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Feruloyl esterases as a tool for the release of phenolic compounds from agro-industrial by-products

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Abstract—Agro-industrial by-products are a potential source of added-value phenolic acids with promising applications in the food and pharmaceutical industries. Here two purified feruloyl esterases from *Aspergillus niger*, FAEA and FAEB were tested for their ability to release phenolic acids such as caffeic acid, *p*-coumaric acid and ferulic acid from coffee pulp, apple marc and wheat straw. Their hydrolysis activity was evaluated and compared with their action on maize bran and sugar beet pulp. The specificity of both enzymes against natural and synthetic substrates was evaluated; particular attention was paid to quinic esters and lignin monomers. The efficiency of both enzymes on model substrates was studied. We show the ability of these enzymes to hydrolyze quinic esters and ester linkages between phenolic acids and lignin monomer.

Keywords: Feruloyl esterase; *Aspergillus niger*; Phenolic acids; Agro-industrial substrates

1. Introduction

Plant cell walls are three-dimensional structures formed with a polysaccharide network of which cellulose, hemicelluloses and pectin are the most important components and it is well established that they contain hydroxycinnamic acids covalently linked to polysaccharides through ester linkages.[1] The role of hydroxycinnamates in the plant cell walls has been widely studied. They have been implicated in the regulation of cellular expansion and plant defense and they may reduce the digestibility of cell wall by restricting accessibility to carbohydrates. They are found in numerous plants and in significant quantities in agro-industrial derived by-products. Ferulic acid is abundant in wheat bran (0.5% w/w), sugar beet pulp (0.8% w/w) and maize bran (around 3.0% w/w).[2–4] It is linked to different positions on the arabinose sugar in wheat bran and sugar beet.[5,6] Moreover, cross-links through diferulic bridges are found both in heteroxylans and pectin tissues thus playing an important role in the structure of non-lignified cell walls.[4] *p*-Coumaric acid was found in significant amounts in maize bran (0.33% w/w).[4] Hydroxycinnamic acids occur widely in the cell walls of lignified plants such as cereal straws where they are linked through ester and ether bridges to polysaccharides and/or lignin.[7] Wheat straw contains, respectively, 1.24% and 0.66% of ferulic and *p*-coumaric acids. In plant material, hydroxycinnamic acids are also found as soluble conjugates of quinic acid named chlorogenic acids.[8,9] The commonest chlorogenic acid is 5-*O*-caffeoyl quinic acid. It is present in particularly high concentrations in numerous food and beverages such as coffee, pears,

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potato tubers and apple, and as a consequence in derived by-products. Analysis of the phenolic compounds in coffee pulp (the solid residue from coffee processing) indicated that chlorogenic acid was the main constituent (around 40%).[10] Apples have also been extensively studied. Values of 200 mg/kg total chlorogenic acid are reported but composition varies markedly with variety, cider varieties being richer than culinary.[11] The cider industry generates a residue that contains at least 500 mg/kg 5-*O*-caffeoyl quinic acid. The industrial use of hydroxycinnamates has attracted growing interest for several years since they and their conjugates were shown to be bioactive molecules, possessing potential antioxidant activities and health benefits.[12] The removal of these phenolic compounds and the breakdown of the ester linkages between polymers allows numerous exploitation for industrial and food applications.

