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Release of ferulic acid from maize bran and derived oligosaccharides by *Aspergillus niger* esterases

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We have examined two *A. niger* esterases (FAE-III (ferulic acid esterase III) and CinnAE (cinnamic acid esterase)) for their ability to release ferulic acid from maize bran, a particularly rich source of ferulic acid. However, even though both enzymes exhibited significant activites on novel feruloylated oligosaccharides derived from maize bran (including FAXX (*O*-(5-*O*-feruloyl-α-L-arabinofuranosyl)-(1→3)-*O*-β-xylopyranosyl-(1→4)-D-xylopyranose)), neither esterase was efficient in removing ferulic acid from the whole bran, even in the presence of other carbohydrases. It is shown that the kinetics of ferulic acid release from feruloylated oligosaccharides was influenced by (a) the nature of the sugar–sugar linkage, and (b) the type of sugar present. The results suggest that enzymic release of ferulic acid from maize bran is limited by physical and steric factors, not by the chemical nature of the linkage.

INTRODUCTION

Ferulic acid is the most abundant hydroxycinnamic acid in the plant world, and as such, is a common constituent of animal forage. It is linked to arabinose or galactose residues in the pectic or hemicellulosic component of the cell walls of bamboo shoots, coastal bermuda grass, sugar cane bagasse, barley straw, wheat bran, sugar beet pulp, spinach and maize (Ralph & Helm, 1993).

Ferulic acid comprises 0.5% (w/w) of wheat bran (Ralet *et al.*, 1990), 0.8% of sugar beet pulp (Micard *et al.*, 1994) and 3.1% of maize bran (Saulnier *et al.*, 1995*a*). The removal of ferulic acid by enzymic methods would allow the exploitation of the acid for industrial and food applications (Gross-Falconnier, 1991; Graf, 1992; Cheetham, 1993).

Efficient enzymic removal of ferulic acid from plant cell wall materials, such as wheat bran, has been shown to require the synergistic interaction between an esterase and an endoxylanase (Faulds & Williamson, 1991, 1993*a*, 1993*b*, 1994; Ferreira *et al.*, 1993). Hydrolysis of more complex cell wall material, such as sugar beet pectin, involves certain specific esterases, and requires a larger battery of cell wall degrading enzymes (unpublished results). *Aspergillus niger* and *Pseudomonas fluorescens* esterases have also been shown to hydrolyse feruloylated oligosaccharides derived by acid hydrolysis from wheat bran and sugar beet pulp (Ralet *et al.*, 1994; Faulds *et al.*, 1995).

The ferulic acid content in maize bran is higher than that in either wheat bran or in sugar beet pulp, but maize bran xylan is more highly substituted with xylose, arabinose and galactose residues (Chanliaud *et al.*, 1995; Saulnier *et al.*, 1995*a*). In this paper, we examine both the ability of an *A. niger* ferulic acid esterase (FAE-III; Faulds & Williamson, 1994) and *A. niger* cinnamic acid esterase (CinnAE; unpublished results) to release ferulic acid from maize bran with and without the addition of carbohydrates, and also the action of cinnamoyl esterases on two novel feruloylated oligosaccharides derived from maize bran (Saulnier *et al.*, 1995*b*).

MATERIALS AND METHODS

Plant material

Micronised maize brans were provided by ULICE (France). Brans (100 g) were dispersed in distilled water (1 l), Termamyl 120L (20 ml) added, and the mixture maintained in a boiling water bath for 60 min. The

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residue was recovered by filtration on a glass sintered funnel, washed with water and dried by washing with ethanol, and then acetone, before placing in an oven at 40°C for 24 h. 74 g was recovered. De-starched bran (10 mg) was treated with 1 M NaOH for 24 h (room temperature and in the dark). The soluble fraction, after centrifugation at 9500*g* for 5 min, was neutralised with 2 vol 1 M HCl and ferulic acid levels analysed by HPLC (Faulds & Williamson, 1991), which indicated an alkali-extractable ferulic acid content in this preparation of 2.5% (w/w).

The feruloylated oligosaccharides, XAF (*O*-β-D-xylopyranose-(1→4)-*O*-β-D-xylopyranosyl-(1→2)-(5-*O*-feruloyl)-α-L-arabinofuranosyl) and GXAF (*O*-L-galactopyranose-(1→4)-*O*-β-D-xylopyranosyl-(1→2)-(5-*O*-feruloyl)-α-L-arabinofuranoyl) (Fig. 1), were isolated from acid-hydrolysed maize bran and characterised as described previously (Saulnier *et al.*, 1995*b*).

