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Degradation of feruloylated oligosaccharides from sugar-beet pulp and wheat bran by ferulic acid esterases from *Aspergillus niger* †

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Abstract

The activity of two forms of ferulic acid esterase (FAE) from *Aspergillus niger* on a synthetic feruloylated substrate (methyl ferulate) and on 11 different feruloylated oligosaccharides from sugar-beet pulp and wheat bran was determined. The enzymes exhibited different specificities for the various feruloylated substrates and were more active on certain substrates of cell-wall origin than on methyl ferulate. Both enzymes preferred the arabinose residue to which ferulic acid is attached in the furanose form. FAE-I had no clear preference for the type of linkage involved between the ferulic acid units and the oligosaccharide chain. In contrast, FAE-III had a clear requirement for ferulic acid to be attached to O-5 of the Ara*f* ring while no catalysis was observed when ferulic acid was attached to O-2. Both enzymes showed maximum activity on feruloylated trisaccharides. An increase in the length of the oligosaccharide chain did not preclude catalysis, but feruloylated oligosaccharides of a dp > 3 were hydrolysed at a reduced rate. Our results support the hypothesis that different kinds of ferulic acid esterases exist with different specificities for the oligosaccharide chain of the feruloylated substrates.

Keywords: Beet pulp; Wheat bran; Ferulic acid; Ferulic acid esterases

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1. Introduction

Ferulic acid is one of the major phenolic acids present in cell walls of several plants of monocot families, including Gramineae [1–7], and of the dicot Chenopodiaceae family [8–15]. The roles of these wall-bound feruloyl groups have not yet been totally elucidated. However, it has been shown that the amount of ferulic acid released during saponification of cell walls was correlated with an increase in microbial [16] and enzymic [17] degradations of cell-wall polysaccharides. Although certain phenolic acids are undigestible or even toxic to many soil and ruminant bacteria [18], some microorganisms can utilize phenolic acids as carbon sources [19]. Anaerobic rumen fungi produce high levels of both *p*-coumaroyl and feruloyl esterases [20]. A *p*-coumaroyl [6,21] and two feruloyl [22] esterases have been recently purified to homogeneity from the anaerobic fungus *Neocallimastix* MC-2. Feruloyl esterase activity has been reported in culture supernatants of *Streptomyces olivochromogenes* [23], *Schizophyllum commune* [24], and *Aspergillus niger* [25]. Furthermore, ferulic acid esterases have recently been purified to homogeneity from *Streptomyces olivochromogenes* [26], *Pseudomonas fluorescens* [27], and *Aspergillus niger* [28,29], and the specificities of these ferulic acid esterases for a range of methyl ester derivatives of cinnamoyl and benzoyl acids have been examined. With the exception of the ferulic acid esterase from *Pseudomonas fluorescens*, which showed similar catalytic activities for methyl ferulate, methyl coumarate, methyl cinnamate, and methyl caffeate, all the ferulic acid esterases could be differentiated by their characteristic substrate specificities, each enzyme recognising specific substitutions on the phenolic ring. In the case of *Aspergillus niger*, two forms of ferulic acid esterase have been isolated from a commercial source of pectinase. One, designated FAE-I, had a molecular mass of 150 kDa, and the second, designated FAE-II, a molecular mass of 29 kDa [28]. FAE-I was specific for ferulic, *p*-coumaric, and caffeic acids, whereas FAE-II was specific for ferulic and sinapinic acids. It was therefore assumed that FAE-I had a clear requirement at C-5 of the phenolic ring for no substitution while FAE-II had a preference for a methoxyl group at C-3 of the phenolic ring [29]. Another ferulic acid esterase, designated FAE-III, was purified directly from a culture supernatant of *Aspergillus niger* [29]. This enzyme showed similar substrate specificities to FAE-II but had much higher specific activities.

In general, ferulic acid esterases are active on plant cell-wall feruloylated polysaccharides only in the presence of carbohydrases, although there are exceptions [29]. During the course of the reaction, carbohydrases produce low molecular weight substrates [26–30], which suggests that the length of the oligosaccharide chain to which ferulic acid is attached plays an important role in the ferulic acid esterase activity. The importance of the nature of the oligosaccharide chain and of the position to which ferulic acid is attached has not been studied to our knowledge. For that purpose, we have produced different feruloylated oligosaccharides from the degradation of wheat bran and sugar-beet pulp.

Isolated feruloylated oligosaccharides from *Gramineae* cell walls consistently showed ferulic acid linked to O-5 of L-Ara*f* side-chains of arabinoxylans [18]. In

dicots, ferulic acid residues were shown to be linked to the pectic side-chains [8,11–15]. In the accompanying papers [31,32], we report on the isolation and characterisation of various feruloylated oligosaccharides from sugar-beet pulp. We have shown [14] that half of the feruloyl groups present in the pulp were linked to O-2 of L-Ara*f* residues of the main core of α-(1 → 5)-linked arabinan chains. The other feruloyl groups were found to be linked to O-6 of D-Gal*p* residues of the main core of β-(1 → 4)-linked type I galactan chains.