Feruloyl esterases (EC 3.1.1.73), members of the carboxylic ester hydrolases sub-class of enzymes, have been found to cleave the ester linkage between hydroxycinnamic acids and sugars. These enzymes have been purified and characterized from a wide range of microorganisms, including bacteria and fungi (*Pseudomonas fluorescens*, *Penicillium funiculosum*, *Talaromyces stipitatus*, *Aspergillus niger*).[13–17] Recently, they were organized into four functional classes termed types A, B, C and D, which take into account substrate specificities against synthetic methyl esters of hydroxycinnamic acids, growth substrate requirements of the microorganisms and protein sequence identity.[18] Each feruloyl esterase has its own specificity with regard to the release of specific cinnamic acids. Two major enzymes were purified from *A. niger*, FAEA and FAEB (CinnAE), which are today classified as types A and C, respectively. Type C feruloyl esterases hydrolyze the four methyl esters of hydroxycinnamic acids generally used as model substrates (methyl ferulate, methyl sinapinate, methyl *p*-coumarate and methyl caffeate) but not diferulic compounds. In comparison, type A feruloyl esterases do not hydrolyze methyl ester of caffeic acid but are able to release diferulic compounds. These enzymes have both been overproduced in *A. niger*.[19,20] Studies of the ability of feruloyl esterases to hydrolyze natural substrates have mainly focused on FAEA and the release of ferulic acid. Different materials have been used such as sugar beet pulp, maize bran, wheat bran or oat hulls for the most important.[16,17,21–23] The pure enzymes are generally very slightly active when used directly on the raw substrate. An alternative strategy is to use suitable enzyme mixtures, including carbohydrate hydrolases and pretreatment of the raw material resulting in partial degradation of the more complex structures, as already reported in the release of ferulic acid from maize bran.[24] Besides, only a few studies have focused on the ability of feruloyl esterases to release *p*-coumaric and caffeic acids from natural substrates.

Our objectives in this study were to examine the different roles of two purified feruloyl esterases from *A. niger* FAEA and FAEB in the release of phenolic acids such as caffeic, ferulic and *p*-coumaric acid from natural agro-industrial residues. In order to better understand the mechanism of action of the two enzymes, we enlarged the panel of substrates. Apple marc and coffee pulp were chosen for their high content of caffeic acid and *p*-coumaric acid; in parallel, maize bran and sugar beet pulp were used for their content in ferulic acid. Special attention was also paid to wheat straw, which is typified by the presence of ferulic and *p*-coumaric acids linked to polysaccharides and/or lignin. In parallel, specificity against synthetic and model substrates was also determined.

2. Results and discussion

2.1. Hydrolysis of coffee pulp and apple marc

Coffee pulp and apple marc were selected for their high content of chlorogenic acids such as 5-*O*-caffeoyl quinic acid. Total amounts of caffeic, *p*-coumaric and ferulic acids were first determined after alkaline hydrolysis and HPLC analysis (Table 1). Coffee pulp and apple marc contained, respectively, 2.66 and 0.33 mg/g caffeic acid, 0.08 and 0.27 mg/g *p*-coumaric acid and 0.24 and 0.18 mg/g ferulic acid. Coffee pulp was rich in caffeic acid and ferulic acid, while apple marc contained greater amounts of *p*-coumaric acid. Initial chlorogenic acid content of both by-products was not visualized by this method since it was immediately hydrolyzed in caffeic acid and quinic acid. Before the action of the enzymes, no free phenolic acids could be detected. After incubation of both by-products with purified feruloyl esterases FAEA and FAEB, significant amounts of phenolic acids were released as shown in Figure 1. Only FAEB was active in the release of *p*-coumaric and caffeic acids from coffee pulp and apple marc. One hundred per cent of the alkali-extractable caffeic acid in the case of coffee pulp, and 83% in the case of apple marc were freed. Also, 73% and 34% of total *p*-coumaric acid was released. In comparison, FAEA had no significant effect. These results are consistent with the respective specificities

Table 1. Phenolic acid composition of agro-industrial by-products

Material	Caffeic acid	*p*-Coumaric acid	Ferulic acid
Coffee pulp	2.66	0.08	0.24
Apple marc	0.33	0.27	0.18
Steam-exploded wheat straw	0	2.13	1.35
Autoclaved maize bran	0	3.12	31.22
Sugar beet pulp	0	0	6.4

Values were determined after alkaline hydrolysis and expressed as mg/g dry material. Data are means of triplicate analysis.

