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Short Communication

Product patterns of a feruloyl esterase from *Aspergillus nidulans* on large feruloyl-arabino-xylo-oligosaccharides from wheat bran

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ABSTRACT

A purified feruloyl esterase (EC 3.1.1.73) from *Aspergillus nidulans* produced in *Pichia pastoris* was used to study the de-esterification of large feruloyl oligosaccharides consisting of 4 to 20 pentose residues and (xylose plus arabinose) and one ferulic acid residue. The feruloyl oligosaccharides were prepared from total oligosaccharidic hydrolysates from wheat bran treated with a purified endoxylanase from *Thermobacillus xylanilyticus*. The feruloyl esterase showed similar specific activity but an affinity about 3.5-fold higher towards feruloyl oligosaccharides than towards methyl ferulate. Mass spectrometry analysis of the products after long-term enzymatic hydrolyses showed that the esterase was able to hydrolyze the largest feruloyl oligosaccharides and therefore could act alone on feruloyled xylans. Consequently, the feruloyl esterase from *A. nidulans* could be useful for the enzymatic deconstruction of xylans in plant cell walls.

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1. Introduction

Wheat straw and bran, corn stover and cobs are abundant renewable resources for lignocellulosic bioconversion. Major hemicelluloses are xylans, β,1–4 polymers of D-xylose residues which can be substituted at *O*-3 or *O*-2 by α-L-arabinose or at *O*-2 by α-D-glucuronic acid or its 4-*O*-methyl ether (Biely, 1985). By ester bonds, cinnamic and acetic acids can link the arabinose and xylose residues, respectively. Enzymatic depolymerization of these substrates involves endo- and exo-enzymes acting on xylans (Biely, 1985). The use of debranching enzymes including α-L-arabinofuranosidases (E.C. 3.2.2.55.), β-D xylosidases (EC 3.2.1.37), α-D glucuronidases (EC 3.2.1.139), feruloyl esterases (FAE, E.C. 3.1.1.73) and acetylesterases (EC 3.1.1.6) together with endoxylanases (EC 3.2.1.8) can significantly improve the enzymatic hydrolysis of cell walls into pentoses from ammonia-fiber expansion pretreated corn stover (Banerjee et al., 2010). The synergic action of FAE-III from *Aspergillus niger* with a xylanase from *Trichoderma viride* leads to the removal of 95% of the ferulic acid (FA) from wheat bran (Faulds and Williamson, 1995). FAEs could play significant roles for the deconstruction of gramineaceaous biomass but alone usually produce only small amounts of free FA from natural substrates (Shin et al., 2006). Studies on FAEs have generally used synthetic substrates, such methyl cinnamates, methyl sinapate, methyl caffeate or feruloyl oligosaccharides (Ralet et al., 1994; Crepin et al., 2004). In the present study, the performance of a purified feruloyl esterase from *Aspergillus nidulans*, (AnidFAE) on large feruloyl-arabino-xyloologosaccharides isolated from enzymatic hydrolysis of wheat bran (degree of polymerisation, DP from 4 to 20 pentoses) was investigated. This enzyme was chosen since *A. nidulans* is an ubiquitous fungus often found on diverse plant materials and is therefore a good model for cell wall degrading enzyme studies and also because the enzyme was found to be active on wheat arabinoxylans (Bauer et al., 2006).

2. Methods

2.1. Production of A. nidulans feruloyl esterase

FAE from *A. nidulans* (AN5267.2) was produced in the yeast *Pichia pastoris* via a methanol inducible expression system (Bauer et al., 2006). The strain was obtained from the Fungal Genetics Stock Center (University Missouri, Kansas City). A culture of 150 ml was grown in Buffered Minimum Glycerol medium (BMGY, Invitrogen) at 30 °C at 220 rpm. After 36 h, the cells were harvested by centrifugation (5000g, 4 °C, 15 min) and resuspended in the same volume of BMMY (Buffered Minimum Methanol medium). After 72 h at 30 °C at 220 rpm in baffled flasks, the culture was centrifuged and the supernatant recovered. Proteins from the supernatant were precipitated with 90% ammonium sulfate at 4 °C. After centrifugation (10,000g, 20 min, 4 °C), the pellet was resuspended in 2 ml of Tris buffer pH8.2, 300 mM NaCl, 50 mM imidazole and dialysed for 12 h against 1 L of the same buffer. The feruloyl esterase was purified to homogeneity by metal chromatography (HiTrap Chelating HP columns, GE Healthcare) according to the manufacturer's protocol. Analysis by SDS–PAGE of the purified protein

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showed that its molecular mass was around 28 kDa and its concentration was estimated to be 6.4 μg of protein per ml based on the comparison of the intensities of AnidFAE bands and of standard BSA bands and image analysis (Gel Doc XS Quantity One software, Bio-Rad).