In the present study, we report on the degradation of these various feruloylated oligosaccharides from sugar-beet pulp by two forms of ferulic acid esterase from *Aspergillus niger*, a high molecular weight form (FAE-I) isolated from a commercial enzymic preparation [28] and a low molecular weight form (FAE-III) directly purified from a culture of *Aspergillus niger* [29]. Methyl ferulate was used as reference substrate for the ferulic esterase activity. Furthermore, the degradation of three feruloylated oligosaccharides from wheat bran was investigated. The dependences of ferulic acid esterase activity on the presence of a sugar and on its structure, on the type of linkage involved, and on the length of the oligosaccharide chain are presented.

The origin, nomenclature, and structural characteristics of the feruloylated oligosaccharides prepared from sugar-beet pulp and wheat bran are summarised in Table 1.

Table 1
Origin, nomenclature, and structural characteristics of the feruloylated oligosaccharides

	Origin	Nomenclature	Structure
Monosaccharides	Sugar-beet pulp	Ara_1F (Ara*p* 2 ← F.A.)	2-*O*-(*trans*-Feruloyl)-L-arabinopyranose
		Gal_1F	?6-*O*-(*trans*-Feruloyl)-D-galactopyranose
	Wheat bran	Ara_1F (Ara*f* 5 ← F.A.)	5-*O*-(*trans*-Feruloyl)-L-arabinofuranose
Disaccharides	Sugar-beet pulp	Ara_2F	*O*-[2-*O*-(*trans*-Feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose
		Gal_2F	*O*-[6-*O*-(*trans*-Feruloyl)-β-D-galactopyranosyl]-(1 → 4)-D-galactopyranose
Trisaccharides	Sugar-beet pulp	Ara_3F	*O*-α-L-Arabinofuranosyl-(1 → 3)-*O*-[2-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 5)-L-arabinofuranose
	Wheat bran	Xyl_2Ara_1F	*O*-[5-*O*-(*trans*-Feruloyl)-α-L-arabinofuranosyl]-(1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose
Tetrasaccharides	Wheat bran	Xyl_3Ara_1F	*O*-β-D-Xylopyranosyl-(1 → 4)-*O*-[5-*O*-(*trans*-feruloyl)-α-L-arabinofuranosyl]-(1 → 3)-*O*-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose
Hexasaccharides	Sugar-beet pulp	Ara_6F	?
Heptasaccharides	Sugar-beet pulp	Ara_7F	?
Octasaccharides	Sugar-beet pulp	Ara_8F	?

2. Results

Spectrophotometric assay of ferulic acid esterase activity.—The enzymic assays were performed at pH 6, which is the optimum pH [28,29] of the ferulic acid esterases, and at 37°C. In agreement with previously published data [31,33], the difference in absorption maxima at pH 6 of free ferulic acid and of methyl ferulate (Fig. 1) permitted spectrophotometric monitoring of the conversion of esterified into free ferulic acid. As shown in Fig. 1, difference spectroscopy at pH 6 between methyl ferulate and free ferulic acid revealed a difference maximum at 335 nm. At this wavelength, methyl ferulate showed an extinction coefficient (19524 M^{-1} cm^{-1}) approximately 4.4 times that of free ferulic acid (4409 M^{-1} cm^{-1}). Identical difference maxima were observed for all the feruloylated oligosaccharides studied. Up to 130 μM, esterified and free ferulic acids obeyed the Lambert–Beer law.

Hydrolysis of the ester linkage can be calculated by the following equation:

$$\text{hydrolysis}\ (\%) = \frac{A_{335} - \epsilon_l C_0}{\epsilon_f - \epsilon_l} \times 100/C_0$$

where A_{335} = absorbance at 335 nm and pH 6, ϵ_l = extinction coefficient of linked ferulic acid at 335 nm (19524 M^{-1} cm^{-1}), ϵ_f = extinction coefficient of free ferulic acid at 335 nm (4409 M^{-1} cm^{-1}), and C_0 = initial concentration of feruloyl ester (M).

