCC) were employed in our laboratory, the sole isolable product was the O-acylated N,N'-dicyclohexylisourea 6, the putative intermediate in the desired coupling. This isolated compound failed to react with 4-nitrophenol in the presence of triethylamine and 4-dimethylaminopyridine, demonstrating that it was insufficiently electrophilic to react with this weak nucleophile. Carrying out the reaction in ethyl acetate gave a mixture wherein NMR spectroscopy showed the presence of the desired 4-nitrophenyl ester 5 but no conditions could be found to separate 5 from co-product dicyclohexylurea. Attempted coupling using N,N'-diisopropylcarbodiimide (DIC) was investigated. Here the corresponding co-product (N,N'-diisopropylurea) would be expected to be more polar and more easily separated chromatographically; however, the coupling reaction itself proved to be very inefficient.

A new method was developed, wherein coupling of **1** with 4-nitrophenol was achieved using one equivalent of water-soluble carbodiimide (WSC, 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide.HCl) in ethyl acetate in the presence of tertiary amine base. Unreacted WSC and the urea co-product were washed out of the product mixture with aq. citric acid, to which 4-nitrophenyl esters are relatively stable, and unreacted carboxylic acid and nitrophenol were removed with dilute aq. sodium hydrogen carbonate. Further purification by column chromatography yielded off-white crystals of 4-nitrophenyl ferulate **5**. Negative-ion ESI-MS showed a molecular ion of m/z 314 [M - H]⁻. The formation of the ester was demonstrated by the downfield shift of the doublet for the nitrophenyl 2,6-protons by some 0.5 ppm in the ¹H NMR spectrum, relative to 4-nitrophenol. Corresponding downfield shifts were also seen for the ¹³C signals for the nitrophenyl 2,6-C₂, assignments having being confirmed by HSQC and HMBC 2-D spectra.



Fig.1.Release of 4-nitrophenol after incubtion of a commercial preparation of ferulic acid esterase (Depol 740L) with 4-nitrophenyl ferulate**5** and with 4-nitrophenyl coumarate **8**at a range of pH values.

This method was applied to the reaction of *para*coumaric acid 7 with WSC and 4-nitrophenol to yieldpale yellow crystals of 4-nitrophenyl *para*-coumarate **8**.Purification by normal phase chromatography was challenging as the product had an R_f value similar to that of 4-nitrophenol. To overcome this obstacle, two equivalents of *para*-coumaric acid had to be used to ensure that the 4-nitrophenol was fully consumed. The ¹H NMR spectrum was consistent with previous studies [20]. Negative-ion ESI-MS showed a molecular ion of *m/z* 284 [M - H]⁻, which corresponds to a molecular formula C₁₆H₁₃NO₆. MS² of the molecular ion *m/z* 284 gave *m/z* 138, arising from nitrophenoxide. As for **6**, the ¹H NMR spectrum confirmed the formation of the ester through the downfield shift of the signal for the nitrophenyl 2,6-protons.

These nitrophenyl esters were used in the development of a colorimetric assay. They proved to be substrates for a crude commercial preparation of ferulic acid esterase (Depol 740L) (Fig.1). This enzyme preparation, derived from Humicola species, is claimed to have an optimal pH range between 4 and 6 and has been demonstrated to hydrolyse ferulate esters and to release caffeic acid from coffee bean [Depol^m 740L-D740L. Factsheet. www. biocatalysts.com/component/]. Incubation of this enzyme preparation with 4-nitrophenyl ferulate5 and with 4-nitrophenyl coumarate 8 at 25°C for 30 min resulted in the release of free 4-nitrophenol. The enzymatic reactions were conducted in McIlvaine buffer at pH 4.5, pH 5.5 and pH 6.5, as a preliminary experiment to confirm the pH range for the enzyme. The substrates 5 and 8 were shown to be stable under the reaction conditions, with no background release of 4-nitrophenol, by incubation of the esters in the absence of enzyme at these pH values for 24 h at room temperature; no degradation was observed, showing that both substrates are stable over this pH range. The data confirmed that there is good activity across this range of pH values (Figure 1) and suggested that the ferulate ester 5 may be a somewhat better substrate than the coumarate ester 8 for the feruloyl esterase in the Depol 740L preparation.

These nitrophenyl ester substrates **5,8** were applied to a time-course study, in which the incubations were carried out in reaction volumes of 5.0 mL to permit measurement of the release of 4-nitrophenol by absorbance and simultaneous measurement by HPLC of the formation the corresponding ferulic or coumaric acid. The data from the absorbance and HPLC methods were compared. In this study, samples were taken at a range of timepoints. The enzymatic reaction was effectively stopped in each sample by adjusting to pH to ca. 8.0 by addition of Na₂HPO₄; this also improved the sensitivity of the absorption measurement by converting the 4-nitrophenol to yellow 4-nitrophenoxide ion.