Enzymes

Aspergillus niger FAE-III was purified from oat spelt xylan-grown cultures of *A. niger* CBS 120.49, as described previously (Faulds & Williamson, 1994). *A. niger* CinnAE was purified from sugar beet pulp-grown cultures of *A. niger* CS180 (unpublished results). CinnAE differs from FAE-III in both physical properties and substrate specificities on model substrates and feruloylated oligosaccharides.

Driselase, a basidiomycete enzyme preparation, was obtained from Sigma Chemical Co., and purified by the method of Borneman *et al.* (1990). The protein concentration of the purified mixture was 9.8 mg/ml.

Endo-β-1,4-xylanase from *Trichoderma viride* and α-L-arabinofuranosidase from *A. niger* were purchased from Megazyme (Aust) Pty Ltd.

One unit (U) of enzyme activity is expressed as the amount of enzyme releasing 1 μmol of product/min under the given conditions. For the cinnamoyl esterases, these are pH 6.0 and 37°C.

Assays

Release of free ferulic acid from de-starched maize bran (DSMB) was measured on reverse phase HPLC as previously described for de-starched wheat bran (Johnson *et al.*, 1988), using 20 mg DSMB/ml assay. Release of free ferulic acid from the feruloylated oligosaccharides XAF and GXAF was measured spectrophotometrically at 335 nm by the method of Ralet *et al.* (1994), using extinction coefficients of 13,800 and 22,800/M/cm, for XAF and GXAF, respectively. K_m and V_{max} measurements were determined by the method of Wilkinson (1961), using 0–0.5 mM XAF and 0–0.3 mM GXAF, and 5 mU FAE-III or 6.1 mU CinnAE. Protein was determined using the Coomassie Protein Assay Reagent from Pierce.

RESULTS AND DISCUSSION

Hydrolysis of de-starched maize bran

No ferulic acid was detected when CinnAE (33 mU) was incubated with DSMB, even after 24 h. 0.3% of the total level of ferulic acid was released when FAE-III (13 mU) was used. This level is very low, corresponding to 66 μg ferulic acid per gram maize bran compared to 30 μg ferulic acid per gram wheat bran (0.6%) in only 30 min by the same amount of the same enzyme (Faulds & Williamson, 1994), which increased to 4% after 24 h (200 μg per gram wheat bran; unpublished results). The complex nature of the maize bran (Saulnier *et al.*, 1995*a*) may thus limit the accessibility of the esterases to the feruloylated regions.

To facilitate hydrolysis, *T. viride* endoxylanase (0.02–2 U), or *T. viride* endoxylanase (1 U) and *A. niger* α-L-arabinofuranosidase (0.03–2 U), was added to the DSMB/FAE-III assay. In the presence of xylanase, FAE-III was able to increase the amount of ferulic acid released from 0.26 to 0.6%. The presence of α-arabinofuranosidase failed to increase the amount of free acid released, suggesting more complex carbohydrate-degrading enzymes were required. One such complex mixture, Driselase, was thus used in conjunction with FAE-III or CinnAE. After 24 h, with the addition of 1 mg purified Driselase, FAE-III (26 mU) released 2.3% of the ferulic acid from DSMB, and CinnAE (33 mU) released 0.93%.

Thus, the esterases were able to release a small amount of ferulic acid after limited hydrolysis of DSMB. One possible explanation could be due to the number of substitutions on the heteroxylan backbone on maize, the presence of highly branched xylose in the side-chain, and the presence of a linkage between arabinose and xylose near the ferulic acid group. Viëtor and co-workers (1994) found that with barley arabinoxylans, the presence of 2-*O*-Ara_f-Xyl_p appeared to block the action of *Aspergillus awamori* endoxylanase I, leading to lower degradation of barley arabinoxylans when compared to wheat arabinoxylans (Gruppen *et al.*, 1992). Thus, with maize bran heteroxylans, the endoxylanase and accessory enzymes present in Driselase may be blocked by the linkages and substitutions present on the backbone, and FAE-III can only act on easily accessible regions. This difference in linkage between wheat bran and maize bran may also explain why *T. viride* endoxylanase has no effect on ferulic acid release, but has a big effect on wheat bran when in conjunction with *A. niger* FAE-III (Faulds & Williamson, 1994).