Fig. 2 illustrates the change in the absorption spectrum of Ara_1F from wheat bran incubated with FAE-I as a function of time. Initially, a peak at 322 nm was observed, corresponding to the esterified feruloyl groups. The absorbance at 335 nm decreased with increased reaction time and all curves passed through an

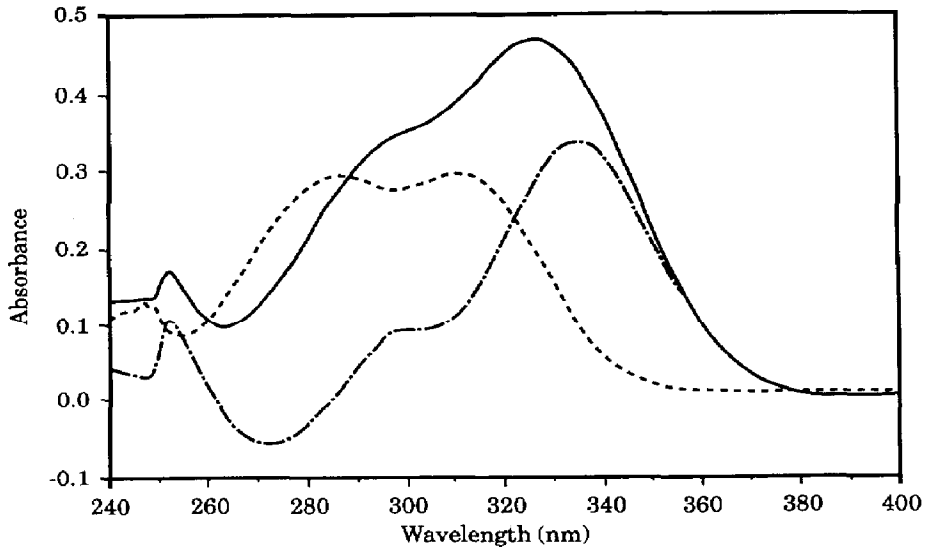

Fig. 1. Absorption spectra of esterified and free ferulic acid (22.5 μM): ———, methyl ferulate; - - - - - -, *trans*-ferulic acid; ·—·—·, difference spectrum.

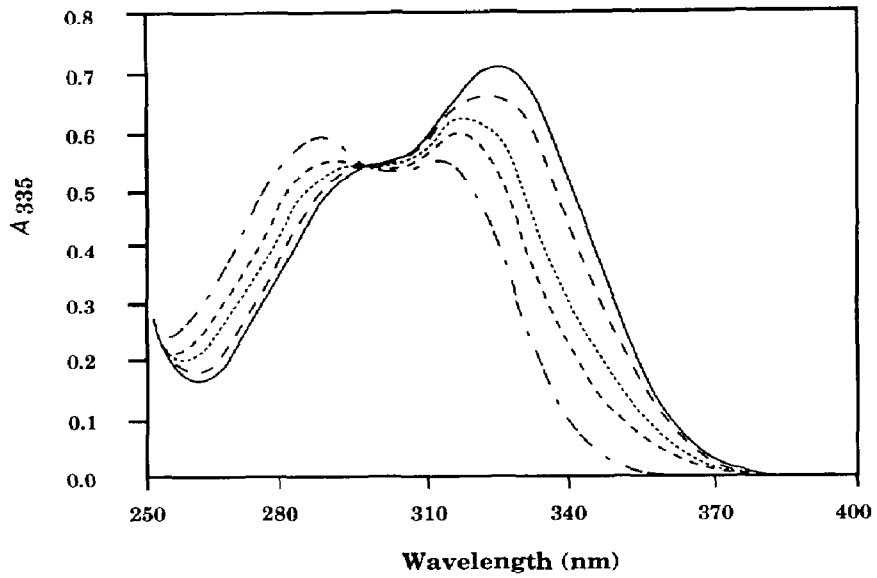

Fig. 2. Changes in absorption spectra during hydrolysis. FAE I was incubated with feruloylated arabinose monosaccharides (30 mM) from wheat bran in 0.1 M MOPS buffer at pH 6: ———, 0 min; — — —, 5 min; - - - - - -, 15 min; – – –, 30 min; - — - — -, free ferulic acid.

isosbestic point at ~290 nm. It was calculated that 9.6, 29.3, and 46.9% of the esterified feruloyl groups initially present were converted into free ferulic acid after 5, 15, and 30 min, respectively. Confirmation of ester hydrolysis was performed by HPLC, and both spectrophotometric and HPLC methods gave similar results.

Activity of FAE-I on methyl ferulate and pure feruloylated oligosaccharides.—Enzyme kinetics for ferulic acid esterases were determined by continuously monitoring the decrease in esterified ferulic acid at 335 nm. Fig. 3 shows the activity of FAE-I on the different feruloylated substrates. The K_m, V_{max}, and efficiency (K_m/V_{max}) values are presented in Table 2.

(a) Activity on methyl ferulate. Methyl ferulate appeared to be a relatively poor substrate for FAE-I as only 12.2% of ferulic acid was released after 30 min. A K_m of 0.286 mM and a V_{max} of 2 U/mg were found, leading to an efficiency of 7 min^{-1} mg^{-1}. The K_m value observed was lower than that previously found using the same enzyme [28]. This is probably due to the different incubation temperatures used (37 and 50°C).