2.2. Production of feruloyl arabino-xylo-oligosaccharides and characterization by MALDI MS

Wheat bran was obtained from a flour mill (ARD, Pomacle, France). Endoxylanase (E.C. 3.2.1.8) was produced from *Termobacillus xylanilyticus* (Touzel et al., 2000) and purified to homogeneity as previously described (Zilliox and Debeire, 1998). Feruloyl oligosaccharides were obtained from the enzymatic end-reaction products obtained from the hydrolysis of wheat bran by the purified endoxylanase according to the protocol previously described (Lequart et al., 1999), with some minor modifications. The enzymatic hydrolysate was applied to an anion-exchange column (40 × 1.6 cm) filled with Dowex 1 × 2, 100–200mesh (OH^-) (Alfa Aesar, Germany). After the elution of neutral and charged xylo-oligosaccharides with ammonium formate (0.8 M) and extensive washing with distilled water, feruloyl oligosaccharides were eluted with 80% EtOH. Fractions of 20 ml were collected, analysed by TLC (Benamrouche et al., 2002) and fractions were pooled according to the size of the oligosaccharides they contained. Three fractions were recovered from the column, the heaviest fraction F1, then F2 and F3.

Mass spectrometry analyses of F1, F2 and F3 were performed in the reflectron mode using a Bruker Reflex IV Time of Flight instrument (Bruker) equipped with a nitrogen pulsed laser (337 nm). The matrix used was 2–5 dehydroxybenzoic acid (Fluka) with a concentration of 20 mg/mL in water/acetonitrile (80/20, v/v). One microliter of matrix was mixed with 1 μL of sample solution directly on the MALDI target and dried at room temperature. Each recorded mass spectrum consisted of the sum of 100 shots at a single position using the positive ion detection mode. External calibration was realised with the peptide calibration standard solution from Bruker.

2.3. Determination of kinetics parameters

Activities of AnidFAE were determined by analysing the release of ferulic acid by spectrophotometric assays as previously described (Ralet et al., 1994) with methyl ferulate (MFA) from Apin Chemicals. F1 and F3 were chosen as the largest and smallest DP fractions. The extinction coefficients of FA and MFA under the conditions of the assays were 3808 and 9030 $M^{-1}\,cm^{-1}$, respectively. The mixtures' extinction coefficients of oligosaccharides were determined on the basis of total neutral sugar contents using the phenol/sulfuric acid method (Dubois et al., 1956), and from the average of the molecular masses according to MALDI-MS spectra (1277 and 835 Da for F1 and F3, respectively). The oligosaccharides were assumed to belong to the same molecular species with xylose and arabinose as pentoses and including one ferulic acid per molecule (Table 1). The calculated values of the extinction coefficients of F1 and F3 were, respectively, 16,135 and 17,240 $M^{-1}\,cm^{-1}$. The enzymatic reaction mixtures contained potassium phosphate buffer pH 7 (50 mM) and the assays were performed at 37 °C with appropriate dilution of the enzyme. One unit of enzyme (IU) is defined as the amount of enzyme required to produce 1 μmole of product per minute. The enzymatic activities were determined by measuring the decrease in absorbance at 335 nm corresponding to the diminution of linked-ferulic acids. K_m and k_{cat} were calculated using Lineweaver–Burk plots from the initial-rate of recombinant AnidFAE with MFA, F1 and F3 as substrates. The substrates concentrations ranged from 25 μM to 1.5 mM.

2.4. Long-term hydrolyses of feruloyl arabino-xylooligosaccharides by AnidFAE and characterization of the reaction products by MALDI-MS

F1, F2 and F3 (2.65, 3.80 and 3.30 mg/ml of total neutral sugars, respectively), were hydrolyzed by FAE (0.01 IU/ml) at 37 °C in

Table 1
Positive-ion *m/z* of the three oligosaccharidic fractions recovered from hydrophobic chromatography before and after hydrolysis by AnidFAE.

1A substrates[a]			1B products[a]					
$[M+Na]^+$	F1% [b]	F2% [b]	F3% [b]	$F1_{FAE}$% [b]	$F2_{FAE}$% [b]	$F3_{FAE}$% [b]	Pentoses[c]	FA
701					**4.3**	**18.9**	5	0
745		5.9	10.5		2.6	5.7	4	1
833				**13.8**	**15.2**	**30.7**	6	0
877	2.2	16.2	39.8		6.8	13	5	1
965				**17.2**	**14.3**	**13.4**	7	0
1009	8.2	26.1	31.2		8.6	9.4	6	1
1097				**16.7**	**8.8**	**4.7**	8	0
1141	13.4	21.6	12.2		7.1	4.2	7	1
1229				**17.7**	**6.9**		9	0
1273	15.1	12.6	4.5		4.4		8	1
1361				**13.5**	**5.2**		10	0
1405	17.1	7.7	1.7		3.1		9	1
1493				**9.2**	**3.7**		11	0
1537	12.1	4.7	0.1		1.9		10	1
1625				**5.9**	**2.4**		12	0
1669	8.9	2.5			1.5		11	1
1757				**3.9**	**1.2**		13	0
1801	6.3	1.8			0.7		12	1
1890				**2**			14	0
1933	4.6	0.9			0.5		13	1
2065	3.4						14	1
2197	2.4						15	1
2329	2.0						16	1
2461	1.4						17	1
2593	1.2						18	1
2725	0.8						19	1
2857	0.5						20	1

[a] Substrates (F1, F2 and F3) and products ($F1_{FAE}$, $F2_{FAE}$ and $F3_{FAE}$) of the enzymatic reactions.
[b] Percentage of each oligosaccharide of defined DP in each fraction. Desesterified feruloyl oligosaccharides are in bold.
[c] Pentoses: sum of xylose and arabinose (for more data, see Lequart et al. (1999)).

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