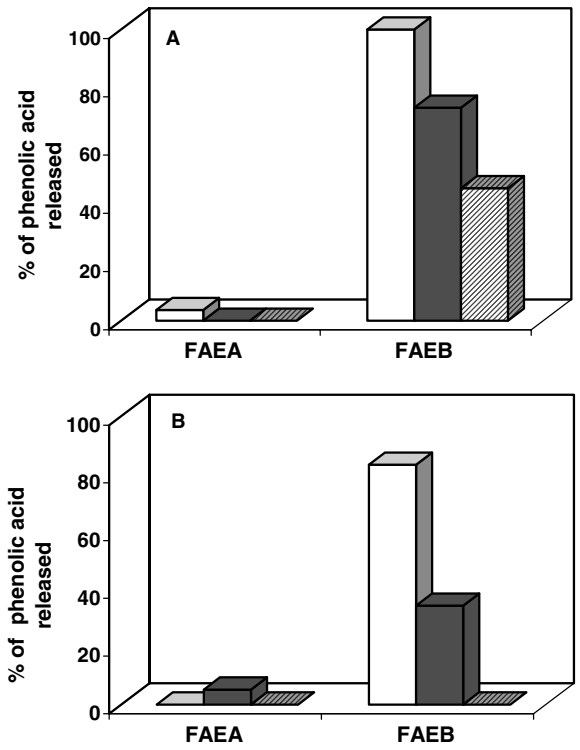

Figure 1. Effect of FAEA and FAEB on the release of phenolic compounds from coffee pulp and apple marc. Caffeic acid (□), *p*-coumaric acid (■) and ferulic acid (▨) released from coffee pulp (A) and apple marc (B). Results are expressed as the percentage of the total initial amount of each phenolic acid (extractable after alkaline hydrolysis). The standard deviation was less than 5% from the mean of the value.

of both enzymes. FAEA is known to be active on ferulic and sinapic sugar-esters, while FAEB, is active on caffeic and *p*-coumaric sugar-esters.[25] However, in coffee pulp and apple marc, *p*-coumaric and caffeic acids occur as conjugates of quinic acid. Hence our results show for the first time that this type of ester linkage can be hydrolyzed by FAEB. A significant amount of ferulic acid was also released from coffee pulp by FAEB when FAEA had no effect. Although ferulic acid esters were shown to be poor substrates for FAEB, the nature of the ester partner may have been important. In terms of molar yield, the amount of caffeic acid released from coffee pulp by FAEB was higher than the amount of hydrolyzed chlorogenic acid (results not shown). It is well known that coffee contains other conjugates of caffeic acid such as dicaffeoyl quinic acid and also minor compounds such as esters of feruloyl-caffeolquinic acids, which could explain this result, suggesting that FAEB could be active on esters other than caffeoyl quinic acid. Recently a new chlorogenic acid hydrolase was purified and characterized from *A. niger*. This enzyme specifically catalyzed the hydrolysis of chlorogenic acid.[26] With a broader specificity, FAEB appeared to be as efficient as chlorogenic acid hydrolase in hydrolyzing chlorogenic acid present in agricultural by-products.

2.2. Hydrolysis of wheat straw

Lignocellulose is the main component of wheat straw, which is a compact structure of cellulose (35–40%) and hemicellulose (20–30%) in close association with lignin (8–15%).[27] Hydroxycinnamic acids, particularly ferulic acid and *p*-coumaric acid, occur widely in the cell walls of graminaceous plants such as wheat straw.[7] Chemical treatments have been reported for the release of these compounds. Mild alkaline hydrolysis serves to release ester-linked ferulic and *p*-coumaric acid, and acid hydrolysis cleaves the alkyl aryl ether bond.[28] In this work, alkaline hydrolysis was used to determine the amount of ester-linked hydroxycinnamic acids (Table 1). We found 2.13 and 1.35 mg/g for *p*-coumaric and ferulic acids, respectively, and these values agree with previous reports.[2] Owing to the complex structure of lignocellulosic material, mechanical pre-treatment has been shown to be an important tool to degrade cell wall structure and make substrates more accessible to enzymes.[29,30] Steam explosion of wheat straw causes partial destructuring of the lignocellulosic complex, and was used in this study. Incubation of steam-exploded wheat straw with FAEA and FAEB caused significant release of phenolic acids, but FAEB was more efficient than FAEA for the release of both *p*-coumaric acid and ferulic acid (Fig. 2A). Sixteen per cent of the alkali-extractable *p*-coumaric acid and 58% of the ferulic acid were released with purified FAEB and without the help of accessory enzymes. Considering that wheat straw is a very bioresistant material, these results are of considerable interest. Increasing enzyme amounts did not give better results. Also, the direct addition of both enzymes did not demonstrate a synergistic effect, indicating that they probably hydrolyze the same types of ester linkages (data not shown). Release of *p*-coumaric acid from various cell wall materials has been previously reported for *p*-coumaroyl esterases from *Aspergillus awamori*, *Penicillium pinophilum* and the anaerobic fungus *Neocallimastix*.[31–33] In addition, experiments were often conducted with non-purified enzymes, or with feruloyl esterases in association with xylanases. The ability of *Aspergillus niger* feruloyl esterase to release *p*-coumaric acid from oat hulls was verified in both the presence and absence of xylanase.[34] No extensive release of free *p*-coumaric acid was obtained under such conditions. The same experiments were carried out for the release of ferulic acid; 4.7% was freed with feruloyl esterase FAEA alone and 68% in combination with xylanase.[22] These results indicate a weak action of FAEA alone on the complex cell walls of oat hulls. *P. pinophilum* *p*-coumaroyl esterase was able to release only 0.4% of *p*-coumaric acid from Italian ryegrass cell walls, indicating that certain types of plant cell walls are more accessible than others.[32]