(b) Activity on feruloylated arabinose oligosaccharides from sugar-beet pulp. The hydrolysis kinetics (Fig. 3) showed that the initial rates of degradation were linear up to 15–20% of ferulic acid released. Although all the feruloylated arabinose oligosaccharides from sugar-beet pulp were hydrolysed by FAE-I, there was a clear preference for feruloylated di- and tri-saccharides (Ara_2F and Ara_3F) from which 41.7 and 71.0%, respectively, of the ferulic acid was released after 30 min. The feruloylated arabinose monosaccharide Ara_1F (Ara*p* 2 ← F.A.) was hydrolysed at a lower rate (14.1% of ferulic acid released after 30 min). No significant differ-

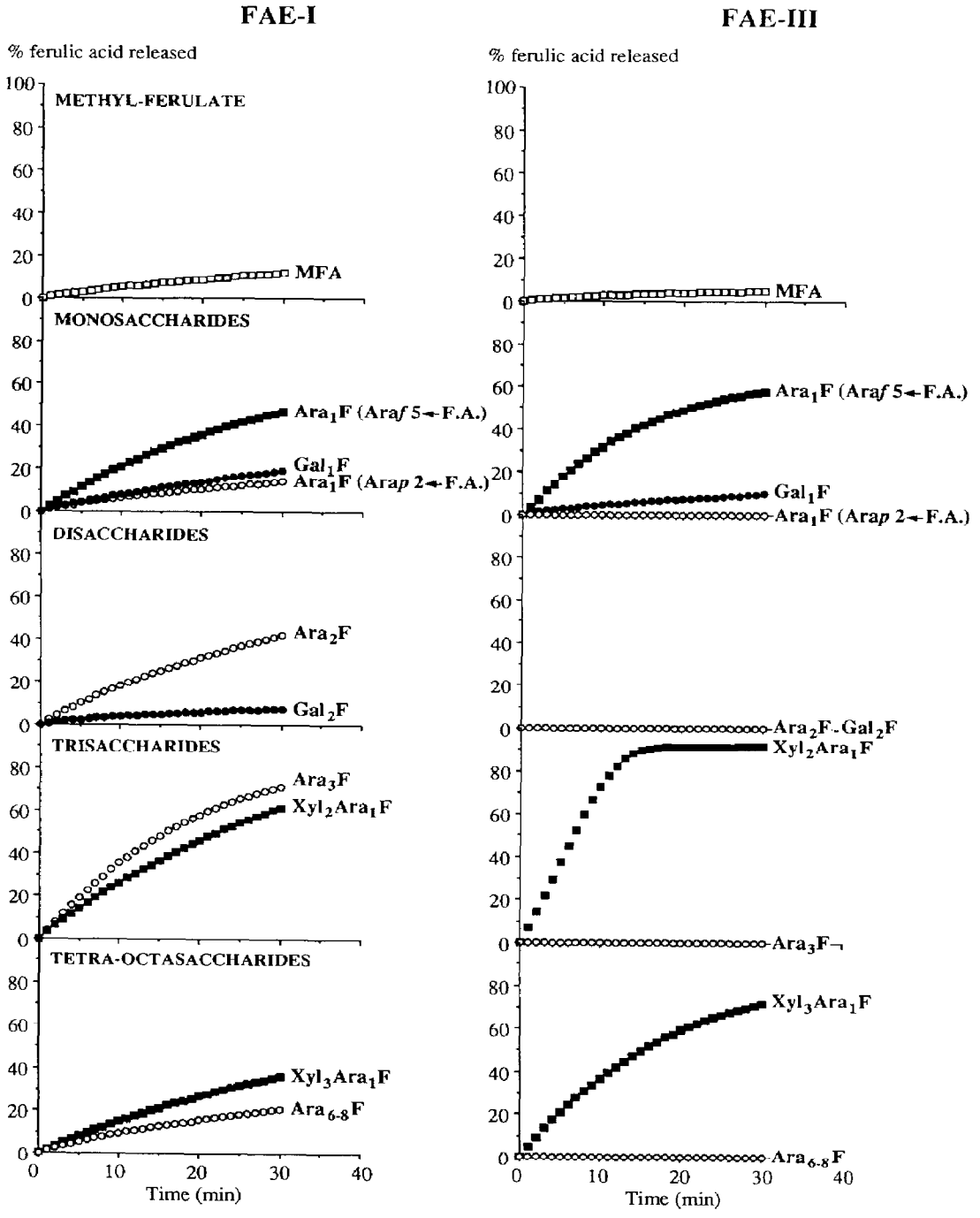

Fig. 3. Rates of hydrolysis of feruloylated substrates by ferulic acid esterases: □, methyl ferulate; ○, feruloylated arabinose oligosaccharides from sugar-beet pulp; ●, feruloylated galactose oligosaccharides from sugar-beet pulp; ■, feruloylated oligosaccharides from wheat bran.