Fig. 2. Time-course of hydrolysis of 4-nitrophenyl ferulate **5** by incubation with a commercial preparation of ferulic acid esterase (Depol 740L). The concentrations of ferulic acid were determined by HPLC and the concentrations of 4-nitrophenol were determined by UV absorbance at 410 nm. The initial concentration of **5** was 1.0 mM.

The time-course in Figure 2 shows that *para*nitrophenyl ferulate **5** was a substrate for the commercial preparation of feruloyl esterase, releasing 4-nitrophenol, as detected by absorbance. The production of the hydrolysis co-product ferulic acid was also examined by HPLC (Fig. 2). The two methods showed similar results but it is notable that the method using UV absorbance was much more repeatable, as shown by the smaller error bars at each timepoint.



Fig. 3. Time-course of hydrolysis of 4-nitrophenyl coumarate **8** by incubation with a commercial preparation of ferulic acid esterase (Depol 740L). The concentrations of coumaric acid were determined by HPLC and the concentrations of 4-nitrophenol were determined by UV absorbance. The initial concentration of **8** was 1.0 mM.

Figure 3 shows the corresponding data for hydrolysis of the nitrophenyl coumarate 8. It is immediately apparent, from the lower rate of cleavage, that the additional aryl methoxy group is required for binding of this substrate to the enzyme, indicating that the ferulovl esterase does indeed have selectivity for hydrolysis of feruloyl esters. Nevertheless, there is still sufficient activity for this substrate to have some potential for detection of the enzyme. As for the feruloyl series, the enzymatic hydrolysis was also assayed by measuring the absorbance of the nitrophenoxide ion and by measuring the coumaric acid product by HPLC (Fig.3). As before, there was correlation between the absorbance results and the HPLC data. These data again reinforce the greater precision of the UV absorbance method, with much smaller standard deviations at each point.



Fig. 4. Time-course of hydrolysis of ethyl ferulate by incubation with a commercial preparation of ferulic acid esterase (Depol 740L). Data are given for the concentrations of ferulic acid determined by HPLC. The initial concentration of ethyl ferulate was 1.0 mM.



Fig. 5. Time-course of hydrolysis of ethyl coumarate by incubation with a commercial preparation of ferulic acid esterase (Depol 740L). Data are given for the concentrations of ethyl coumarate and coumaric acid determined by HPLC.

To compare the ability of the feruloyl esterase to hydrolyse nitrophenyl esters against the hydrolysis of the ethyl ester used in previous assays [26], similar time-course experiments were carried out using ethyl ferulate and ethyl coumarate. Clearly, the ethanol produced is not chromogenic, so the assay used HPLC determination of the product ferulic acid. The data (Fig. 4) suggest that the nitrophenyl ester and the ethyl ester are hydrolysed at similar rates by the enzyme.

The enzymatic hydrolysis of the nitrophenyl coumarate **8** was compared with hydrolysis of the corresponding ethyl ester (Fig. 5), showing that the nitrophenyl ester and the ethyl ester are cleaved at similar rates by the enzyme but confirming that the coumarates (ethyl and nitrophenyl) are poorer substrates than the corresponding ferulates.



Fig. 6. Comparison of hydrolysis of ferulate ester **5** and ethyl ferulate after incubation of stem extracts of transformed *Miscanthussinensis* plants. Nitrophenol was measured by the absorbance method and ferulic acid was measured by HPLC *Miscanthus sinensis* plants were transformed with a fungal ferulic acid esterase gene and included a transformant showing high expression (FAE+++) and one with low expression (FAE+). An untransformed plant was used as a control (FAE-).

Taken together, the data show that 5 is an excellent chromogenic substrate for feruloyl esterase, with potential for use in detecting this enzymatic activity in biological preparations. The slower cleavage of 8 points towards some

Chemistry & Biology Interface

selectivity [27].

This potential application of 5 was explored in representative plant material. Transgenic lines of Miscanthus sinensis have been developed, which have been transformed to incorporate an FAE gene from Aspergillus niger [20,21]. Using these lines with different levels of expression of FAE, the results obtained for enzymatic activity using our new colorimetric assay were compared with results from the HPLC assav with the substrate ethyl ferulate used previously (Fig. 6) [26]. The new rapid colorimetric method using 5 gave similar or higher values, compared to the formation of ferulic acid from ethyl ferulate as measured by HPLC. The application of 5 for the measurement of FAE activity was further demonstrated with transgenic lines of maize (Zea mays) and tall fescue (Festuca arundinacea) transformed with two different FAE gene constructs (Table 1) [21].