Driselase was found to release approximately 45% of solubilised feruloyl groups from maize shoots as FAXX (*O*-(5-*O*-feruloyl)-α-L-arabinofuranosyl)-(1→3)-*O*-β-xylopyranosyl-(1→4)-D-xylopyranose: Kato & Nevins, 1985), only after preliminary hydrolysis of the maize with 30 mM oxalic acid followed by a long (120 h)

incubation of the insoluble bran with Driselase. The remaining 55% remained uncharacterised. This suggests that the oxalic acid hydrolysed the maize sufficiently for the Driselase to slowly break up the remaining polymer. However, this level of hydrolysis has not so far been achieved using enzymes alone.

Hydrolysis of feruloylated oligomers

As the release of ferulic acid from intact maize bran was limited, the basis for the poor hydrolysis was examined. We tested whether it was due to the nature of the ester linkage or to steric and physical factors in the maize cell wall. The nature of the ester linkage between ferulic acid and the sugar has previously been shown to influence hydrolysis of feruloylated oligosaccharides derived from wheat bran and sugar beet pulp (Ralet *et al.*, 1994; unpublished results). The ability of these two fungal enzymes to hydrolyse feruloylated compounds identified in maize bran, such as Ara_1F (5-*O*-feruloyl-α-L-arabinofuranose; Saulnier *et al.*, 1995*b*) and FAXX (Ohta *et al.*, 1995; Kato & Nevins, 1985) has previously been demonstrated (Ralet *et al.*, 1994; unpublished results). However, their ability to hydrolyse two novel feruloylated oligosaccharides from maize bran, XAF and GXAF, has not been determined.

XAF and GXAF were isolated by acid hydrolysis and gel filtration chromatography (Saulnier *et al.*, 1995*b*), and their structures are shown in Fig. 1. FAXX constitutes ~45% of the ferulic acids in maize shoot cell walls (Kato & Nevins, 1985), while XAF, GXAF and Ara_1F represent ~33% in maize bran cell walls (Saulnier *et al.*, 1995*b*). Wheat bran isolates corresponding to FAXX and Ara_1F were hydrolysed by both FAE-III (Ralet *et al.*, 1994) or CinnAE (unpublished results). In XAF, xylose is (1→2) linked to the arabinose residue, in contrast to wheat bran, where arabinose is linked (1→3) to xylose. Xylose is also present in the side-chains of maize xylans. Arabinose is the reducing sugar in maize, xylose the reducing sugar in wheat bran. Characterisation of feruloylated oligosaccharides isolated from maize bran has shown the presence of galactose linked (1→4) to xylose residues (Saulnier *et al.*, 1995*b*). To date, such a linkage has not been found in wheat bran.

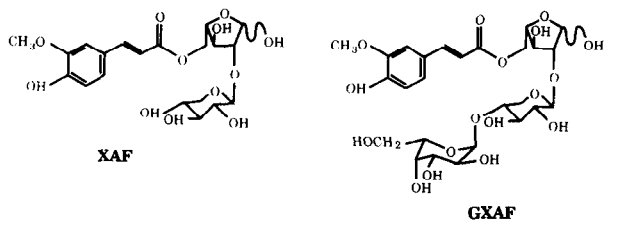

Fig. 1. Structures of two novel feruloylated oligosaccharides isolated from maize bran (from Saulnier *et al.*, 1995*b*). XAF, (*O*-β-D-xyl_p(1→2)-[(5-*O*-(feruloyl)-L-Ara_f)]; GXAF,(*O*-L-Gal_p)-(1→4)-*O*-β-D-xyl_p-(1→2)-(5-*O*-(feruloyl)-L-ara_f)

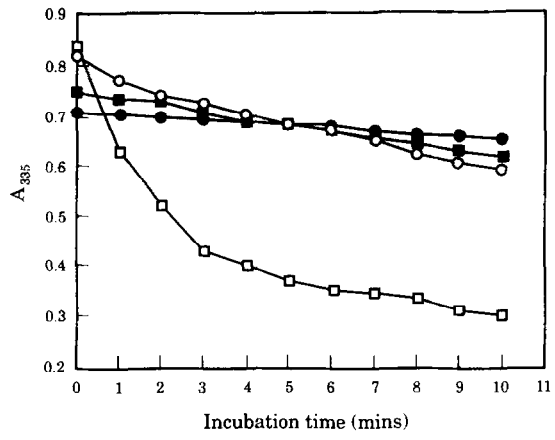

Fig. 2. Release of ferulic acid from XAF and GXAF (33 μM) by *A. niger* esterases (5 mU). ΔA_{335} of 0.1 during XAF and GXAF hydrolysis by the esterases corresponds to 22% and 13% hydrolysis, respectively, of the relevant substrate. □, XAF with FAE-III; ■, XAF with CinnAE; ○, GXAF with FAE-III; ●, GXAF with CinnAE.