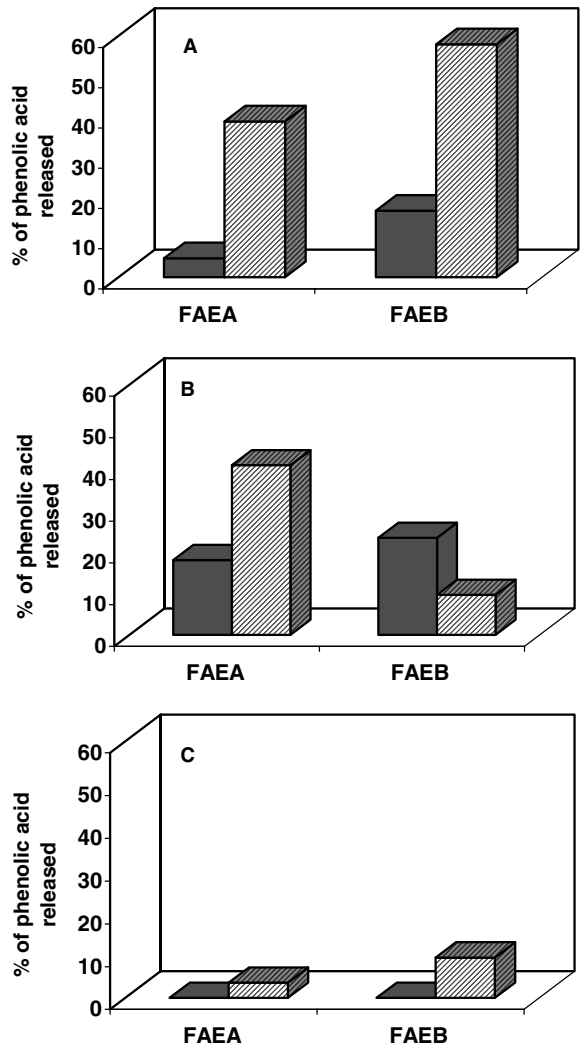

Figure 2. Effect of FAEA and FAEB on the release of phenolic compounds from wheat straw, maize bran and sugar beet pulp. *p*-coumaric acid (■) and ferulic acid (▨) released from wheat straw (A), maize bran (B) and sugar beet pulp (C). Results are expressed as the percentage of the total amount of each phenolic acid present in the substrate before the addition of the enzymes. The standard deviation was less than 5% from the mean of the value.