Table 2
Affinity (K_m), maximum velocity (V_{max}), and efficiency (V_{max}/K_m) of FAE-I

Substrates	Nomenclature	Number of points	K_m (mM)	SD [a]	V_{max} (U/mg)	SD [a]	V_{max}/K_m (min^{-1} mg^{-1})
Methyl ferulate	MFA	11	0.286	(0.062)	2.0	(0.3)	7.0
Monosaccharides	Ara_1F (Ara*p* 2 ← F.A.)	10	0.478	(0.126)	4.0	(0.9)	8.4
	Gal_1F	5	0.333	(0.121)	3.6	(1.2)	10.7
	Ara_1F (Ara*f* 5 ← F.A.)	10	0.129	(0.012)	5.1	(0.3)	39.5
Disaccharides	Ara_2F	9	0.300	(0.034)	8.5	(0.8)	28.3
	Gal_2F	13	1.023	(0.114)	4.6	(0.6)	4.5
Trisaccharides	Ara_3F	9	0.085	(0.006)	6.0	(0.2)	70.6
	Xyl_2Ara_1F	9	0.133	(0.009)	5.4	(0.3)	40.6
Tetrasaccharides	Xyl_3Ara_1F	9	0.138	(0.020)	3.6	(0.3)	26.1
Hexasaccharides	Ara_6F	10	0.500	(0.166)	4.4	(1.3)	8.9
Heptasaccharides	Ara_7F	10	0.435	(0.153)	5.3	(1.3)	12.2
Octasaccharides	Ara_8F	10	0.400	(0.097)	4.0	(0.8)	10.0

[a] Standard deviation.

ences in the rate of hydrolysis were observed between the feruloylated oligosaccharides of greater dp (Ara_6F, Ara_7F, and Ara_8F) (17.2–20.3% of ferulic acid released after 30 min).

Michaelis–Menten kinetics were observed with respect to substrate concentration and two typical double-reciprocal plots are shown in Fig. 4. K_m and V_{max} values for Ara_1F (Ara*p* 2 ← F.A.), Ara_6F, Ara_7F, and Ara_8F were not significantly different and an efficiency of 8.4–12.2 min^{-1} mg of $protein^{-1}$ was observed. The affinity of the enzyme for these substrates was in the same range as that observed for methyl ferulate, but the V_{max} was 2 times higher. Lower K_m values and slightly higher V_{max} values were observed for Ara_2F and Ara_3F, leading to an efficiency of 28.3 and 70.6 min^{-1} mg of $protein^{-1}$, respectively. FAE-I showed

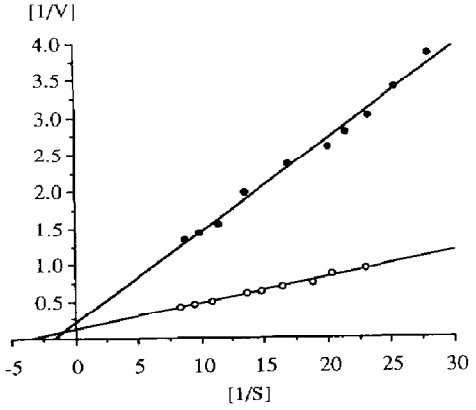

Fig. 4. Lineweaver–Burk double-reciprocal plots for hydrolysis of feruloylated mono- and di-saccharides from sugar-beet pulp by *Aspergillus niger* FAE-I: ●, Ara_1F; ○, Ara_2F; [S] = concentration of esterified ferulic acid (mM); [V] = rate of hydrolysis in μmol min^{-1} mg $protein^{-1}$.

maximum affinity for the feruloylated trisaccharide (Ara_3F); the affinity for the feruloylated disaccharide (Ara_2F) was moderate and close to that observed for the feruloylated monosaccharide Ara_1F (Ara*p* 2 ← F.A.). The highest maximum velocity was observed with Ara_2F.

(c) Activity on feruloylated galactose oligosaccharides from sugar-beet pulp. The hydrolysis kinetics (Fig. 3) showed that the feruloylated galactose monosaccharide (Gal_1F) was hydrolysed in a similar manner to the feruloylated arabinose monosaccharide Ara_1F (Ara*p* 2 ← F.A.) and 19.2% of the ferulic acid was released after 30 min. The feruloylated galactose disaccharide (Gal_2F) was hydrolysed at a very low rate and only 7.6% of the ferulic acid was released after 30 min. The K_m and V_{max} values observed for Gal_1F were not significantly different from those obtained for Ara_1F (Ara*p* 2 ← F.A.), showing that these two feruloylated monosaccharides, although different in nature, were recognised and hydrolysed in a similar way by FAE-I. Gal_2F was hydrolysed at a similar V_{max}, but the K_m of the enzyme for this substrate was ~ 3 times lower than that observed for the feruloylated monosaccharides.