These preliminary evaluation studies demonstrate the utility of these nitrophenyl esters as chromogenic substrates for measuring the activity of feruloyl and coumaryl esterases in biological materials. The nitrophenyl esters are readily accessible synthetically through an optimised route, avoiding the pitfalls of approaches reported previously. The colorimetric assay has been demonstrated to be equally sensitive to HPLC-DAD assays based on detection of ferulic acid product. The two principal benefits of the new colorimetric assay, compared with HPLC assays which measure release of ferulic acid, are speed (obviating the time required for sample preparation and reducing sample run time) and the potential for adaptation to a high-throughput 96-well plate format, with automation. This will greatly facilitate identification of enzymes with potential to increase the efficiency of release of sugars from lignocellulosic biomass, reducing costs of processing.

Table 1: Release of 4-nitrophenol afterincubation of plant extracts with 4-nitrophenylferulate

Transgenic or wild-type plant material ^a	Release of 4-nitrophenol (nmol gFW ⁻¹ min ⁻¹)
Maize L ^b – FAE1 ^c	4.08
Maize S ^b – FAE1	0.72
Maize S Control	0.12
Maize L – Control	<LoD ^d
Fescue L – FAE2 ^c	4.32
Fescue L – FAE3 ^c	0.24
Fescue L – FAE4 ^c	0.12
Fescue L – Control	< LoD

^aMaize (*Zea mays*) and tall fescue (*Festuca arundinacea*) plants were transformed with a fungal ferulic acid esterase gene (FAE); untransformed plants were included as controls.

 $^{b}L = leaf, S = stem$

^cPlants designated FAE1, FAE2, FAE3 and FAE4 were transformed with pIGB6, pINH1D, pJQ5 and pINH1D + pJQ5 constructs, respectively.

^d LoD = Limit of detection.

ACKNOWLEDGEMENTS

This work was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) Institute Strategic Programme Grant on Energy Grasses & Biorefining (BB/J0042/1). We gratefully acknowledge support for BEACON from the European Regional Development Fund through the Welsh European Funding Office, part of the Welsh Assembly. We also thank Thomas Wilson, Dr. Manfred Beckmann and Kathleen Tailliart (Aberystwyth University) for useful discussions on LC-ESI-MS/MS and FT-ICR-MS and Dr. Tim Langdon (Aberystwyth University) on the transgenic lines.

References

M. Kleinert, T. Barth, Phenols from lignin. Chem. Eng. Technol.31 (2008) 736-745.

- F. Cherubini, The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. Energ. Convers. Manage.51 (2010) 1412-1421.
- L.M. Chauvigne-Hinés,L.N. Anderson, H.M. Weaver, J.N. Brown, P.K. Koech, C.D. Nicora, B.A. Hofstad, R.D. Smith, M.J. Wilkins, S.J. Callister, A.T. Wright, A suite of activity-based probes for cellulose degrading enzymes. J. Am. Chem. Soc. 134 (2012) 20521-20532.
- P.A. Kroon, M.T. Garcia-Conesa, I.J. Fillingham, G.P. Hazlewood, G. Williamson, Release of ferulic acid dehydrodimers from plant cell walls by feruloyl esterases. J. Sci. Food Agric. 79 (1999) 428-434.
- L.R. Lynd, P.J. Weimer, W.H. van Zyl, I.S. Pretorius, Microbial cellulose utilization: Fundamentals and biotechnology. Micro. Mol. Biol. Rev. 66 (2002) 506-577.
- A.E. Fazary, Y.-H. Ju, Feruloyl esterases as biotechnological tools: current and future perspectives. Acta Biochim. Biophys. Sinica 39 (2007) 811-828.
- L. Marmuse, M. Asther, E. Fabre, D. Navarro, L. Lesage-Meessen, M. Asther, M. O'Donohue, S. Fort, H. Driguez, New chromogenic substrates for feruloyl esterases. Org. Biomol. Chem. 6 (2008) 1208-1214.
- J. Ralph, S. Quideau, J.H. Grabber, R.D. Hatfield, Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. J. Chem. Soc., Perkin Trans. (1994) 3485-3498.
- M.T. Garcia-Conesa, P.A. Kroon, J. Ralph, F.A. Mellon, I.J. Colquhoun, L. Saulnier, J.-F. Thibault, Williamson, G. A cinnamoyl esterase from *Aspergillus niger* can break plant cell wall cross-links without release of free diferulic acids. Eur. J. Biochem. 266 (1999) 644-652.
- V. Mastihuba, L. Kremnický, M. Mastihubová, J.L. Willett, L. Côté, A spectrophotometric assay for feruloyl esterases. Anal. Biochem. 309 (2002) 96-101.
- V. Puchart, M. Vršanská a, M. Mastihubová, E. Topakas, C. Vafiadi, C.B. Faulds, M. Tenkanen, P. Christakopoulos, P. Biely, Substrate and positional specificity of feruloyl esterases for monoferuloylated and monoacetylated 4-nitrophenyl glycosides. J. Biotech. 127 (2007) 235-243.
- W.S. Borneman, L. Ljungdahl, R.D. Hartley, D.E. Akin, Isolation of *p*-coumaroyl esterase from the anaerobic fungus *Neocallimastix* strain MC-2. Appl. Env. Microbiol. 58 (1991) 2337-2344.
- T. Koseki, S. Fushinobu, Ardiansyah, H. Shirakawa, M. Komai, Occurrence, properties, and applications of feruloyl esterases. Appl. Microbiol. Biotechnol. 84 (2009) 803-810.
- P.A. Kroon, C.B. Faulds, G. Williamson, Purification and characterization of a novel esterase induced by growth of *Aspergillus niger* on sugar-beet pulp. Biotechnol. Appl. Biochem. 23 (1996) 255-262.
- 15. E. Allerdings, J. Ralph, H. Steinhart, M. Bunzel, Isolation and structural identification of complex feruloylated heteroxylan side-chains from maize bran. Phytochemistry