Ferulic acid is known to be ester-linked to arabinoxylans and as lignification is initiated, ether linkage to lignin occurs through the phenolic oxygen at the β-position of coniferyl alcohol.[7] It is also suggested that ferulic acid ester–ether bridges play a predominant role in cross-linking between lignin and polysaccharides in wheat straw, increasing cell wall rigidity. Besides this, a predominant amount (around 70%) of *p*-coumaric acid is ester-linked to the cell wall component, mainly to lignin. It is extensively esterified at the γ position, on the side chain of lignin monomers, but *p*-coumaric acid is not involved in ester–ether bridges.[35] Although it is not possible in our case to tell whether our esterases are active on ester linkages between the phenolic acids and hemicellulose or lignin, our results indicate unambiguously that these bonds are partly accessible for purified FAEA and FAEB. The role of *A. niger* FAEA in wheat straw pulp bleaching has already been demonstrated.[19] FAEA was used in synergy with other plant cell wall degrading enzymes such as laccases and xylanases, and results indicated clearly an improved delignification in the presence of FAEA. Our results show clearly that both feruloyl esterases are able to cleave ester linkages commonly found in wheat straw, allowing a better accessibility to lignin degrading enzymes.

2.3. Hydrolysis of sugar beet pulp and maize bran

Sugar beet pulp, maize and wheat brans are classical substrates often used for the determination of esterase specificities. They are quite different, sugar beet pulp being made up of primary cell walls rich in pectins, whereas brans contain secondary cell walls, rich in heteroxylans. In sugar beet pulp, ferulic acid is mainly esterified to O-2 of arabinofuranose residues and to O-6 of galactopyranose residues.[6] In maize and wheat brans, the major polysaccharides are heteroxylan, arabinofuranose being esterified at O-5 by ferulic acid. Maize bran also contains high levels of ferulic dimers, which limits enzymatic degradation.[5] In this work maize bran was preferred to wheat bran owing to its higher content of ferulic and *p*-coumaric acids. It was previously shown that autoclaving treatment of the bran improved the solubilization of feruloylated oligosaccharides, which are substrates for feruloyl esterases.[36] In our conditions, 40% of ferulic acid was released with FAEA against 8% with FAEB from the autoclaved fraction of maize bran (Fig. 2B). Similarly, FAEB is able to release ferulic acid from sugar beet pulp (8%), whereas FAEA displayed no significant activity (Fig. 2C). Also, significant amounts of *p*-coumaric acid were released from maize bran by both enzymes, FAEB being more efficient than FAEA. Some of these results agree with previous findings indicating that FAEA is mainly active on O-5 ester linkages[37] and FAEB on O-2 or O-6 ester linkages.[38] They also clearly indicate the ability of both pure enzymes to release *p*-coumaric acid from the autoclaved fraction of maize bran. It was previously shown that large amounts of ferulic acid could be released from sugar beet pulp and autoclaved maize bran (up to 79% and 97%, respectively).[39] However, the enzymes used were commercial enzyme mixtures or crude *A. niger* culture medium, both of which exhibited significant pectin-, hemicellulose- and cellulose-degrading activities. De Vries et al.[17] compared the effect of FAEB and FAEA on sugar beet pulp. A maximal amount of 5% ferulic acid was released with FAEB using sugar beet pulp fraction pre-treated with rhamnogalacturonan hydrolase and rhamnogalacturonan acetylesterase A to increase the accessibility to the substrate. Using *P. funiculosum* feruloyl esterase, 30% ferulic acid was released, but very

high levels of enzymes were necessary (100 U/g).[14] No release of ferulic acid from wheat bran was shown using the purified ferulic acid esterase of *Streptomyces olivochromogenes* and only 4% with *A. niger* FAEA.[40,13] In parallel, numerous studies have been conducted using wheat bran as a substrate, but most experiments have been carried out on arabinoxylan enriched fractions or in the presence of accessory enzymes.[14,17,41,42] To our knowledge only one report indicates a significant release of both ferulic acid and *p*-coumaric acid from steam-extracted xylan of wheat straw using *A. awamorii* feruloyl and *p*-coumaroyl esterases.[31]