(d) Activity on feruloylated oligosaccharides from wheat bran. All the feruloylated oligosaccharides from wheat bran [Ara_1F(Ara*f* 5 ← F.A.), Xyl_2Ara_1F, and Xyl_3Ara_1F] were hydrolysed by FAE-I, but with a slight preference for the feruloylated trisaccharide (Xyl_2Ara_1F); 46.9, 61.3, and 36.5% of the ferulic acid was released after 30 min for these feruloylated mono-, tri-, and tetra-saccharides, respectively. As for feruloylated oligosaccharides from sugar-beet pulp, the initial rates of degradation were linear up to ~ 20% of ferulic acid released.

The enzyme showed the same K_m for all the feruloylated oligosaccharides from wheat bran. A similar V_{max} was observed for Ara_1F (Ara*f* 5 ← F.A.) and Xyl_2Ara_1F, but the V_{max} for Xyl_3Ara_1F was slightly lower.

Only slight differences were observed for the V_{max} values within the different feruloylated oligosaccharides derived from cell walls, the higher values being observed for the feruloylated arabinose disaccharide from sugar-beet pulp and for the feruloylated trisaccharides from sugar-beet pulp and wheat bran. The V_{max} observed for the synthetic substrate (methyl ferulate) was lower than that observed for the natural substrates. The enzyme showed a significantly different affinity for the various substrates with a clear preference for the feruloylated arabinose trisaccharide from sugar-beet pulp, followed by the feruloylated oligosaccharides from wheat bran. Methyl ferulate, feruloylated monosaccharides and disaccharides from sugar-beet pulp, and feruloylated oligosaccharides with large dp also from beet pulp were relatively poor substrates.

Activity of FAE-III on methyl ferulate and pure feruloylated oligosaccharides.— The K_m, the V_{max}, and efficiency (V_{max}/K_m) of FAE-III on the feruloylated substrates are presented in Table 3. The hydrolysis kinetics are shown on Fig. 3.

(a) Activity on methyl ferulate. As already shown for FAE-I, methyl ferulate appeared to be a relatively poor substrate for FAE-III and only 4.9% of ferulic acid was released after 30 min. A K_m of 2.08 mM and a V_{max} of 175 U/mg were found, leading to an efficiency of 84 min^{-1} mg^{-1}.

(b) Activity on feruloylated oligosaccharides from sugar-beet pulp. FAE-III had

Table 3
Affinity (K_m), maximum velocity (V_{max}), and efficiency (V_{max}/K_m) of FAE-III

Substrates	Nomenclature	Number of points	K_m (mM)	SD [a]	V_{max} (U/mg)	SD [a]	V_{max}/K_m (min^{-1} mg^{-1})
Methyl ferulate	MFA	14	2.080	(0.19)	175	(14)	84
Monosaccharides	Ara_1F (Ara*p* 2 ← F.A.)		n.d. [b]		n.d.		
	Gal_1F	5	5.000	(1.000)	200	(50)	40
	Ara_1F (Ara*f* 5 ← F.A.)	12	0.075	(0.007)	130	(6)	1728
Disaccharides	Ara_2F		n.d.		n.d.		
	Gal_2F		n.d.		n.d.		
Trisaccharides	Ara_3F		n.d.		n.d.		
	Xyl_2Ara_1F	20	0.019	(0.002)	114	(4)	7600
Tetrasaccharides	Xyl_3Ara_1F	11	0.063	(0.012)	206	(23)	3270
Hexasaccharides	Ara_6F		n.d.		n.d.		
Heptasaccharides	Ara_7F		n.d.		n.d.		
Octasaccharides	Ara_8F		n.d.		n.d.		

[a] Standard deviation.
[b] Not detectable.

no activity on any of the feruloylated arabinose oligosaccharides and even the use of 10 times more concentrated enzyme did not lead to hydrolysis. A low activity was observed on feruloylated galactose monosaccharide (Gal_1F), and 9.7% of the ferulic acid was released after 30 min of hydrolysis. No activity was observed on feruloylated galactose disaccharide (Gal_2F) for the concentration of enzyme tested, but the use of 10 times more concentrated enzyme allowed observation of a slight hydrolysis of that substrate up to 5.5% of ferulic acid released after 30 min of hydrolysis.

The small amount of Gal_1F available [31,32] did not permit checking of more than 5 substrate concentrations, and this explains the somewhat higher standard deviations obtained for the K_m and V_{max} values. FAE-III showed a high V_{max} but a very low K_m for Gal_1F, leading to an efficiency of ~ 40 min^{-1} mg^{-1}. The very low activity of FAE-III on Gal_2F did not permit accurate measurement of the apparent K_m and V_{max}.

(*c*) *Activity on feruloylated oligosaccharides from wheat bran.* All the feruloylated oligosaccharides from wheat bran were extensively and rapidly hydrolysed by FAE-III; 57.5 and 72.0% of the ferulic acid was released after 30 min of hydrolysis for the feruloylated monosaccharide Ara_1F (Ara*f* 5 ← F.A.) and the feruloylated tetrasaccharide Xyl_3Ara_1F, respectively. The feruloylated trisaccharide Xyl_2Ara_1F was almost totally (> 90%) hydrolysed after 15 min of incubation with FAE-III. For Ara_1F (Ara*f* 5 ← F.A.) and Xyl_3Ara_1F, the initial hydrolysis rate was linear up to ~ 30% of ferulic acid released, whereas for Xyl_2Ara_1F, it was linear up to 75% of ferulic acid released.