Figure 2 shows the rates of the reaction of 5 mU of either FAE-III or CinnAE on XAF and GXAF. The hydrolysis of XAF by FAE-III was rapid (initial rate = 93.0 U per mg protein), with 52% of the total ferulic acid level being released within 4 min. The rate of release of ferulic acid from the same substrate by CinnAE was 10-fold lower (0.77 U per mg protein) with 4.8% hydrolysis in 4 min. Similarly, the activity of FAE-III on GXAF (23.8 U per mg protein: 11.8% hydrolysis in 4 min) was significantly higher than that found for CinnAE (0.40 U g^{-1} and 3.1%, respectively), but both enzymes demonstrated a preference for the feruloylated disaccharide.

Calculations of kinetic constants (K_m and V_{max}) revealed that even though both enzymes had a higher catalytic efficiency on XAF, CinnAE had a greater affinity for the disaccharide whereas FAE-III had a greater affinity for the trisaccharide (Table 1). Furthermore, the larger magnitude of difference observed in the catalytic efficiency between the two enzymes (~100-fold) was due to the differences in V_{max} and not in the values for K_m. It has been shown that the spectrophotometric activities on many other substrates common to both enzymes are much higher using FAE-III than with CinnAE, except in the case of Ara_1F from sugar beet (Faulds & Williamson, 1994; Ralet *et al.*, 1994; unpublished results).

A comparison of the catalytic efficiencies of FAE-III from this work and previously reported work on feruloylated oligosaccharides from wheat bran and sugar-beet pulp (Ralet *et al.*, 1994) reveals that the type of sugar ester-linked to ferulic acid clearly influences the ability of the enzyme to hydrolyse ferulic acid (Table 2). Ferulic acid is ester-linked to arabinose at C-5 in maize bran, but the attachment of xylose to the C-2 of arabinose reduces the efficiency compared to a C-3 attach-

Table 1. K_m and V_{max} values for the hydrolysis of XAF and GXAF from maize bran by *A. niger* FAE-III and CinnAE

	FAE-III		CinnAE	
Substrate	XAF	GXAF	XAF	GXAF
K_m (mM)	0.383 (±0.08)	0.162 (±0.02)	0.125 (±0.01)	0.223 (±0.04)
V_{max} (U/mg protein)	938 (±138)	176 (±19)	3.80 (±0.27)	3.94 (±0.71)
Catalytic efficiency (V_{max}/K_m)	2448	1086	30.4	17.7

Table 2. The catalytic efficiency of *A. niger* FAE-III for various feruloylated oligosaccharides

Substrate	Nomenclature	Catalytic efficiency
Substrate	MFA	84
Monosaccharide	GalF	40
	AraF	1728
	($AraF_5$←FA)	
Disaccharide	XAF	2448
Trisaccharide	GXAF	1086
	FAXX	7600
Tetrasaccharide	FAXXX	3270

ment, as found in wheat bran. Whereas the presence of two xylose sugars increased the rate of hydrolysis of FAXX derived from wheat bran (also found in maize bran), solution of L-galactose for the terminal xylose reduced the catalytic efficiency 7-fold.

From these results and from the work on wheat bran-derived oligosaccharides, we can conclude that: (a) the position of the ester linkage between ferulic acid and the primary sugar; (b) the position of the linkage between the primary and secondary sugars; (c) the length of the oligosaccharide; and (d) the type of sugars present, all influence the release of ferulic acid by *A. niger* FAE-III and CinnAE from plant cell wall material.

In summary, less than 1% of alkali-extractable ferulic acid was released from DSMB by two *A. niger* esterases. The presence of carbohydrase mixtures only slightly increased this release. However, novel feruloylated oligosaccharides isolated from maize bran were good substrates for the enzymes, suggesting steric and physical factors limit hydrolysis of the intact maize bran. The type of sugar and nature of sugar–sugar linkages also were found to strongly influence the rate of hydrolysis.

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