2.4. Determination of substrate specificities on model compounds

To investigate the specificity of FAEA and FAEB more precisely, and to confirm the different results obtained on agro-industrial substrates, several model compounds were chosen. 5-*O*-Caffeoyl quinic acid (AC) and *p*-coumaroyl quinic acid (CQ) were used, as they are naturally present in coffee pulp and apple marc. An ester of *p*-coumaric acid and coniferyl alcohol (CC) was synthesized, representative of the ester linkage between hydroxycinnamic acid and lignin monomer. FA (*O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]) and FAX (*O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1→3)D-Xyl*p*) were typical esters found in maize bran, and methyl esters of hydroxycinnamic acids served as reference substrates. Under such conditions, we had examples of ester linkages between hydroxycinnamic acids and sugars (arabinose), quinic acid and lignin monomer. Kinetic constants K_m and k_{cat} were measured, and catalytic efficiencies (k_{cat}/K_m) were determined for both enzymes and each substrate (Table 2). It can be observed that FAEB exhibited a broader specificity than FAEA. In our conditions FAEB was active on all substrates except MSA. This result is in contradiction with the last classification of feruloyl esterases,[18] but is consistent with all previous data, which indicated without ambiguity that FAEB was not able to hydrolyze MSA.[43,44] FAEA was not active on methyl and quinic esters of *p*-coumaric and caffeic acids. FAEB was active on the two quinic acid esters (AC and CQ), which explains its ability to release caffeic and *p*-coumaric acids efficiently from coffee pulp and apple marc. Surprisingly, FAEB was also active on FA and FAX, which explains the release of ferulic acid from maize bran. In addition, we show here for the first time the ability of FAEA and FAEB to hydrolyze ester linkages between hydroxycinnamic acid and lignin components (CC) present in wheat straw. Considering catalytic efficiencies of FAEB, as expected, the greatest values were obtained for esters of caffeic acid and *p*-coumaric acid. However, quinic esters (CQ and AC) were not efficiently hydrolyzed. This result indicates the major role of the structure of the molecular moiety esterified to the cinnamic acid, as already mentioned. Similar results were observed with esters of ferulic acid, where methyl ester was a poor substrate for FAEA compared with FA and FAX, which matched natural substrates. Recently, the three-dimensional structure of an inactive mutant of type-A feruloyl esterase from *A. niger* in a complex with a feruloylated trisaccharide was determined at 2.5 Å resolution.[45] Although the FA moiety is clearly visible in the active site, prompting the authors to discuss the implication of several amino acids in the catalytic activity, the carbohydrate part of the substrate was not visible. They suggest that tight binding of the carbohydrate is not required for catalysis. However, kinetic results clearly indicate that the non-phenolic partner plays an essential role, probably for the orientation of the hydrolyzable bond.

Table 2. Substrate specificity of *A. niger* FAEA and FAEB for different model substrates

Substrates	FAEA			FAEB		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
Ferulic acid esters						
MFA	16	0.308	0.52×10^5	4	0.163	0.25×10^5
FA	121	0.061	1.97×10^6	28	0.132	2.12×10^5
FAX	95	0.045	2.11×10^6	8	0.044	1.79×10^5
Sinapic ester						
MSA	61	0.103	5.95×10^5	ND	—	—
p-Coumaric esters						
MpCA	ND	—	—	69	0.02	3.43×10^6
CC	20	0.237	0.85×10^5	140	0.044	3.21×10^6
CQ	ND	—	—	48	0.163	2.9×10^5
Caffeic esters						
MCA	ND	—	—	234	0.158	1.48×10^6
AC	ND	—	—	63	0.245	2.56×10^5

ND: activity not detected.
MFA, methyl ferulate; MSA, methyl sinapinate; M*p*CA, methyl *p*-coumarate; MCA, methyl caffeate. AC, chlorogenic acid, CQ, 5-*O*-*p*-coumaroylquinic acid; FA, *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]; FAX, *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1→3)D-Xyl*p*; CC, coniferyl *p*-coumarate.