As shown in Table 3, the apparent maximum velocity of hydrolysis seemed to increase slightly with increase in the dp of the feruloylated wheat bran substrates. The apparent K_m values were similar for Ara_1F (Ara*f* 5 ← F.A.) and Xyl_3Ara_1F. It was, however, much lower for Xyl_2Ara_1F and it can be calculated that the

affinity of FAE-III for this feruloylated trisaccharide is 4 times higher than that observed for the feruloylated mono- and tetra-saccharides. The efficiency of the enzyme was very high for all the feruloylated oligosaccharides; on Xyl_2Ara_1F, it was 4.4 times higher than that on Ara_1F (Ara*f* 5 ← F.A.), and 2.3 times higher than that on Xyl_3Ara_1F.

FAE-III showed a much higher maximum velocity on feruloylated substrates than FAE-I, which indicates that it has a particularly high specific activity. FAE-III appeared to be very specific towards feruloylated oligosaccharides from wheat bran. Feruloylated arabinose oligosaccharides from sugar-beet pulp were not hydrolysed at all by this enzyme and feruloylated galactose oligosaccharides were hydrolysed only to a very limited extent. It appeared that the maximum velocity values were in the same range (factor 1.7) for all the substrates, whereas drastic differences were observed for the affinity of the enzyme for the feruloylated substrates (factor 260).

3. Discussion

The two ferulic acid esterases were more active on selected substrates of cell-wall origin than on methyl ferulate, which shows that ferulic acid esterases have a certain specificity for the oligosaccharide chain to which ferulic acid is attached in the cell-wall polysaccharides. Borneman et al. [22] have shown that two feruloyl esterases from *Neocallimastix* MC-2 hydrolysed methyl ferulate at low rates compared to feruloylated tri- and tetra-saccharides from coastal Bermuda grass.

The ferulic acid esterases could be differentiated by their specificities for the natural feruloylated substrates of different origin. FAE-I hydrolysed all the feruloylated oligosaccharides tested, and V_{max}/K_m for the best and the worst substrates only varied 16-fold. FAE-III had a much more rigorous specificity, as only certain of the feruloylated oligosaccharides tested were hydrolysed; a factor of 190 was found for the efficiency between the best and the worst substrates. It has to be noted that, for both enzymes, the maximum velocity was fairly similar for all the substrates (factor 4.2 and 1.7 for FAE-I and FAE-III, respectively), whereas the affinity values were dramatically different (factor 12 and 260 for FAE-I and FAE-III, respectively). This implies that the binding of the sequence of sugars of the substrate to the active site of the enzyme is the rate-limiting step of the enzymic reaction.

For both enzymes, there seemed to be a clear preference for the arabinose residue to which ferulic acid is attached to be in the furanose form; this was particularly obvious for FAE-I which hydrolysed feruloylated Ara*f* mono- and di-saccharides at a much higher rate than feruloylated Ara*p* and Gal*p* monosaccharides. This enzyme had a similar efficiency on feruloylated Ara*p* and Gal*p* monosaccharides, which suggests that the nature of the sugar esterifying the feruloyl group is not of fundamental importance. The position to which ferulic acid is attached did not seem to affect largely the activity of FAE-I. Although there was

a slight preference for the feruloylated trisaccharide for sugar-beet pulp in which ferulic acid is attached to O-2 of the Ara*f* ring, the feruloylated trisaccharide from wheat bran in which ferulic acid is attached to O-5 was efficiently hydrolysed. FAE-III, in contrast, hydrolysed only feruloylated arabinose oligosaccharides from wheat bran and not at all those from sugar-beet pulp. This enzyme had a clear requirement for ferulic acid to be attached to O-5 of the Ara*f* ring and no catalysis was observed when ferulic acid was attached to O-2. This could be explained by the extra length of the linkage present between the sugar ring and the phenolic ring when the feruloyl group is attached to O-5 of the Ara*f* ring. The active site of the enzyme might be unable to bind the phenolic ring unless it is at a sufficient distance from the sugar ring. This hypothesis is supported by the fact that feruloylated galactose oligosaccharides, in which ferulic acid is attached to O-6 of the Gal*p* ring, can bind to the active site of the enzyme.

Both enzymes, but it is particularly obvious for FAE-III, showed maximum activity on feruloylated trisaccharides. The affinity for feruloylated oligosaccharides of lower dp was similar or slightly lower for FAE-I and much lower for FAE-III (factor 4 between the feruloylated mono- and tri-saccharides from wheat bran). An increase in the length of the oligosaccharide chain did not preclude catalysis, but feruloylated oligosaccharides of dp > 3 were hydrolysed at a reduced rate because the ferulic acid esterases exhibited a lower affinity for them. This suggests the presence of subsites for three sugar rings in or close to the active site.