67 (2006) 1276-1286.

- A. Chyba, V. Mastihuba, M. Mastihubová, Synthesis of 4-nitrophenyl caffeate and its use in assays of caffeoyl esterases. Anal. Biochem. 445 (2014) 49-53.
- S. Hegde, P. Srinivas, G. Muralikrishna, Single-step synthesis of 4-nitrophenyl ferulate for spectrophotometric assay of feruloyl esterases. Anal. Biochem. 387 (2009) 128-129.
- A.M. Ramos de la Pena, J.C. Contreras Esquivel, Methods and substrates for feruloyl esterase activity detection; a review. J. Mol. Catal. B Enzymatic 130 (2016) 74-87.
- M. Cernerud, J. Reina, J. Tegenfeldt, C. Moberg, Chiral polymers via asymmetric epoxidation and asymmetric dihydroxylation. Tetrahedron: Asymmetry 7 (1996) 2863-2870.
- G. Cevasco, S. Thea, Participation of an extended *p*-oxo ketene intermediate in the dissociative alkaline hydrolysis of aryl 4-hydroxycinnamates. J. Org. Chem. 59 (1994) 6274-6278.
- M.M. de O Buanafina, M. T. Langdon, B. Hauck, S. Dalton, E. Timms-Taravella, P. Morris, Targeting expression of a fungal ferulic acid esterase to the apoplast, endoplasmic reticulum or Golgi can disrupt feruloylation of the growing cell wall and increase the biodegradability of tall fescue (*Festuca arundinacea*). Plant Biotech J. 8 (2010) 316–331.
- S.J. Dalton, A. Winters, T. Langdon, E. Timms-Taravella, I. Donnison, Genetic transformation of *Miscanthus sinensis* with FAE'. Plant Transformation Technologies II Conference, Vienna, Austria, 19-22 February (2011).
- S.J. Dalton, Biotechnology of *Miscanthus*, in Biotechnology of neglected and underutilized crops. Ed: Shri Mohan Jain, S. Dutta Gupta. (2013) 243-294; Springer Verlag. ISBN 978-94-007-5499-7
- M. Mastihubová, V. Mastihuba, L. Kremnický, J.L. Willett, L. Côté, Chemoenzymatic preparation of novel substrates for feruloyl esterases. SynLett 10 (2001) 1559-1560.
- S.A. Snyder, F. Kontes, Explorations into neolignan biosynthesis: Concise total syntheses of Helicterin B, Helisorin, and Helisterculin A from a common intermediate. J. Am. Chem. Soc. 131 (2009) 1745-1752.
- G.G. Pérez-Morales, A. Ramírez-Coronel, O. Guzmán-López, F. Cruz-Sosa, I. Perraud-Gaime, S. Roussos, G. Saucedo-Castañeda, Feruloyl esterase activity from coffee pulp in solid-state fermentation. Food Tech. Biotech. 49 (2011) 352-358.
- A. Vrnai, T.H.F. Costa, C.B. Faulds, A.M.F. Milagres, M. Siika-aho, A. Ferraz, Effects of enzymatic removal of plant cell wall acylation (acetylation p-coumaroylation and feruloylation) on accessibility of cellulose and xylan in natural (non-pretreated) sugar cane fractions. Biotech. Biofuels 7 (2014) 153.