2.5. Conclusion

Plant cell walls are largely exploited in various industrial sectors such as animal nutrition and the pulp and paper industry. They are also a source of natural biomolecules such as sugars and phenolic acids, which are attracting growing interest, for example, as aroma precursors and antioxidants, and in bioethanol production. Owing to their complex structures, of which some, such as lignin, are highly bioresistant polymers, the degradation of cell walls requires the action of a wide spectrum of enzymes. A better knowledge of the specificity of these enzymes would help to specify optimized enzyme cocktails. Among these enzymes, feruloyl esterases allow the direct release of phenolic acids from agro-industrial

Table 3. Specificities of feruloyl esterases against different kinds of hydroxycinnamic acid esters

Agro-industrial by-product	Targeted ester linkage	Phenolic compound released R-C(=O)-OH	Feruloyl esterase	
			FAEA	FAEB
Coffee pulp	R-C(=O)-O- quinic acid	Caffeic acid	–	++
Apple marc		*p*-Coumaric acid	–	++
		Ferulic acid	–	++
Wheat straw	R-C(=O)-O- 2-coniferyl alcohol	*p*-Coumaric acid	+	++
		Ferulic acid	+	++
Sugar beet pulp	R-C(=O)-O- 2-arabinose / R-C(=O)-O- 6-galactose	Ferulic acid	+	++
Maize bran	R-C(=O)-O- 5-arabinose	*p*-Coumaric acid	++	+
		Ferulic acid	++	+

by-products. Our results are summarized in Table 3 and they clearly indicate that depending on the nature of the substrate, the choice of the feruloyl esterase is of fundamental importance. Due to its broader specificity, FAEB appears to be a more promising tool.

3. Experimental

3.1. Agro-industrial substrates

Coffee pulp was obtained from the production co-operative Bénéficio de café (Mexico) and apple marc from the company Val de Vire (France) and the Unité INRA de Recherches Cidricole de Rennes (France). Autoclaved maize bran, produced according to a previously described process[24,36] and sugar beet pulp were obtained from Agro-Industries Recherche et Développement (Pomacle, France). Steam-exploded wheat straw was obtained from SAF-ISIS (Souston, France) and prepared according to the procedure described by Tabka et al.[46]

3.2. Enzyme assays

FAEA and FAEB were obtained from *A. niger* after homologous overexpression of the corresponding genes. Recombinant esterases were produced and purified as previously described.[19,20] The purity of both enzymes was controlled by SDS-PAGE analysis and silver nitrate staining. The absence of xylanase activity was checked by measuring the release of xylose from 1% birchwood xylan in 50 mM sodium-citrate buffer pH 5.5 at 45 °C according to time. Reducing sugars were determined by the 3,5-dinitrosalicylic acid method with xylose as standard.[47,48]

Esterase activity was assayed by a continuous spectrophotometric method as previously described,[37] using a range of model substrates such as methyl esters of hydroxycinnamic acids that is methyl ferulate (MFA), methyl sinapinate (MSA), methyl *p*-coumarate (M*p*CA) and methyl caffeate (MCA) (Apin chemicals Ltd, Oxon, UK). Chlorogenic acid (AC) was purchased from Sigma–Aldrich Corporation (St Louis, USA) and activity was measured by the same procedure. Activities were expressed in nanokatals (nkat), 1 nkat being the amount of enzyme that catalyzes the release of 1 nmol of substrate per second. Kinetic constants (k_{cat}, K_m) were calculated from initial rate data determined at various substrate concentrations using the Lineweaver–Burk plots and linear regression analysis. Protein concentration was determined according to Lowry et al.[49]

3.3. Preparation of model substrates

3.3.1. 5-*O*-*p*-Coumaroyl quinic acid (Fig. 3A). Phenolic compounds were extracted from cider using ethyl acetate according to the method of Salih et al.[50] The extract rich in *p*-coumaroyl quinic acid was evaporated under diminished pressure and redissolved in water before fractionation by preparative HPLC. HPLC was performed using two high pressure Dynamax® SD 300 (Rainin, Oakland, USA) model pumps with a 100 mL WT1 titanium head, connected to a variable UV/vis detector and a 5 mL manual injector (Rheodyme® L.P.). A Merck (Darmstadt, Germany) C-18 reversed phase column (Lichrospher 100 RP-18, 12 μm, 200 × 50 mm) maintained at 21 °C was used. The detector wavelength was set at 280 nm. The mobile phase, at a flow rate of 40 mL/min, was a mixture of two solvents: A, water with 2.5% AcOH, and B, acetonitrile. Separation was achieved by elution gradient using an initial composition from 0 to 3 min of 97% solvent A. Solvent B was then increased to 9% at 13 min, 11% at 18 min and 30% at 45 min. The signal was acquired with EZ-Chrom Station software (Scientific, Software Inc, CA, USA). *p*-Coumaroyl quinic acid enriched fraction eluted at 32 min was collected, concentrated under diminished pressure and redissolved in solvent A. A second purification step was performed using the same chromatographic system equipped with a prepLC radial