Several ferulic acid esterases could be obtained from *Aspergillus niger*. These enzymes were shown to have different specificities for the phenolic rings [28,29] and we have shown that FAE I and FAE III also have different specificities for the oligosaccharide chain esterifying the feruloyl groups. The growth medium seems to play a major role in the type of ferulic acid esterase produced by the microorganisms. When *Aspergillus niger* is grown on pectins or pectin-rich material, a greater number of forms of ferulic acid esterase seem to be produced [28]. When grown on cereal-derived products, only one form of ferulic acid esterase is produced (FAE-III) [29], and this form is unable to hydrolyse feruloylated oligosaccharides derived from sugar-beet pectins. FAE I, like many other ferulic acid esterases from other sources [20,22,24,26], has no activity on feruloylated polysaccharides from cell walls. From our study, by extrapolation from the results on the larger feruloylated oligosaccharides, it appears that this inactivity of FAE-I type enzymes might be due to physical phenomena such as the accessibility of the substrate.

4. Experimental

Sugar-beet-pulp feruloylated oligosaccharides.—Sugar-beet pulp was hydrolysed enzymatically (Driselase) and by acid to yield a series of feruloylated oligosaccharides. Feruloylated di-, tri-, hexa-, hepta-, and octa-saccharides of arabinose (Ara_2F, Ara_3F, Ara_6F, Ara_7F, and Ara_8F, respectively) as well as feruloylated galactose disaccharide (Gal_2F) were obtained after hydrolysis of the pulp with the fungal carbohydrase mixture (Driselase) [31,32]. Feruloylated arabinose (Ara_1F)

and galactose (Gal_1F) monosaccharides were obtained through mild acid hydrolyses [31,32].

Wheat-bran feruloylated oligosaccharides.—Destarched wheat bran (10 g) was incubated in 1 L with 1 mg/mL Driselase in distilled water at 37°C for 48 h [6,29]. The suspension was boiled for 10 min and centrifuged at 5000 *g* for 10 min. The supernatant solution was concentrated in vacuo to 10 mL, filtered (0.45 μm), and applied to a column (74 × 2.1 cm) of Sephadex LH-20 equilibrated with water. The elution at 30 mL/h with the same solvent yielded two main feruloylated fractions. These fractions have been collected, freeze-dried, and analysed by ^{13}C, ^{1}H, and COSY NMR spectroscopy [32]. The spectra obtained showed unambiguously that these two peaks comprised highly pure *O*-β-D-Xyl*p*-(1 → 4)-*O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1 → 3)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (Xyl_3Ara_1F) and *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1 → 3)-*O*-β-D-Xyl*p*-(1 → 4)-D-Xyl*p* (Xyl_2Ara_1F).

Destarched wheat bran (10 g) was incubated in 0.1 M trifluoroacetic acid (1 L) at 100°C for 3 h. The acid-soluble fraction was recovered by filtration, the solvent evaporated in vacuo, and the residue dissolved in 10 mL of distilled water. After filtration (0.45 μm), the solution was injected on Biogel P-2 and the feruloylated arabinose monosaccharide eluted at 2.1 column volumes as already described for sugar-beet pulp [31]. This fraction was collected, freeze-dried, and analysed by ^{13}C, ^{1}H, and COSY NMR spectroscopy. The NMR data were fully consistent with this compound being 5-*O*-(*trans*-feruloyl)-L-Ara*f* as shown by earlier workers [2,3,33].

Preparation of the enzymes.—FAE-I was obtained from commercially available "pectinase" (Sigma Chemical Co. P-5146) [28]. FAE-III was obtained from an *Aspergillus niger* culture grown on oat spelt xylan [29]. Both enzymes were purified to homogeneity using hydrophobic interaction and ion-exchange chromatography [28,29].

Assays for ferulic acid esterase activity. The assays with FAE-I were performed using a 33 μM solution of feruloylated substrates in MOPS pH 6 and a concentration of enzyme of 0.908 μg of protein/mL of assay. The assays with FAE-III were performed using a 33 μM solution of feruloylated substrates in MOPS pH 6 and a concentration of enzyme of 0.044 μg of protein/mL of assay. The decline in absorbance at 335 nm was monitored continuously in the spectrophotometer with the sample compartment maintained at 37°C. One unit of activity (1 U) is defined as the amount of enzyme releasing 1 μmol of free ferulic acid/min under these conditions. Protein was estimated using the Coomassie Protein Assay reagent from Pierce.

The effect of substrate concentration on ferulic acid esterase activity was examined at 37°C in MOPS pH 6. The K_m and V_{max} were determined from Lineweaver–Burk plots and by the method of Wilkinson [35]. The latter method provides an estimate of the standard error.

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