compression module (Waters, Milford M.A. USA) a pre-column (25 × 10 mm) and a (25 × 100 mm) column of C-18 reversed phase (Nova pack HR 6 μm 60 A, Waters, Milford M.A. USA). The identity of *p*-coumaroyl quinic acid was confirmed by HPLC-DAD[51] and using a MS/MS LCQ Deca (Thermo Finnigan) in negative mode equipped with an electrospray ionization source. The structure of this compound is given in Figure 3A.

3.3.2. Coniferyl *p*-coumarate (Fig. 3B). Coniferyl *p*-coumarate was a gift of Dr. Rakotomanomana (INRA, Avignon, France). The characteristics of the product were in agreement with previous results.[52]

The structure of this substrate is given in Figure 3B.

3.3.3. *O*-[5-*O*-(*trans*-Feruloyl)-α-L-Ara*f*] (FA) and *O*-[5-*O*-(*trans*-feruloyl)-α-L-Ara*f*]-(1→3)D-Xyl*p* (FAX). The two feruloylated oligosaccharides were purified according to Saulnier et al.[36] The hydrolysis of the substrates was confirmed by HPLC analysis; the reaction products were identified by their retention times and UV–vis spectra.

3.4. Determination of total phenolic acid content of agro-industrial by-products

Initial phenolic acid content of agro-industrial by-products was determined after alkaline hydrolysis with 2 M NaOH at 35 °C for 30 min according to Saulnier et al.[5]

Enzymatic hydrolysis of agro-industrial by-products was performed as follows: dry materials (200 mg) were autoclaved in 2 mL of water at 110 °C for 30 min. The mixture was then incubated with 2 nkat of purified enzyme in 2 mL of 200 mM MOPS buffer pH 6, containing 0.02% (w/v) sodium azide. Hydrolysis was performed overnight at 37 °C with stirring (100 rpm). The mixture was centrifuged at 4000*g* for 10 min and 1 mL of supernatant was acidified to pH 2 with HCl, and extracted twice with EtOAc. After drying, samples were dissolved in 50% MeOH (v/v). HPLC analysis was performed on an HP 1050 instrument (Hewlett–Packard, Rockville, MD) equipped with a variable UV/visible detector and a 34-position autosampler-autoinjector. Separations were carried out as previously described.[53]

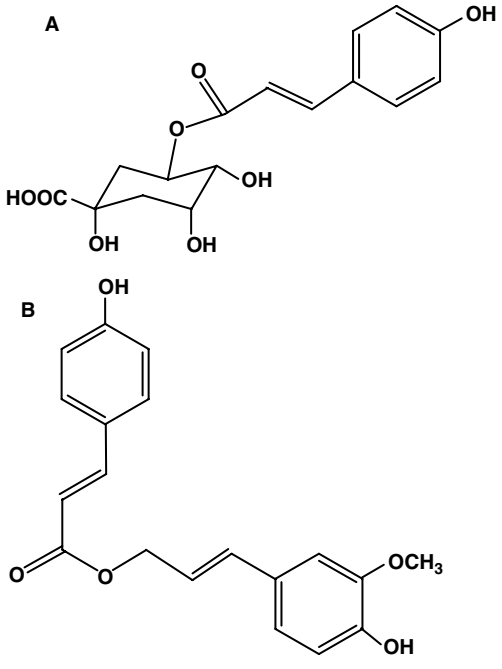

Figure 3. Structure of two *p*-coumaroyl esters used in this study. 5-*O*-*p*-Coumaroylquinic acid (A) and coniferyl *p*-coumarate (B) were, respectively, extracted and synthesized as described in Experimental.

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